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Franck Mauvais-Jarvis *Editor*

Sex and Gender Factors Affecting Metabolic Homeostasis, Diabetes and Obesity

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Franck Mauvais-Jarvis
Editor

Sex and Gender Factors Affecting Metabolic Homeostasis, Diabetes and Obesity

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Foreword

As children, we were taught to look both ways before crossing the road, as a matter of safety for ourselves and for others. Clearly, looking for traffic in only one direction can be dangerous. Yet in biomedical research, neglecting the larger picture and looking only one way – that is, at male cells and animals in preclinical studies and men in clinical studies – was largely the norm until recently. This biased focus persists in some cases, especially in the realm of preclinical research, despite the pervasiveness of sex differences in human anatomy, physiology, and disease.

I first became interested in sex and gender differences in health and disease as a clinician and researcher in ophthalmology, given the female-biased prevalence of many types of ocular diseases and the common roles of sex hormones in those diseases. My interests in sex and gender influences on health and disease, and on sex and gender disparities in research studies, grew from this starting point, eventually leading me to my current role as director of the Office of Research on Women's Health (ORWH) at the US National Institutes of Health (NIH). As part of NIH's initiative to enhance reproducibility through rigor and transparency, ORWH has been leading the development of a policy requiring NIH-funded scientists to consider sex as a biological variable in basic, preclinical, translational, and clinical research.

While myriad sex and gender differences have been documented across the medical disciplines, they are particularly prominent in certain areas. For example, sex and gender differences appear to be more the rule than the exception when it comes to energy balance, fat distribution, obesity, and metabolic disorders, such as diabetes. Although such sex and gender differences are often well known from a bird's-eye view, many of the details underscoring the differences still need to be investigated, given the historical overreliance on male cells and animals and the underreporting of sex- and gender-specific results in the medical literature.

Fortunately, a promising shift to correct these shortfalls is already underway for research on metabolic homeostasis, obesity, and diabetes. Dr. Franck Mauvais-Jarvis, professor of endocrinology at the Tulane University School of Medicine, has championed a move to include both sexes in preclinical research in these areas of medicine. As editor of this book, Dr. Mauvais-Jarvis brings together leading

colleagues in the field, who cover a comprehensive array of subjects pertaining to metabolic homeostasis and the causes and treatments of diabetes and obesity. These topics include the epidemiology of diabetes and obesity; key sex differences in energy metabolism, diabetes, and obesity; the roles of sex hormones in energy balance, metabolic homeostasis, body fat distribution, muscle function, and feeding behavior; and hormone replacement therapies for men and women.

A goal of NIH is for all biomedical research to consider sex and gender (as appropriate) so as to automatically "look both ways" to see the whole picture. Failure to consider both sexes in preclinical research incompletely informs the premise and design of subsequent clinical studies. A focus on only one sex of cells and research animals also results in missed opportunities for discoveries that ultimately benefit everyone, for example, "protective effects" of certain factors in one sex, which may have therapeutic relevance for everyone. The foundation of knowledge arising from biomedical research helps answer important questions about disease presentation, clinical care, and the efficacy and safety of therapeutics. Failure of preclinical or clinical research to be inclusive with regard to sex and gender offers a subset of the population with a foundation that lags behind the foundation available to the rest.

This book synthesizes an emerging roadmap for considering sex and gender in metabolic homeostasis, diabetes, and obesity. It also exemplifies many important insights that such an approach offers for improved, evidence-based medicine. Publication of the book could not be more timely, owing to the growing global burden of obesity and diabetes, which exhibit important sex/gender differences in prevalence, pathophysiology, and consequent risks.

National Institutes of Health, Janine Austin Clayton
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2017

Preface

Due to the inherent biological differences between the two sexes, multiple aspects of glucose homeostasis and energy balance are regulated differently in male and female mammals and influence both the development of diabetes and obesity and the response to drugs. In 1993, the National Institutes of Health Revitalization Act mandated the inclusion of women in clinical trials, but many investigators still do not analyze the results by sex. In addition, most researchers avoid studying female rodents due to the added complexity of research plans. The consequence is a generation of data that risks being relevant to only males. NIH director Francis Collins and associate director for research on women's health Janine Clayton finally asked scientists to consider sex in preclinical research, to ensure that women get the same benefit of medical research as men. The NIH released a notice on new rules to promote the study of animals and cells from both sexes to prevent the overreliance on male animals in preclinical studies. In March 2017, I organized (with the help of Debbie Clegg and Art Arnold) the first Keystone Symposium on "Sex and Gender Factors Affecting Metabolic Homeostasis, Diabetes and Obesity" in Lake Tahoe, CA, to connect interdisciplinary groups of scientists who otherwise would not have an opportunity to meet. Participants came from a wide range of basic and clinical backgrounds but shared a common focus of studying sex differences in metabolic disease. The meeting was a success and generated new knowledge and ideas on the importance of sex and gender biology and medicine, from a molecular standpoint to the population level. This book was conceived as a necessary follow-up to this meeting. Each chapter was written by participants who are experts in their respective fields of sex differences in metabolic homeostasis and disease.

New Orleans, LA, USA — Franck Mauvais-Jarvis

Introduction

This book is organized to describe major sex differences in metabolic homeostasis and disease as well as the role of estrogens and androgens. In the first chapter, I introduce epidemiological evidence that the incidence and prevalence of both types of diabetes, as well as obesity and metabolic syndrome, exhibit a clear global gender bias. Gender differences affect many aspects of metabolism that are involved in the pathogenesis of dysglycemia and adiposity. The most obvious examples are body composition, as discussed by Miriam Bredella in chapter "Sex Differences in Body Composition," adipose tissue biology and distribution reviewed by Susan Fried and coworkers in chapter "Cellular Mechanisms Driving Sex Differences in Adipose Tissue Biology and Body Shape in Humans and Mouse Models," and insulin secretion and action presented by Rita Basu and coworkers in chapter "Men Are from Mars and Women Are from Venus: Sex Differences in Insulin Action and Secretion." Sex differences are also apparent at both the clinical and preclinical levels in obesity-induced inflammation (reviewed by Durga Singer and coworkers in chapter "The Role of Sex and Sex Hormones in Regulating Obesity-Induced Inflammation"), the effects of obesity-induced hyperleptinemia on cardiovascular function (analyzed by Eric Belin De Chantemelle in chapter "Sex Differences in Leptin Regulation of Cardiovascular Function in Health and Metabolic Diseases"), and the role of the microbiome and immunity in type 1 diabetes (discussed by Jayne Danska and coworkers in chapter "Sex Effects at the Ramparts: Nutrient- and Microbe-Mediated Regulation of the Immune-Metabolic Interface"). There are also major sex differences in liver biology and function related to estrogen receptor-α (ERα) induction by feeding, as argued by Adriana Maggi and coworkers in chapter "Sexual Dimorphism of Estrogen Action in Mouse Liver." Jose Garcia and coworkers review sex differences in skeletal muscle metabolism and wasting in chapter "Sex Differences in Muscle Wasting," and Holly Ingraham analyzes sex differences in the development of hypothalamic neural circuits controlling energy homeostasis in chapter "Origins and Functions of the Ventrolateral VMH: An Organized Neuronal Cluster Orchestrating Sex Differences in Metabolism and Behavior."

The major contributors of sex differences in glucose and energy homeostasis are the gonadal hormones estrogens and androgens after the onset of puberty. Starting

with estrogens, I review the effects of menopause and estrogens in glucose homeostasis in women in chapter "Menopause, Estrogens and Glucose Homeostasis in Women," and John Stafford covers estrogen's regulation of liver lipid metabolism in women in chapter "Role of Estrogens in the Regulation of Liver Lipid Metabolism." The actions of ERα in the muscle are critical to whole-body metabolic homeostasis in females, analyzed by Andrea Hevener in chapter "The Role of Skeletal Muscle Estrogen Receptors in Metabolic Homeostasis and Insulin Sensitivity," while the effects of estrogens on body weight regulation in men are addressed by Katya Rubinow in chapter "Estrogens and body weight regulation in men." Some of the most powerful actions of estrogens (at least experimentally) relate to the central regulation of energy balance and the protection of pancreatic islets. To analyze these effects separately, Miguel Lopez and coworkers review the stimulatory actions of estrogens in brown adipose tissue thermogenesis (chapter "Estradiol Regulation of Brown Adipose Tissue Thermogenesis"), Yong Xu dissects the role of brain estrogens in feeding behavior (chapter "Brain Estrogens and Feeding Behavior"), and Karen Briski and coworkers focus on the effects of estrogens on the body's response to hypoglycemia (chapter "Sex Differences and Role of Estradiol in Hypoglycemia-Associated Counter-Regulation"). The effects of estrogens on islet and insulin production are analyzed by Mauvais-Jarvis and coworkers in chapter "The Role of Estrogens in Pancreatic Islet Physiopathology." Estrogen actions are mediated by different pools of nuclear and extranuclear ERs as well as the G protein-coupled estrogen receptor (GPER). In chapter "Nuclear and Membrane Actions of Estrogen Receptor-α: Contribution to the Regulation of Energy and Glucose Homeostasis," Pierre Gourdy and coworkers analyze the contribution of nuclear and membrane actions of ERα in the regulation of energy and glucose homeostasis, while Eric Prossnitz and coworkers review the effects of GPER in metabolic homeostasis in chapter "G Protein-Coupled Estrogen Receptor (GPER) and Sex-Specific Metabolic Homeostasis." Finally, in chapter "Gender-Dependent Role of Steroid Sulfatase and Estrogen Sulfotransferase in Metabolic Homeostasis," Wen Xie provides insights on tissue estrogen activation and inactivation and the gender-dependent role of steroid sulfatase and estrogen sulfotransferase in metabolic homeostasis.

Another critical aspect of sex-specific metabolic homeostasis and disease involves the effects of androgens in men and women. On one end of the spectrum, the impact of testosterone deficiency and 5α-reductase inhibitors (which prevent the conversion of testosterone into the potent androgen dihydrotestosterone) on metabolic dysfunction in men is presented by Abdulmaged Traish in chapter "Negative Impact of Testosterone Deficiency and 5α-Reductase Inhibitors Therapy on Metabolic and Sexual Dysfunction in Men," and Farid Saad discusses the therapeutic benefits of testosterone therapy on glucose homeostasis in hypogonadal men in chapter "Testosterone Therapy and Glucose Homeostasis in Men with Testosterone Deficiency (Hypogonadism)." On the other end of the spectrum, androgen excess during development and prepubertal and adult life is believed to predispose to polycystic ovarian syndrome (PCOS), the most common endocrine disease in women of reproductive age. The role of androgen excess during development and adulthood in the genesis of the metabolic abnormalities observed in PCOS is analyzed in sheep

by Vasantha Padmanabhan in chapter "Prenatal Testosterone Programming of Insulin Resistance in the Female Sheep," in nonhuman primates by Charles Roberts and coworkers in chapter "Sex Differences in Androgen Regulation of Metabolism in Nonhuman Primates," and in women by Hector Escobar-Morreale and coworkers in chapter "The Role of Androgen Excess in Metabolic Dysfunction in Women."

Finally, a critical emerging field in endocrinology is the metabolic impact of cross hormone therapy in transgender individuals. This increasingly important issue is covered by Deborah Clegg and coworkers (chapter "Sex, Gender, and Transgender: Metabolic Impact of Cross Hormone Therapy").

The knowledge compiled in this book is intended to be a catalyst to open interdisciplinary avenues of research into gender-specific treatments for metabolic diseases.

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Part I
Sex Differences in Diabetes and Obesity

Epidemiology of Gender Differences in Diabetes and Obesity

Franck Mauvais-Jarvis

Abstract Some aspects of glucose homeostasis and energy balance are regulated differently in males and females. This review discusses the most fundamental gender differences in diabetes and obesity, including the prevalence of impaired fasting glucose and impaired glucose tolerance, the prevalence and incidence of type 2 and type 1 diabetes, as well as the prevalence of metabolic syndrome and obesity. These gender-specific differences in glucose homeostasis and energy balance represent a source of factors that should be studied to develop gender-based therapeutic avenues for diabetes.

Introduction

Epidemiology is defined as the study of the patterns, causes, and effects of health and disease in selected populations. It is central to public health and shapes policy decisions and evidence-based medicine by identifying risk factors for disease and targets for disease prevention. Increasing evidence suggests that sex and gender affect the pathophysiology, incidence, prevalence, course, and response to therapy of many diseases. Sex differences in physiology and disease are of fundamental importance because they represent gender-related biological factors that might lead to better prevention and therapy. Some aspects of glucose homeostasis and energy balance are regulated differently in men and women. This chapter provides an overview of the most fundamental gender differences in diabetes and obesity. These include the prevalence of impaired fasting glucose and impaired glucose tolerance, the prevalence and incidence of type 2 and type 1 diabetes, as well as the prevalence of metabolic syndrome and obesity. These gender-specific differences in glucose homeostasis and energy balance represent a source of factors that may lead to the development of gender-based therapeutic avenues for metabolic disease.

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Gender Differences in Glucose Homeostasis

There are considerable gender differences in the response to an oral glucose challenge (glucose tolerance test, OGTT). Women have lower fasting plasma glucose (FPG) and higher plasma glucose 2 h following an OGTT (2-h PG) (Sicree et al. 2008). These differences are positively correlated to height and have been hypothesized to be secondary to smaller muscle mass and associated glucose uptake, for a fixed charge of 75 g of glucose during the OGTT in women (Sicree et al. 2008). In fact, men and women have almost identical HbA1c values, suggesting that chronic postprandial glucose excursions are similar in men and women and that the 2-h PG may be a consequence of the fixed glucose load during the OGTT. Alternatively, gonadal hormones may be responsible for this gender difference in glucose homeostasis. Indeed, menopausal estrogen therapy decreases FG while impairing glucose tolerance (Van Genugten et al. 2006; Mauvais-Jarvis et al. 2017).

Insulin sensitivity also differs by sex. Healthy women have lower skeletal muscle mass, higher adipose tissue mass, more circulating free fatty acids, and higher intramyocellular lipid content than men of the same age, all factors that should promote insulin resistance in women compared to men. Yet, women exhibit similar insulin sensitivity as men. Women are even resistant to free fatty acid-induced insulin resistance (Frias et al. 2001). When matched for physical fitness, during a hyperinsulinemic, euglycemic clamp, systemic insulin sensitivity is higher in women than men as a result of higher glucose disposal by skeletal muscles (Nuutila et al. 1995). However, it is important to keep in mind that physical fitness is a critical parameter for insulin sensitivity in women. Women with lower fitness than men exhibit insulin resistance (Basu et al. 2006). Yet, these same women exhibit comparable glucose disposal to men because of the enhanced ability of glucose to promote its own disposal in an insulin-dependent manner (glucose effectiveness) (Basu et al. 2006). Women also tend to have enhanced postprandial insulin and C-peptide concentrations (despite similar plasma glucose) after a meal test, suggesting increased insulin secretion compared to men (Basu et al. 2006). In fact, the disposition index is higher in women than in men, reflecting greater insulin secretion for a given level of insulin action (Basu et al. 2006). The mechanisms that facilitate glucose homeostasis in women compared to men are unclear, but the differences could be due at least in part to the beneficial effect of endogenous estradiol before menopause (Mauvais-Jarvis et al. 2017).

Gender Differences in Type 2 Diabetes

The prevalence of prediabetic syndromes such as impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) is also characterized by a sex difference in all populations studied. Indeed, IFG is male biased while IGT is more prevalent in women (Glumer et al. 2003; Sicree et al. 2008; Van Genugten et al. 2006; Williams

et al. 2003). The reason for these sex differences in early dysglycemia is unknown but could involve the effect of gonadal hormones. Indeed, menopausal hormone therapy with estrogens decreases fasting glucose while impairing glucose tolerance (Van Genugten et al. 2006; Mauvais-Jarvis et al. 2017). The prevalence of type 2 diabetes is also characterized by a sex difference. Overall, the global prevalence of diabetes is higher in men, but there are more women with diabetes than men (Wild et al. 2004). This sex difference in diabetes prevalence is reversed depending upon the stage of reproductive life (Wild et al. 2004); that is, there are more diabetic men before the age of puberty, while there are more diabetic women after the age of menopause. The combined effect of a greater number of elderly women than men in most populations and the increasing prevalence of diabetes with age is the given explanation for this observation. Recent data from the National Health and Nutrition Examination Survey (NHANES) over two decades revealed that diabetes and prediabetes (including undiagnosed cases) affect almost half of the adult US population and exhibit a sex bias (Menke et al. 2015). When diabetes was not previously diagnosed, the authors used two definitions of diabetes corresponding to different subsets of data: The first included three criteria, combining the hemoglobin A_{1c} (HbA_{1c}) with FPG and 2-h PG; the second included the HbA_{1c} and FPG only, without the 2-h PG. From 2011 to 2012, the study reported a significant male predominance in the prevalence of total diabetes (13.6% vs 11.4%, men vs women) and a trend toward an increase in the prevalence of prediabetes (39.1% vs 33.8%, men vs women), only when the 2-h PG was not used in the definition. This is an important observation because, as discussed above, more men show impaired FPG and more women show impaired glucose tolerance (assessed by 2-h PG). Thus, the exclusion of the 2-h PG values in the calculation of diabetes prevalence may have underestimated the prevalence of diabetes and prediabetes cases in women.

An important male predominance (75%) is also observed in ketosis-prone diabetes (classified as idiopathic type 1 diabetes by the American Diabetes Association), a severe form of type 2 diabetes mostly encountered in non-Caucasian subjects and with a propensity to acute insulin dependence and diabetic ketoacidosis alternating with periods of remission (Mauvais-Jarvis et al. 2004; Umpierrez et al. 2006). In ketosis-prone diabetes, women are relatively protected unless they are in an anovulatory or hypoestrogenic state (Mauvais-Jarvis et al. 2004; Louet et al. 2008).

Gender Differences in Type 1 Diabetes

There is a gender difference in the incidence of type 1 diabetes (T1D). Interestingly, T1D is the only common autoimmune disease not characterized by a female predominance. In fact, T1D is even characterized by a male predominance in Caucasians (ratio, 1:7) (Gale and Gillespie 2001). The pubertal period is associated with a decreased incidence of T1D in girls (Blohme et al. 1992; Nystrom et al. 1992) who retain more robust residual β-cell function than boys (Samuelsson et al. 2013). This suggests that female gonadal hormones transiently protect against T1D. In fact,

serum levels of the main estrogen, estradiol (E2), and serum estrogen activity are decreased in adolescents with T1D, suggesting that these individuals could have lost the protective effect of E2 against T1D (Martinez et al. 2016). In addition, E2 has been shown to protect rodent and human islet survival from multiple metabolic and pro-inflammatory injuries in vivo (Tiano and Mauvais-Jarvis 2012; Mauvais-Jarvis 2016). In a recent preclinical study, E2 treatment prevented insulitis and T1D in nonobese diabetic (NOD) mice by restoring the immunomodulatory functions of iNKT cells (Gourdy et al. 2016).

Gender Differences in Metabolic Syndrome and Obesity

The global prevalence of obesity is higher in women than in men in all continents (Kelly et al. 2008). Interestingly, in recent decades, the prevalence of abdominal obesity has increased more in women than in men in the USA (Ford et al. 2004). Today, the prevalence of visceral obesity associated with metabolic syndrome is two to ten times higher in women in many countries around the world (Al-Lawati et al. 2003; Gu et al. 2005; Gupta et al. 2004). Using the data from the National Health and Nutrition Examination Survey (NHANES), a recent study reported that the prevalence of metabolic syndrome in 2012 was significantly higher in women than in men (35.6% vs 30.3%, respectively) (Aguilar et al. 2015). A previous study using the data from the NHANES for the years 1999–2006 (Mozumdar and Liguori 2011) previously reported a significant increase in incidence of metabolic syndrome among US women compared to men. A large increase in the prevalence of metabolic syndrome among women compared with men is a noteworthy finding also described in Chinese adults (17.8% vs 9.8%, respectively) (Gu et al. 2005) and Indian populations (39.9% vs 22.9%, respectively) (Gupta et al. 2004). Importantly, when the individual abnormalities of metabolic syndrome were analyzed, the prevalence of abdominal obesity was dramatically higher in women than in men in the adult population of the USA (58.0% vs 41.1%, respectively) (Mozumdar and Liguori 2011), India (44% vs 25.6%, respectively) (Gupta et al. 2004), and especially China (13.9% vs 1.7%, respectively) (Gu et al. 2005) and Oman (44.3% vs 4.7%, respectively) (Al-Lawati et al. 2003). This female predisposition to central adiposity was observed across all age groups and in both urban and rural areas (Gu et al. 2005).

Conclusions

The role of sex and gender is a fundamental issue in medicine, and there are sex differences in glucose homeostasis, prediabetic syndromes, and type 1 and type 2 diabetes. Sex hormones play a role, at least partially, in these sex differences. Further characterization of these sex-specific differences in glucose homeostasis,

insulin secretion, and insulin action, as well as in the incidence and progression of diabetes, will provide a new source of factors that may help to prevent dysglycemia and inform clinical trials. This knowledge is essential to foster the development of relevant sex-based therapeutic avenues for diabetes. The determinants of these sex differences in the development of the global epidemic of abdominal obesity represent an untapped source of factors that can be harnessed to develop relevant gender-based therapeutic avenues for metabolic syndrome.

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Sex Differences in Body Composition

Miriam A. Bredella

Abstract Body composition differs between men and women. Men have more lean mass, and women have more fat mass than men. Men are more likely to accumulate adipose tissue around the trunk and abdomen, whereas women usually accumulate adipose tissue around the hips and thighs. Less is known about sex differences in ectopic fat depots. Advances in imaging allow the *noninvasive* assessment of abdominal and femorogluteal fat compartments, intramyocellular lipids, intrahepatic lipids, pericardial adipose tissue, and neck adipose tissue including brown adipose tissue and tongue adipose tissue. In this review, sex differences of regional adipose tissue, muscle mass, ectopic lipids, and brown adipose tissue and their effects on cardiometabolic risk will be discussed. In addition, novel imaging techniques to quantify these body composition compartments *noninvasively* will be described.

Introduction

There is a great interest in the potential physiologic differences between males and females that may affect the prevention, diagnosis, and treatment of obesity and diabetes. Although males and females are both susceptible to obesity, the incidence and health consequences differ between the sexes (Power and Schulkin 2008) as do the patterns of fat distribution (Lemieux et al. 1993). Men have more lean mass, and women have more body fat than men of the same BMI, and men are more likely to accumulate adipose tissue around the trunk and abdomen, whereas women usually accumulate adipose tissue around the hips and thighs. Less is known about sex differences in ectopic fat depots. Advances in imaging allow the *noninvasive* assessment of abdominal and femorogluteal fat compartments (Bredella et al. 2010, 2013; Machann et al. 2005), intramyocellular lipids (Bredella et al. 2010; Machann et al.

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2004, 2008; Torriani et al. 2005, 2007), intrahepatic lipids (Machann et al. 2008; Bredella et al. 2010; Dichtel et al. 2016), pericardial adipose tissue (Nichols et al. 2008; Wheeler et al. 2005), and neck adipose tissue including brown adipose tissue (Cypess et al. 2009; Saito et al. 2009; Torriani et al. 2014) and tongue adipose tissue (Godoy et al. 2016). In this review, sex differences of regional adipose tissue, muscle mass, ectopic lipids, and brown adipose tissue and their effects on cardiometabolic risk will be discussed. In addition, novel imaging techniques to quantify these body composition compartments *noninvasively* will be described.

Abdominal Adipose Tissue

Women have a higher percentage of body fat than men of the same BMI and a relatively higher proportion of body fat in the femorogluteal region, compared to more fat in the abdominal region in men (Lemieux et al. 1993). Studies have shown that the distribution of body fat has a greater impact on cardiometabolic risk than excess total adiposity. While the male pattern of abdominal fat accumulation is associated with increased cardiometabolic risk, the female pattern of fat distribution around the femorogluteal area might be relatively protective (Bjorntorp 1985, 1992; Goodpaster et al. 1997; Ohlson et al. 1985; Snijder et al. 2005). Within the abdomen, fat can accumulate in the subcutaneous area, subcutaneous adipose tissue (SAT) or in the deep abdomen, visceral adipose tissue (VAT). Multiple studies have demonstrated that VAT is associated with increased cardiometabolic risk (Bjorntorp 1992; Bjorntorp and Rosmond 1999; Tchernof and Despres 2013). Advances in imaging allow the detailed assessment of subcutaneous and visceral fat compartments (Bredella et al. 2009, 2010; Machann et al. 2005).

Dual-energy X-ray absorptiometry (DXA) is a technique that is routinely used for osteoporosis screening (Blake and Fogelman 2007) and is therefore readily available. It is associated with minimal radiation exposure and is relatively inexpensive. DXA is able to assess body composition, such as fat and lean mass, which has been shown to correlate closely with measures obtained by computed tomography (CT) or magnetic resonance imaging (MRI) in individuals of normal weight (Fuller et al. 1999; Glickman et al. 1985; Levine et al. 1985). However, we have demonstrated that in the extremes of the weight spectrum—obesity and anorexia nervosa—DXA underestimates trunk fat, a surrogate for VAT, as well as thigh fat, and this error increases with increasing weight (Bredella et al. 2010a). Advances in DXA technology now allow the assessment of VAT and abdominal SAT, using an algorithm that is based on changes in gray-scale values and special modeling techniques (Micklesfield et al. 2012). We performed a study testing this new technique in women across the weight spectrum. DXA was less accurate in quantifying VAT and SAT when used in subjects with extremely low weight and more accurate in overweight and obese women (Bredella et al. 2013).

CT and MRI are considered the gold standard for detailed assessment of body composition, including abdominal fat compartments (Abate et al. 1994; Rossner

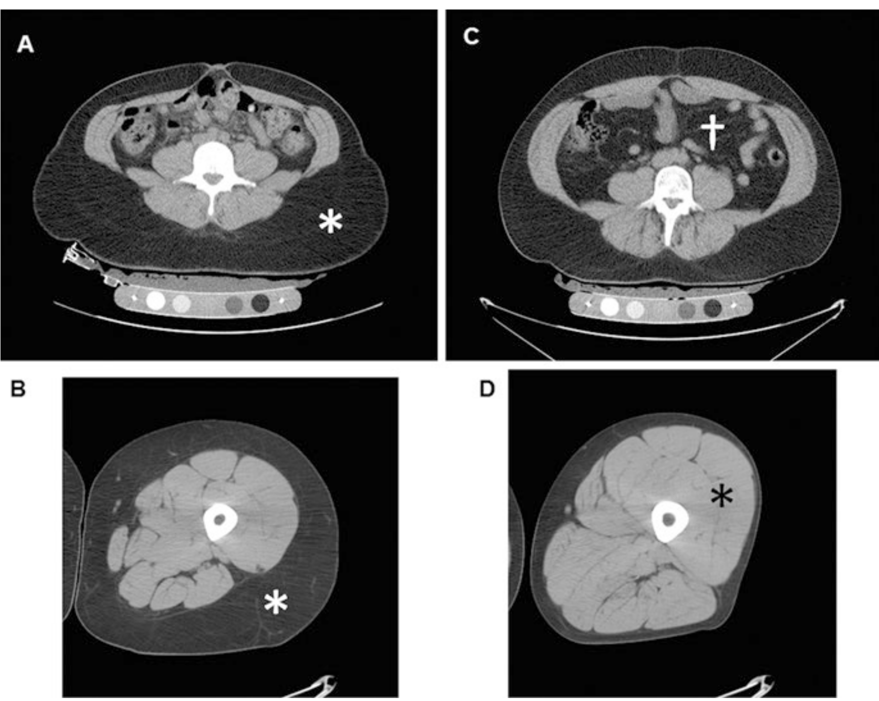

Fig. 1 Body composition of the abdomen and thigh assessed by CT in a 39-year-old woman (**a**, **b**) and 37-year-old man (**c**, **d**) with obesity (BMI, 33 kg/m^2) who were otherwise healthy. The woman has more subcutaneous adipose tissue in the abdomen and thigh (*white asterisks*), while the man has more visceral adipose tissue (*cross*) and muscle mass (*black asterisk*). The woman had a better metabolic profile compared to the man (serum LDL cholesterol 57 mg/dL vs 147 mg/dL; HDL cholesterol 68 mg/dL vs 32 mg/dL; triglycerides 45 mg/dL vs 159 mg/dL; insulin 4.9 μU/mL vs 7.0 μU/mL; HOMA-IR 0.98 vs 7.00)

et al. 1990) (Fig. 1). Studies using CT have shown that men have up to twice as much VAT compared to women (Kvist et al. 1988). Machann et al. (2005) used whole body MRI to assess sex differences in body composition in 150 healthy volunteers (90 women, 60 men) across a wide age range (19–69 years) who were at risk for developing type 2 diabetes mellitus (T2DM). At similar age and BMI, women had significantly higher %total adipose tissue, lower %VAT, and higher %SAT. Women had more fat in the lower extremities compared to men (Machann et al. 2005). In a study examining premenopausal women, Lemieux et al. (1994) found that although women had more total body fat than men, they had lower VAT assessed by CT, and this was associated with a better metabolic risk profile (Lemieux et al. 1994). Fox et al. (2007) examined 3,001 subjects from the Framingham Heart Cohort (1,452 women, 1,549 men, mean age 51 years) who underwent CT of the abdomen. Among men and women, abdominal SAT and VAT were significantly associated with blood pressure, fasting plasma glucose, triglycerides, high-density lipoprotein cholesterol, and increased odds of hypertension, impaired fasting

glucose, T2DM, and the metabolic syndrome, with stronger correlations between VAT and most cardiometabolic risk factors. In women, VAT was more strongly associated with cardiometabolic risk factors compared to men with larger effect sizes. There were significant sex interactions with increasing volumes of SAT and VAT being consistently and more strongly associated with more adverse cardiometabolic risk factors in women than in men (Fox et al. 2007). These studies suggest that although women have more VAT, it confers greater cardiometabolic risk compared to men.

Muscle Mass

Skeletal muscle plays a critical role in regulating glucose homeostasis being responsible for the majority of basal and insulin-stimulated glucose uptake. Impaired insulin action at the level of skeletal muscle is central to the clinical manifestations of insulin resistance and T2DM (DeFronzo and Tripathy 2009). Furthermore, sarcopenia, the age-related decline in skeletal muscle mass, quality, and function, may represent an underappreciated contribution to increased risk of T2DM (Larsen et al. 2016; Park et al. 2009), and patients with T2DM show a greater decline in leg muscle mass, strength, and function compared with healthy controls (Leenders et al. 2013). Lean or fat-free mass can be assessed using DXA (Fuller et al. 1999; Levine et al. 1985); however, we have shown that DXA overestimated thigh muscle mass and this error increases with increasing weight (Bredella et al. 2010). CT and MRI are considered the gold standard for quantifications of skeletal muscle mass (Beneke et al. 1991; Borkan et al. 1983; Engstrom et al. 1991; Mitsiopoulos et al. 1985) (Fig. 1).

Sex differences in muscle mass become apparent during puberty, with boys having larger muscles than girls (Kanehisa et al. 1994; Tanner et al. 1981). Gallagher et al. (1985) assessed sex differences in skeletal muscle mass by DXA in 148 women and 136 men. Men had higher muscle mass than women, and this difference was greater in the upper compared to the lower body. With aging a larger magnitude decrease of muscle was observed in men compared to women (Gallagher et al. 1985). Janssen et al. (1985) performed whole body MRI in 468 men and women from 18 to 88 years. Men had significantly higher skeletal muscle mass than women in both absolute terms and relative terms relative to body mass (38% vs 31%). The sex differences were greater in the upper (40%) than lower (33%) body. Aging was associated with loss of muscle mass, independent of sex, with greater loss of muscle in the lower body (Janssen et al. 1985).

A study examining 1,433 subjects (658 men and 775 women), 60 years or older, who participated in the Fifth Korea National Health and Nutritional Examination Survey 2010, found a higher prevalence of sarcopenic obesity in women compared to men (31.3% vs 19.6%) (Oh et al. 2015). Men and women with sarcopenic obesity had higher fasting insulin, HOMA-IR, and serum triglycerides (Oh et al. 2015). Ochi et al. (2010) examined 496 healthy middle-aged to elderly men and women

with CT of the thigh to assess muscle mass corrected for body weight and carotid ultrasound to assess carotid intima-media thickness (IMT) and brachial-ankle pulse wave velocity (baPWV). High relative muscle area was inversely associated with carotid IMT and baPWV in men but not in women (Ochi et al. 2010). In a study from the National Health and Nutrition Examination Survey III examining 4,652 elderly men and women (mean age 70.6 ± 0.2 years), the prevalence of sarcopenic obesity was lower in women compared to men (18.1% vs 42.9%). However, in women but not in men, sarcopenic obesity was associated with increased mortality, and women with sarcopenia had higher mortality risk compared to men, regardless of the presence of obesity (Batsis et al. 2014). The reported sex differences in muscle mass with higher morbidity and mortality in women emphasize the importance of maintaining muscle mass with aging, especially in women who are at greater risk for developing sarcopenic obesity due to higher fat and lower muscle mass.

Intramyocellular Lipids

Within skeletal muscle, lipids occur between muscle fibers, called extramyocellular lipids (EMCL), and within muscle cells, called intramyocellular lipids (IMCL). IMCL have been shown to play a critical role in the pathogenesis of insulin resistance (Shulman 2000, 2014). IMCL can be quantified using proton MR spectroscopy (1H-MRS), and several studies have shown higher IMCL as determined by 1H-MRS in states of insulin resistance, T2DM, and disorders of lipid metabolism. The ability to distinguish IMCL from EMCL is based on their difference in geometric arrangements within muscle which is associated with different bulk magnetic susceptibility, which leads to a spectroscopic frequency separation between the two pools (Boesch 2007; Boesch and Kreis 2000; Boesch et al. 1997; Goodpaster et al. 2000) (Fig. 2).

Machann et al. (2005) performed 1H-MRS for quantification of IMCL within soleus muscle and whole body MRI for quantification of adipose tissue depots in 150 healthy volunteers. Men had significantly higher IMCL compared to women, despite similar BMI and age. In women but not in men, IMCL were positively associated with total adipose tissue, VAT, and abdominal SAT and inversely associated with lower extremity adipose tissue (Machann et al. 2005).

However, not only the quantity but also the composition of the IMCL pool plays an important role for cardiometabolic risk. Fatty acid composition in patients with insulin resistance and the metabolic syndrome is characterized by high levels of saturated fatty acids and low levels of polyunsaturated fatty acids (Vessby et al. 2002; Warensjo et al. 2005). Therefore, techniques that could assess lipid components and the degree of unsaturation in vivo may provide important information on cardiometabolic risk. The composition of muscle lipids can be quantified using localized 2D correlation spectroscopy (L-COSY) (Thomas et al. 2005; Velan et al. 2007, b). To study sex difference in skeletal muscle composition, Velan et al. (2008) examined eight healthy normal-weight premenopausal women and eight

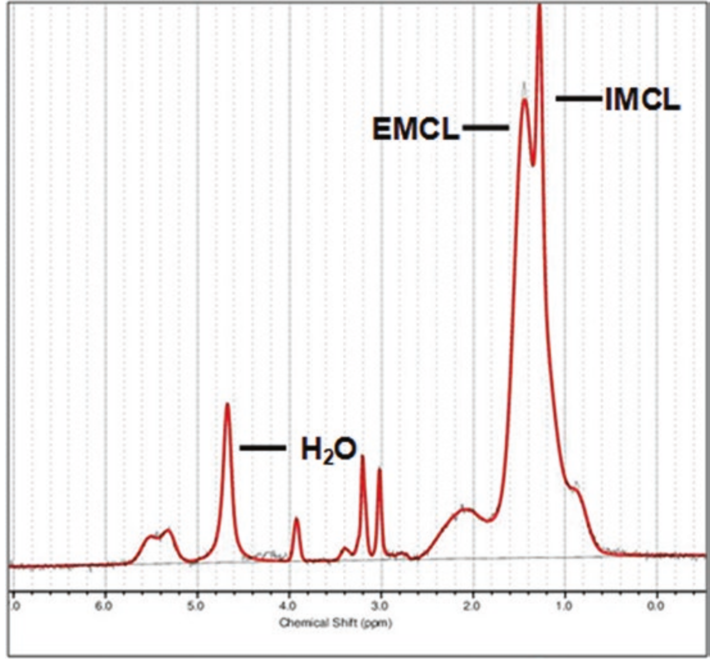

Fig. 2 Proton MR spectroscopy (1H-MRS) of soleus muscle for assessment of intramyocellular lipid content. 1H-MRS spectrum shows IMCL and EMCL resonances. *IMCL* (intramyocellular lipids) methylene protons at 1.3 ppm; *EMCL* (extramyocellular lipids) methylene protons at 1.5 ppm; H_2O residual water signal

normal-weight age-matched men using L-COSY of soleus muscle to determine the amount of saturated and unsaturated fatty acids. Women had a lower degree of unsaturation within IMCL and EMCL compared to men (Velan et al. 2008). These findings might contribute to increased cardiometabolic risk in women.

Intrahepatic Lipids

A complication of obesity is nonalcoholic fatty liver disease (NAFLD), fatty infiltration of the liver in the absence of alcohol use. NAFLD encompasses a spectrum that ranges from simple steatosis to nonalcoholic steatohepatitis (NASH). NASH is associated with the development of fibrosis, cirrhosis, and hepatocellular carcinoma and is expected to become the most common indication for liver transplantation by 2020 (Charlton 2008; Williams et al. 2011; Wree et al. 2013). While sex differences in gastrointestinal diseases are increasingly recognized, there are few data on sex differences in NAFLD. Advances in MRI technology allow the accurate quantification of hepatic lipid content *noninvasively*, and we have developed pulse sequences

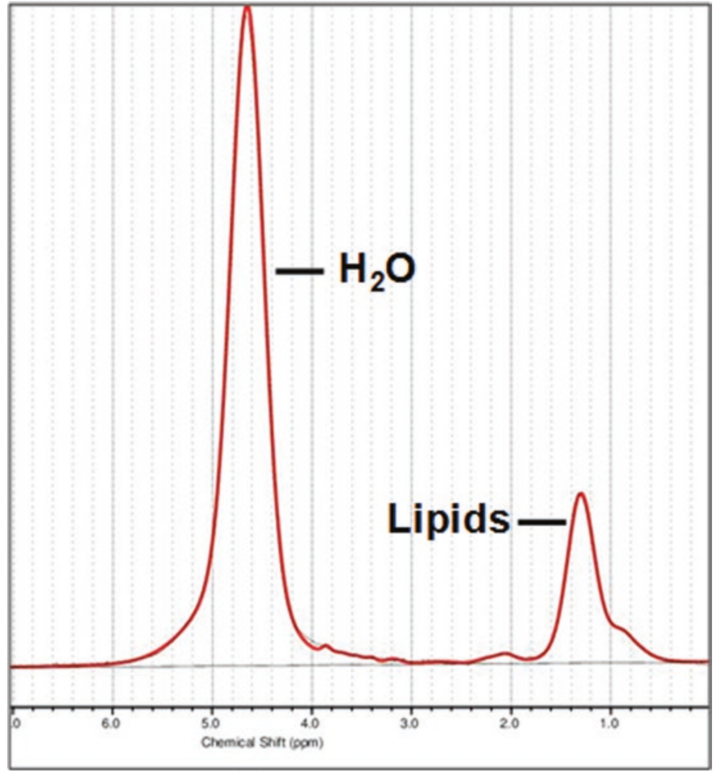

Fig. 3 Breath-hold single voxel proton MR spectroscopy (1H-MRS) of the right hepatic lobe for assessment of intrahepatic lipids. 1H-MRS spectrum shows lipids (1.3 ppm) and unsuppressed water (4.7 ppm) resonances

for proton MR spectroscopy (1H-MRS) that allow assessment of hepatic lipids in a single breath-hold (Bredella et al. 2010; Dichtel et al. 2016) (Fig. 3).

Machann et al. (2005) performed 1H-MRS for quantification of hepatic lipid content in 150 healthy volunteers across a wide age range (19–69 years) who were at risk for developing T2DM. There was no significant difference in hepatic lipid content between men and women at similar BMI. However, in women, intrahepatic lipids were positively associated with age, VAT, and abdominal SAT, while in men intrahepatic lipids only correlated with VAT (Machann et al. 2005). Westerbacka et al. (2004) assessed intrahepatic lipids in 66 men and 66 women using 1H-MRS. There was no significant difference in intrahepatic lipid content between men and women. Intrahepatic lipids were positively associated with measures of serum insulin, independent of age, BMI, and intra-abdominal and subcutaneous fat with no sex differences observed (Westerbacka et al. 2004).

Fatty infiltration of the liver can also be assessed using computed tomography (CT) by measuring liver attenuation in Hounsfield units (HU) which correlates with hepatic lipid content assessed by 1H-MRS (Bredella et al. 2010). North et al. (2012)

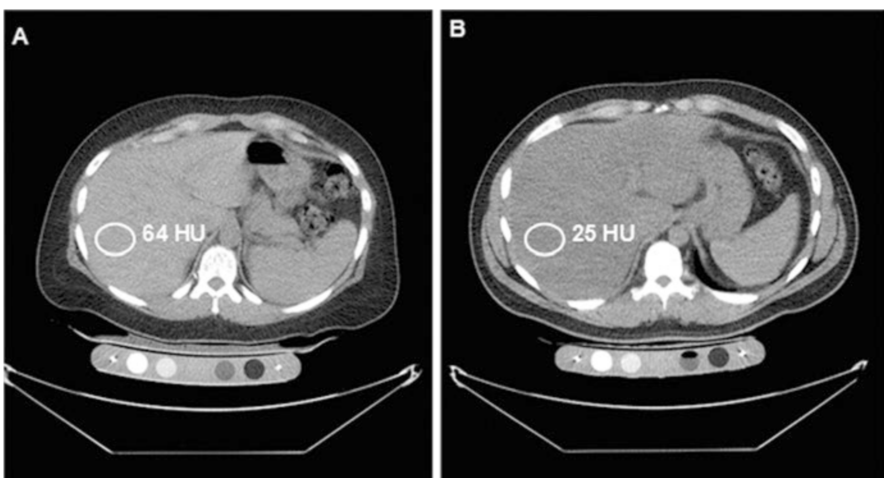

Fig. 4 Non-contrast CT of the liver for assessment of intrahepatic lipid content in the right hepatic lobe in a 39-year-old woman with obesity (BMI, 31 kg/m^2) (**a**) and a 37-year-old man with the same BMI (**b**). CT attenuation was lower in the man compared to the woman, consistent with fatty infiltration. Images are presented by using the same window and level

examined liver attenuation by CT as a marker of fatty infiltration in 1,242 men and 1,477 women who participated in the NHLBI Family Heart Study. Men had significantly lower liver attenuation, consistent with fatty infiltration, compared to women (Fig. 4). In both sexes fatty infiltration was associated with VAT, serum triglycerides, and measures of insulin resistance; however, the association of fatty liver infiltration with VAT and HOMA-IR had a stronger magnitude of effect in women. Fatty infiltration of the liver was associated with alcohol consumption and BMI only in men, while no such associations were observed in women (North et al. 2012).

Lonardo et al. (Lonardo and Trande 2000) studied men and women with and without fatty liver, assessed by ultrasound, to determine sex differences in predictors of fatty infiltration. BMI was an independent predictor of fatty infiltration in either sex. Measures of impaired glucose tolerance were predictors of liver fatty infiltration in women but not in men, while elevations in serum triglycerides were predictors of fatty infiltration in men but not in women. Moreover, central adiposity was a predictor of fatty liver in women but not in men. These findings suggest that there are sex-specific pathways for fatty infiltration of the liver (Lonardo and Trande 2000). An understanding of the underlying mechanisms responsible for these sex differences in intrahepatic fat accumulation may lead to improved therapeutic strategies for the prevention and treatment of NAFLD and NASH.

Pericardial Adipose Tissue

Recent studies have identified pericardial adipose tissue (PAT), the fat around the heart, as a novel risk factor for coronary artery disease (CAD), atrial fibrillation, carotid intima-media thickness, and carotid stiffness (Brinkley et al. 2011; Lee et al. 2016; Rosito et al. 2008; Schlett et al. 2012; Soliman et al. 2010). PAT is a unique fat depot given its anatomic proximity to the myocardium, coronary arteries, and atrial conduction system (Friedman et al. 2014). In addition to storing lipids, PAT also secretes adipokines and inflammatory cytokines (Baker et al. 2006; Cheng et al. 2008) which, given the proximity to the coronary arteries and shared blood supply with the coronary artery wall, may lead to acceleration of atherosclerosis (Fantuzzi and Mazzone 2007; Yudkin et al. 2005). PAT can be accurately quantified using CT (Ding et al. 2008).

The incidence of cardiovascular disease (CVD) differs by sex, and although CVD and heart disease are more prevalent in older men than women (Arnold et al. 2005), women suffering from CVD have a higher mortality compared to men. Differences in PAT volume between men and women may account for some of the observed sex differences in manifestations of CVD.

In a study of 1,155 participants (522 men, 633 women, mean age 63 years) of the Framingham Heart Study, who were free of CVD, Rosito et al. (2008) found significantly higher PAT volume by CT in men compared to women, despite similar age and BMI (Fig. 5). However, PAT was positively associated with systolic and diastolic blood pressure and fasting glucose in women but not in men. In addition, there were significant sex interactions between PAT and serum triglycerides, HDL cholesterol, the presence of hypertension, impaired fasting glucose, T2DM, and the metabolic syndrome, with larger effect sizes in women compared to men, which

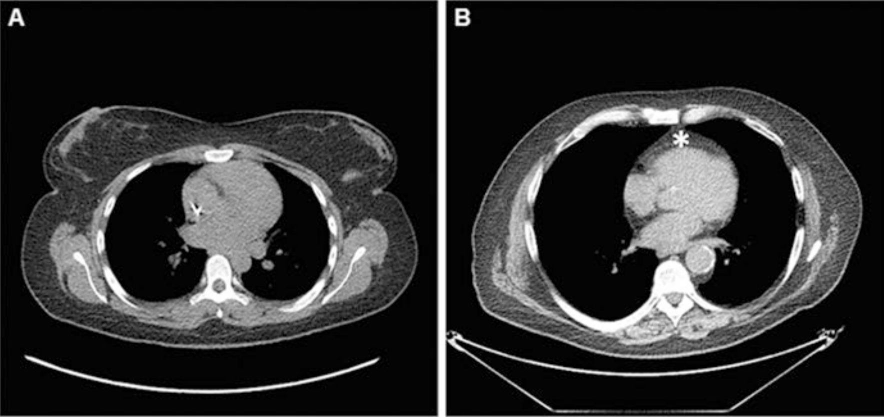

Fig. 5 Non-contrast CT of the chest for assessment of pericardial adipose tissue in a 33-year-old woman with obesity (BMI 31 kg/m^2) (**a**) and an age- and BMI-matched man (**b**). The man had higher pericardial adipose tissue area (*asterisk*) compared to the woman despite the same BMI

were independent of BMI. These data suggest that PAT is associated with more adverse risk factor profiles in women than men (Rosito et al. 2008). In another study examining 1946 participants (1,067 men, 879 women, mean age 44.0 ± 6.4 years) of the Framingham Heart Study, Friedman et al. (2014) examined the relationship between PAT and atrial conduction as measured by P wave indices. In men and women, PAT was significantly associated with P duration in analyses after adjusting for visceral or intrathoracic fat. Among women, PAT was associated with P wave area after adjustment for intrathoracic and visceral fat and with P wave terminal force after adjustment for visceral fat. In multivariable models adjusting for BMI, pericardial fat remained associated with P wave duration and P wave terminal force in women and P wave amplitude and P wave terminal force in men (Friedman et al. 2014). Brinkley et al. (2011) examined 5,770 participants (2,719 men, 3,051 women, mean age 62.1 ± 10.2 years) from the Multi-Ethnic Study of Atherosclerosis (MESA) cohort who underwent CT for PAT and ultrasound for assessment of carotid stiffness. In men and women, PAT was positively associated with parameters of arterial stiffness, independent height, demographics, behavioral factors, blood pressure, metabolic factors, medication use, CRP, BMI, and waist circumference. However, the effect size was larger in women. In an exploratory analysis examining whether the relationship between PAT and carotid stiffness was altered by total or abdominal obesity, the association between PAT and carotid stiffness was twofold stronger in women who were nonobese, while in men there was no difference in the associations among the obesity subgroups. These findings suggest that excess PAT is more detrimental when the overall amount of body fat is low or normal and these changes are more pronounced in women (Brinkley et al. 2011).

Neck Adipose Tissue

Fat accumulation in the neck—usually estimated by neck circumference—has been found to be a strong marker of metabolic disease, independent of BMI or waist circumference (Preis et al. 2010). In contrast to the well-defined abdominal fat compartments, VAT and SAT, less is known about discrete fat compartments in the neck and potential sex differences in neck adipose tissue. In a retrospective study, we determined specific neck adipose tissue compartments by CT in 151 women and 152 men across a wide range of age (mean age 55 ± 17 years; range 18–91 years) and BMI (28 ± 6 kg/m^2, range 16–47 kg/m^2) (Torriani et al. 2014). There were 101 subjects in each BMI category (normal weight, overweight, obese), and there were no age differences between sexes in each category. Neck adipose tissue was assessed by CT. Measures of cardiovascular (CV) risk and the presence or absence of the metabolic syndrome were determined. We identified three discrete fat compartments in the neck: subcutaneous/superficial neck adipose tissue (NATsc), located between the skin and deep cervical fat and two intermuscular fat compartments; posterior cervical neck adipose tissue (NATpost), located between the sternocleidomastoid, scalene, and trapezius muscle; and perivertebral neck adipose tissue

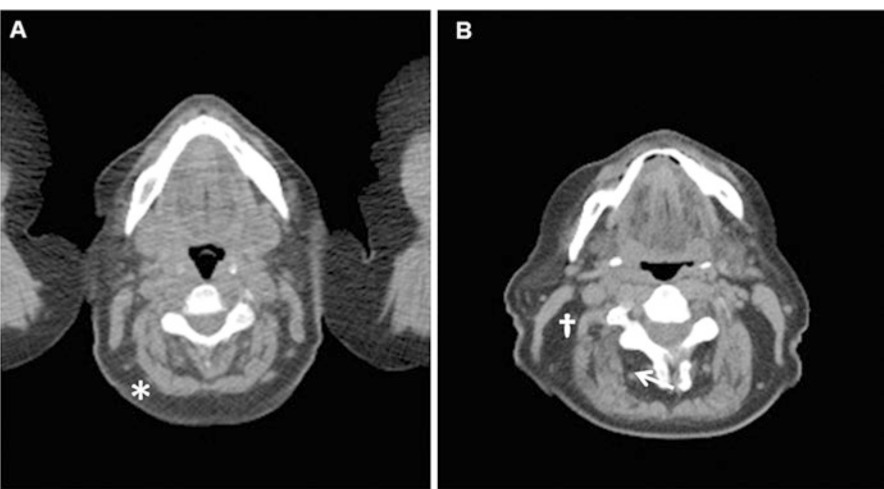

Fig. 6 CT of the neck for assessment of neck adipose tissue (NAT) in a 56-year-old woman and a 56-year-old man with the same BMI (35 kg/m^2). The woman had more subcutaneous NAT (*asterisk*), and the man had more intermuscular adipose tissue [posterior NAT (*cross*) and perivertebral NAT (*arrow*)]. The man but not the woman had serum measures consistent with the metabolic syndrome

(NATperivert), fat interspersed between muscles surrounding the cervical vertebral body. There was no difference in neck adipose tissue in lean men and women. However, overweight and obese women had significantly higher subcutaneous neck fat compared to men, despite being age and BMI matched. Conversely, overweight and obese men had significantly higher intermuscular neck adipose tissue depots (NATpost and NATperivert) compared to obese women (Fig. 6). NATpost and NATsc were associated with measures of cardiometabolic risk, which was stronger in women compared to men. In both sexes, NATsc was associated with the metabolic syndrome even after adjusting for BMI. In both sexes NATpost had the highest prevalence ratio for the metabolic syndrome, but this persisted only in women after additional adjustment for BMI. Also NATperivert only remained a significant predictor of the metabolic syndrome in women after adjustment for BMI (Torriani et al. 2014). Similar to the abdomen, women have more subcutaneous fat in the neck and men more intermuscular fat. Accumulation of neck adipose tissue is associated with higher risk of the metabolic syndrome in women compared to men.

Brown Adipose Tissue

Advances in positron emission tomography (PET) combined with computed tomography (CT) technology allow the *noninvasive* assessment of brown adipose tissue (BAT) in humans. Because of its metabolic activity, BAT can be visualized by [18F]

fluorodeoxyglucose (FDG) uptake using FDG-PET/CT (Chen et al. 2016; Sampath et al. 2016).

BAT is the major site for nonshivering thermogenesis during cold exposure, and BAT is believed to contribute to the control of body temperature, energy expenditure, and adiposity (Cannon and Nedergaard 2004; Lowell and Spiegelman 2000). Nonshivering thermogenesis is mediated by the expression of uncoupling protein 1 (UCP1) which is expressed exclusively in the mitochondrial membrane of BAT (Cannon and Nedergaard 2004).

BAT is regulated by environmental, nutritional, endocrine and neural factors (Thuzar and Ho 2016) and is stimulated during cold exposure. Therefore, personalized cooling protocols are recommended in prospective and longitudinal studies using FDG-PET/CT for the quantification of BAT (Chen et al. 2016). BAT is also regulated by sex steroids (Lopez and Tena-Sempere 2016). Ovariectomy in rats causes atrophy of BAT depots, an effect reversed by estrogen replacement, suggesting that estrogen stimulates BAT mass (Pedersen et al. 2001; Rodriguez-Cuenca et al. 2007).

Several studies in humans performed under thermoneutral conditions have demonstrated sex differences of BAT with significantly higher prevalence and volumes of BAT in women (Cypess et al. 2009; Au-Yong et al. 2009; Ouellet et al. 2011; Zhang et al. 2014). Cypess et al. (2009) analyzed 3,640 consecutive clinical FDG-PET/CTs performed under thermoneutral conditions for various diagnostic reasons and found a higher prevalence of BAT-positive scans in women (7.5%) compared to men (3.1%). Moreover, women had significantly higher BAT mass and activity compared to men (Cypess et al. 2009). Similar results were found in a study by Au-Yong et al. of 3,614 consecutive patients who underwent FDG-PET/CTs under thermoneutral conditions (Au-Yong et al. 2009). Prevalence of BAT-positive scans in women was 7.2% and 2.8% in men (Au-Yong et al. 2009). Ouellet et al. (2011) also found a higher prevalence of BAT in women compared to men in 6,652 clinical FDG-PET/CTs performed under thermoneutral conditions. However, the difference diminished with age, and sex was not an independent determinant of BAT prevalence after adjusting for covariates, such as age, BMI, lean body weight, diabetes, or outdoor temperature. However, BAT mass and activity remained significantly higher in women compared to men, even after controlling for covariates (Ouellet et al. 2011). In the largest study to date, Zhang et al. (2014) examined 31,088 FDG-PET/CTs performed under thermoneutral conditions for routine medical checkup or cancer surveillance and found a significantly higher prevalence of BAT in women (2.36%) compared to men (0.7%). This sex difference was higher in the medical checkup group (female 3.16% vs male 0.77%) and lower in the cancer surveillance group (female 1.59% vs male 0.61%) (Zhang et al. 2014). However, prospective studies employing standardized cooling protocols did not confirm sex differences in BAT (Saito et al. 2009; Yoneshiro et al. 2011). Saito et al. (2009) performed FDG-PET/CTs in 56 healthy volunteers after a 2-h cooling protocol and found no sex difference in BAT prevalence (Saito et al. 2009). In a larger prospective study, Yoneshiro et al. (2011) performed 162 FDG-PET/CTs after a 2-h cooling protocol and found no significant sex difference in the prevalence of BAT (Yoneshiro et al.

2011). These studies suggest that sex differences in BAT observed in the retrospective studies might be due to a difference in sensitivity to environmental temperature (Thuzar and Ho 2016).

Tongue Adipose Tissue

The tongue plays an important role in upper airway patency, and increased tongue size and accumulation of adipose tissue within the tongue have been associated with a higher risk for obstructive sleep apnea (OSA) (Kim et al. 2014; Schwab et al. 1995). Men have a higher prevalence of OSA than women, and sex differences in tongue fat accumulation might represent a mechanism for sex differences in OSA. Godoy et al. (Godoy et al. 2016) assessed CT attenuation in HU as a measure of tongue fatty infiltration and measures of airway patency in 104 women and 102 men across the weight spectrum (range 16–47 kg/m^2, mean 28 ± 6 kg/m^2). Fatty infiltration of the tongue was higher in men compared to women (Fig. 7) and was associated with measures of decreased upper airway patency independent of age and BMI, suggesting higher upper airway, soft tissue burden, and narrower airways in males versus females (Godoy et al. 2016).

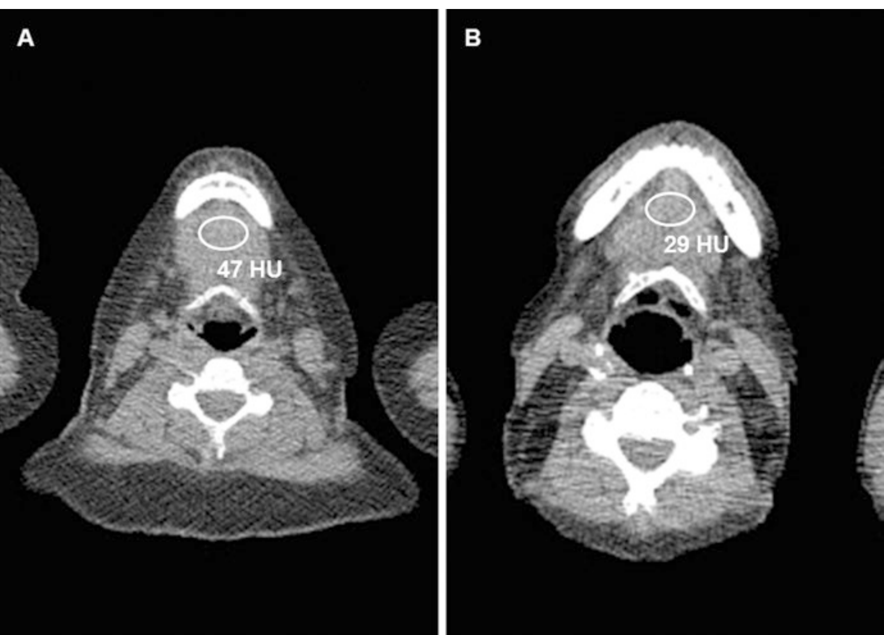

Fig. 7 Non-contrast CT of the neck for assessment of tongue adipose tissue in a 63-year-old woman (BMI, 34 kg/m^2) (**a**) and 63-year-old man with similar BMI (BMI, 35 kg/m^2) (**b**). The man had lower CT attenuation of the tongue compared to the woman, consistent with fatty infiltration. Images are presented by using the same window and level

Summary

Body composition differs between men and women. Men have more VAT and higher inter- and intramuscular adipose tissue and pericardial and tongue adipose tissue, which is associated with increased cardiometabolic risk, despite higher muscle and lean mass. Women on the other hand have more femoral SAT and neck SAT and potentially more BAT. This female pattern of fat distribution is associated with improved cardiometabolic risk at similar BMI. However, ectopic fat deposition within the abdomen, muscle, pericardium, and neck is more strongly associated with adverse cardiometabolic risk in women compared to men.

Disclosure The author has nothing to disclose.

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Cellular Mechanisms Driving Sex Differences in Adipose Tissue Biology and Body Shape in Humans and Mouse Models

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Abstract Sex differences in adipose tissue distribution and the metabolic, endocrine, and immune functions of different anatomical fat depots have been described, but they are incompletely documented in the literature. It is becoming increasingly clear that adipose depots serve distinct functions in males and females and have specific physiological roles. However, the mechanisms that regulate the size and function of specific adipose tissues in men and women remain poorly understood. New insights from mouse models have advanced our understanding of depot differences in adipose growth and remodeling via the proliferation and differentiation of adipose progenitors that can expand adipocyte number in the tissue or simply replace dysfunctional older and larger adipocytes. A limited ability of a depot to expand or remodel can lead to excessive adipocyte hypertrophy, which is often correlated with metabolic dysfunction. However, the relationship of adipocyte size and function varies by depot and sex. For example, femoral adipose tissues of premenopausal women appear to have a greater capacity for adipose expansion via hyperplasia and hypertrophy; although larger, these gluteal-femoral adipocytes remain insulin sensitive. The microenvironment of specific depots, including the composition of the extracellular matrix and cellular composition, as well as cell-autonomous genetic differences, influences sex- and depot-dependent metabolic and growth properties. Although there are some species differences, studies of the molecular and physiological determinants of sex differences in adipocyte growth and function in humans and rodents are both needed for understanding sex differences in health and disease.

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Introduction

Women are more insulin sensitive than men, and before menopause they are at lower risk for metabolic disease. Sex differences in metabolic profiles are correlated with sex-linked variations in the distribution of body fat. Women are likely to have a "pear-shaped" or gynoid fat distribution (usually defined as waist-to-hip ratios <0.8), whereas men tend to deposit more fat within the trunk (Karastergiou et al. 2012). Although in both sexes most body fat is stored in subcutaneous depots (~80–90% of total), men store more fat in visceral depots, i.e., depots located within the intra-abdominal cavity that are physically associated with the digestive tract. Epidemiological and clinical evidence indicate that a gynoid fat distribution, independent of total body fat or its surrogate, body mass index (BMI), is associated with lower risk for metabolic abnormalities, cardiovascular diseases, and total mortality (Canoy et al. 2007; Carey et al. 1997; Chan et al. 1994; Pischon et al. 2008). Thus, substantial research effort has been directed at understanding the mechanisms that modulate sex-dependent variations in depot size and metabolic and endocrine function.

Body shape is largely determined by the mass of adipose depots, which in turn depend on both the size and number of individual adipocytes. Mature adipocytes of every species so far examined seem to have a maximal size, storing up to 2–3 μg lipid per fat cell in the extreme. Adipocyte function varies as a function of its size in cross-sectional studies and when adipocytes of different size are isolated from a single depot. However, there are also depot-dependent effects on adipocyte function that are independent of their size. For example, gluteal-femoral adipocytes of premenopausal women are larger yet are more insulin sensitive in vitro (Johnson et al. 2001) and more efficient at storing fat in vivo (in lower-body obese women (Santosa et al. 2008)) compared to smaller abdominal adipocytes. In general though, when adipocytes within a depot become more hypertrophied, they secrete fewer "good adipokines" such as adiponectin and release more fatty acids (FA) than can be burned, resulting in ectopic fat deposition that disrupts the function of the liver, muscle, and other tissues. Thus, the ability of adipose tissues to expand by hyperplasia may serve a protective function, i.e., provide a "safe storage" for excess energy in the face of overnutrition (Virtue and Vidal-Puig 2010). However, "safe storage" can also be provided by large adipocytes that can remain functionally competent. Thus, it is important to understand the cellular and molecular mechanisms that control both hypertrophic and hyperplastic growth of adipose tissues in a sex- and depot-dependent manner.

In the context of obesity, increased adipocyte size is tightly correlated with cellular and systemic metabolic dysfunction, which leads to chronic tissue and systemic inflammation. These strong associations provide motivation for studies that address the cellular mechanisms regulating adipose expansion, including sex differences in fat storage and mobilization, adipose tissue growth and remodeling, and their physiological determinants such as innervation. We highlight data from human studies when it is available, as well as mouse studies that have provided novel mechanistic insight into sex differences.

Sex Differences in Fat Distribution Are Genetically Regulated

Genome-wide association studies establish a clear genetic component to variations in fat distribution and have identified loci that influence fat distribution in men and women, with stronger effects in the latter (Shungin et al. 2015; Winkler et al. 2015). The contribution of each to phenotypic variations is small, and to date few "hits" have been followed up with functional studies of depot and sex differences. Studies of the genetics of fat distribution have been reviewed recently (Pulit et al. 2017), so we will focus on recent advances in understanding of sex differences in adipose biology at the tissue and cell levels. Additionally, but largely beyond the scope of this review, epigenetic programming by nutrition and hormones can also affect fat distribution toward a more female or male pattern (Keller et al. 2017; MacArtney-Coxson et al. 2017; Fall et al. 2017).

Few Studies Directly Compare Sex Differences in the Adipose Tissue Transcriptome

A first approach to understanding sex differences in adipose tissue has been to catalog variations in gene expression in the major fat depots of human and animal models, most importantly in mice because of the relative ease of testing mechanisms in vivo. Nevertheless there remain significant gaps in these descriptions due to practical details in sample collection from multiple depots from sufficient numbers of male and female subjects that are well characterized with regard to variables known to affect fat distribution, including total and regional body fat, age, and menopausal status. Additionally, adipose tissue is composed of multiple cell types, but few studies have assessed which cell types within an adipose depot drive the differences in gene expression observed.

The most extensive data on the transcriptome of abdominal sc and omental (a visceral depot) adipose tissues from men and women come from the GTEx (Genotype-Tissue Expression) consortium, an ongoing collection of multiple "non-diseased" tissues from recently deceased human donors (Mele et al. 2015). Most of the sex-biased genes in adipose tissue were globally sex biased in all tissues. Genes overexpressed in men were predominantly located in the Y chromosome and in women in the X chromosome. MMP3 was the topmost sex-different gene (higher in males) among autosomal, protein-coding genes. The adipose-specific sex-different genes were the transcriptional regulators SALL1 and AFF2, the immune-related genes CPAMD8 and CYTL1, IP6K3 of the inositol phosphokinase family, the unknown function genes FAM151A and ZNF534, as well as the long noncoding RNA LINC00230A (Mele et al. 2015). As the analyses expand to the target population (from $n = 175$ to approx. 1,000), it is likely that additional sex-biased genes will be identified. Although these RNAseq data are valuable for hypothesis generating and detecting major and consistent sex differences, it is important to note that

limitations of this dataset include lack of phenotypic characterization beyond BMI and age to assess the influence of body fat and its distribution.

Several relatively small clinical studies investigated the transcriptome (mRNA and miRNA) of lower- versus upper-body subcutaneous adipose tissue of men and women highlighting a number of pathways, including development and transcription factor DNA binding (Divoux et al. 2017; Karastergiou et al. 2013; Pinnick et al. 2014; Rantalainen et al. 2011). However, the sex differences have not been analyzed in detail, and studies to date are likely not powered for this comparison. We found that 66 genes (23%) were differentially expressed between abdominal and gluteal only in men, 159 (56%) were depot different only in women, and 59 (21%) were different in both sexes (Karastergiou et al. 2013). Pinnick et al. noted that the obesity-associated changes in the expression of immune and metabolic genes are more pronounced in the abdominal versus the gluteal depot and in men more than in women (Pinnick et al. 2014).

Sex differences in the adipose tissue transcriptome of mice have been compared by microarray studies that found that pathways of inflammation were lower in females and insulin signaling was higher, mostly in the gonadal depot (Grove et al. 2010). However, this study was limited to animals on a high-fat (HF) diet (HFD) so that inflammation predominated as the major difference. A new study from Reue and colleagues detected sex-biased expression of miRNAs in male vs female gonadal fat depots (epididymal vs parametrial) including some implicated in adipogenesis (Link et al. 2017). Systemic studies of mRNAs, miRNAs and lncRNAs in multiple depots of male and female mice fed defined diets across the lifespan will likely soon shed new light on mechanisms driving sex-linked phenotypic differences.

Recent Advances in Understanding Sex Differences in Fat Accumulation

Sex Differences in Regional Adipocyte Metabolism

The traditional function of adipose tissue is to store excess FA as triglycerides in the fed state and release it in proportion to the metabolic needs of other tissues in response to fasting or in exercise. The former is achieved through hydrolysis of FA from circulating chylomicron triglycerides (TG) by lipoprotein lipase (LPL) and their uptake, esterification, and storage within the lipid droplet of the adipocyte. Fat mobilization is mediated through the actions of rate-determining lipases (adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL)) on adipocyte TG. Importantly, differences in the metabolic capacity of adipocytes from upper- vs lower-body adipocytes also influence regional rates of fat storage and mobilization and hence fat distribution.

Recent studies have provided some new insights into how FA are directed toward storage in upper- vs lower-body fat depots of men and women. As previously reviewed, in the fed state, minor sex differences are noted as meal-derived FA are mostly stored in upper-body fat in both men and women (Karastergiou et al. 2012; Karpe and Pinnick 2015; Santosa and Jensen 2015). In the specific case of high-fat, high-calorie meals though, lower-body obese women but not upper-body obese women or men do preferentially store a larger percentage of energy in their gluteal-femoral fat, which has higher activities of key esterification enzymes, acyl-CoA synthetase (ACS), and diacylglycerol acetyltransferase (DGAT). Expression of these enzymes is comparable between sexes in the abdominal sc and visceral fat (Hames et al. 2015; Morgan-Bathke et al. 2015). Additionally, FA uptake also occurs in the postabsorptive state independent of LPL (direct FA uptake). This phenomenon mirrors fat distribution; in other words direct FA uptake is higher in the abdominal depot of men and in the gluteal-femoral depot of women (Karastergiou et al. 2012; Santosa and Jensen 2015; Karpe and Pinnick 2015; Mundi et al. 2014). The importance of the higher fat storage capacity of femoral fat is provided by a small interventional study, in which surgical reduction of femoral fat by 1.1 kg for 1 year led to increased postprandial TG levels without changes in FA storage in the subcutaneous depots (Hernandez et al. 2015).

Lipolysis, the release of FA from adipose tissue in the fasting state to provide energy to other tissues, does not reflect fat distribution: in both men and women, abdominal depots are more lipolytically active. However, there are clear sex differences in metabolic fuel utilization. Lipolysis relative to whole-body requirements, i.e., resting energy expenditure, is higher by approximately 40% in women. Women are also more dependent on FA oxidation during periods of high demand, like exercise and prolonged fasting (Karastergiou et al. 2012; Karpe and Pinnick 2015; Santosa and Jensen 2015; Schmidt et al. 2014), i.e., females tend to rely more on FA to generate ATP that are associated with sex-linked variations in adipocyte rates of lipolysis ex vivo (Karastergiou et al. 2012; Karpe and Pinnick 2015; Santosa and Jensen 2015) and mitochondrial gene expression (Nookaew et al. 2013).

Sex differences in adipose metabolism may in part be driven by sex chromosomes and sex steroids, both estrogen and progesterone (Link and Reue 2017). Much remains to be learned about the role of sex steroids in the regulation of adipose metabolism. This question was recently explored in in vivo human studies of acute (4 weeks) hypogonadism. Suppression of estrogen and progesterone in premenopausal women increased postprandial TG without alterations in fat storage and oxidation (Santosa et al. 2016), whereas suppression of testosterone in men led to increased femoral LPL activity and meal-derived FA storage in the femoral depot, suggesting that androgens tonically inhibit the storage of FA in the lower body of men (Santosa et al. 2017). Longer estrogen deprivation also affects fat distribution by increasing visceral fat (Shea et al. 2015).

Recent Advances in Understanding Adipose Tissue Cellularity, Growth, and Remodeling

Adipose Tissue Expansion via Hyperplasia Depends on the Recruitment of Adipose Progenitor Cells

Based on rodent models and in vitro and in vivo studies in humans, it is clear that cells that possess adipogenic capacity, variably called adipose progenitor cells (APCs) or adipose stem cells (ASCs), reside within adipose tissues and are recruited during development and in response to a positive energy balance. ASCs are characterized as stem cells because they have the potential to also differentiate into osteoblasts or chondrocytes. The lineage of these cells varies as a function of depot and sex (Fried et al. 2015; Sanchez-Gurmaches and Guertin 2014). ASCs can proliferate and differentiate in a stepwise fashion into preadipocytes, i.e., cells that are more committed to adipocyte cell fate and express early markers of adipocyte differentiation but still have a fibroblastic appearance.

Sex and Depot Differences in Expandability: New Studies in Mice It has been suggested that lower-body fat depots in women, and likely some men, serve as a safe reservoir for fat storage, at least in part by an enhanced ability to expand by recruiting new adipocytes. The concept that subcutaneous depots of females have higher capacity for hyperplastic expansion derives from early studies in mice based on mature adipocyte counting methods. Subcutaneous adipose tissues of genetically or diet-induced obese females (Johnson and Hirsch 1972) exhibited higher hyperplastic expansion consistent with more recent lineage-tracing studies (Medrikova et al. 2012).

The capacity for hyperplasia is logically linked to numbers of APCs within a depot. Using flow cytometry to separate out a specific population of CD34+/Sca-1+ APCs, our study (Wu et al. 2017) confirmed the observation of Joe et al. (Joe et al. 2009) that 10-week old *low-fat fed* C57BL/6 female mice have greater number of APCs in both inguinal and gonadal depots than males (Joe et al. 2009). In females, diet-induced obesity produced by a 45% (by calories) HFD for 14 weeks increased APC and mature adipocyte number in the gonadal but not inguinal fat pad. With a longer period of HF feeding or higher fat diet (60% vs 45% by calories), recruitment of female inguinal APCs might occur and eventually achieve an increase in the number of countable mature adipocytes. Consistent with this possibility, Jeffery et al. used a short, 1-week pulse to label proliferating APCs formed during the first week of HF feeding (60% fat by calories) and found transient increases in APC labeling in both the gonadal and inguinal depots of females (Jeffery et al. 2016). After 7 weeks of "chase," new adipocytes were detected in both depots of the females. The authors did not confirm that this was associated with a net increase in mature adipocyte number.

In contrast to the hyperplasia (increased adipocyte number) and the recruitment of new adipocytes observed in the female gonadal fat pad after HFD, in males, the

gonadal fat depot does not increase the number of countable mature adipocytes (Wu et al. 2017; Faust et al. 1978; Van Beek et al. 2015). Thus, it was surprising that studies with pulse labeling or lineage tracing show that HF feeding of C57BL/6 male mice leads to "hyperplasia" as indicated by the appearance of labeled "new adipocytes" in the epididymal (gonadal) fat pad as determined by lineage tracing (Wang et al. 2013; Jeffery et al. 2015). However, these studies did not measure the number of mature adipocytes in the pad to confirm depot expansion via hyperplasia rather than the replacement of dead adipocytes with new ones. We (Wu et al. 2017) found that HFD in males increased the number of APCs per gonadal fat pad, but we could not detect an increase in mature adipocyte number by standard methods. This result is consistent with prior literature that, even with 60% HFD, the male gonadal fat pad becomes inflamed and adipocyte number does not increase (Strissel et al. 2007). Consistent with this observation, Macotela et al. found that APCs from epididymal adipose tissue had a low capacity to differentiate (Macotela et al. 2012). Thus the "new adipocytes" could be simply replacing "large, dysfunctional ones" without a net increase in adipocyte number. This process is very important, as recruitment of adipose progenitors/preadipocytes is critical to the adipose tissue remodeling process and new, mature adipocytes appear fairly rapidly (Strissel et al. 2007). The factors that determine whether growth by hyperplasia vs maintenance of the "health" of the adipose tissue via "replacement" occurs require further study. Simultaneous measures of adipocyte birth and death are needed to determine whether new adipocytes that appear in the male gonadal depot represent an increase in number per pad or the replacement of dead or dying cells (Fig. 1).

A recent study examined adipose progenitors in two perivascular adipose depots of Sprague-Dawley rats and found fewer APCs in these specialized depots associated with the thoracic aorta, but not with the mesenteric resistance arteries (Contreras et al. 2016). Females had fewer APCs, although APC proliferation and adipogenic capacity were similar in both sexes. These sex differences might be especially important to understand sex differences in cardiovascular risk (Link et al. 2017).

Taken together, analysis of dynamic changes in adipose cellularity emphasizes that studies of the turnover of adipose cells in multiple adipose depots are needed. Further, although the so-called "retroperitoneal" depot is small in the mouse, it merits further study as it is anatomically similar in both sexes and hence less susceptible to local factors related to its association with the respective reproductive organs or the presence of mammary tissue in the inguinal sc depot of rodents. Further, the cellularity of the retroperitoneal depot in rats is the most responsive to diet-induced obesity (Faust et al. 1978).

Sex and Depot Differences in the Number of Preadipocytes: Studies in Humans Tchoukalova et al. noted that preadipocytes, defined by expression of several metabolically important mRNAs after isolation and plating of stromal-vascular cells, are more numerous in the abdominal and femoral sc adipose tissues of nonobese women than in men, with a larger sex difference in the latter (Tchoukalova et al. 2010a). Femoral preadipocytes also differentiate more in response to an overfeeding challenge in healthy, normal-weight adults (Tchoukalova

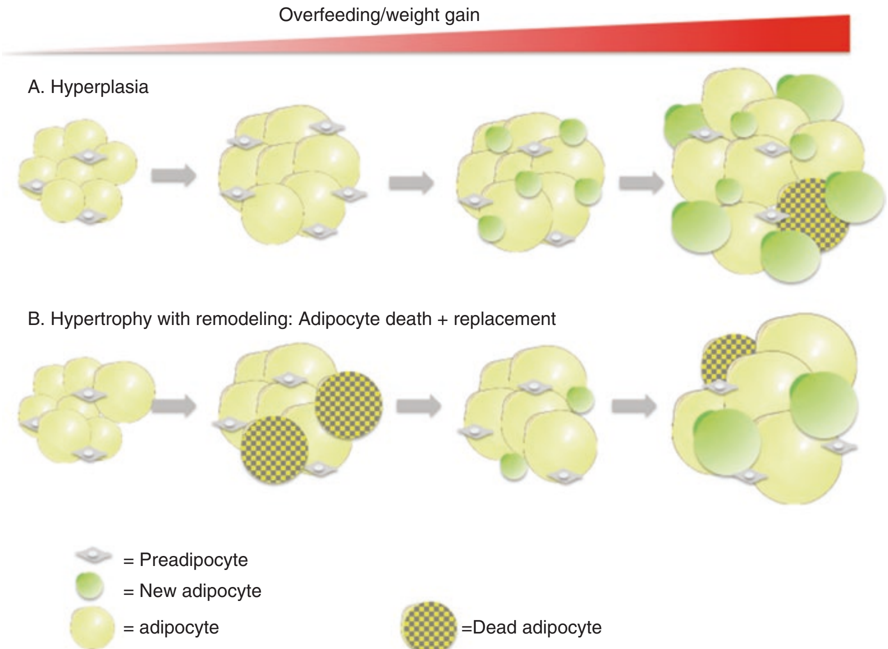

Fig. 1 Hyperplasia vs remodeling of adipose tissue in response to overfeeding. As described in section "Adipose Tissue Expansion via Hyperplasia Depends on the Recruitment of Adipose Progenitor Cells," researchers can detect formation of "new adipocytes" derived from adipose progenitor cells/preadipocytes. Panel **a** shows a depot that has a higher "expansion capacity" due to hyperplasia. It starts with smaller cells compared to those in panel **b**. In response to overfeeding, they initially enlarge via hypertrophy (more lipid stored in each adipocyte). At the same time, preadipocytes proliferate, and some are induced to differentiate as indicated by their *green color*, allowing the tissue mass to increase ~threefold. After overfeeding there is a net increase in the number of adipocytes in the depot. In contrast, *panel b* shows a depot that remodels and can accumulate *without a net increase* in adipocyte number. As the tissue expands to accommodate extra energy, the largest adipocytes die and are *replaced* by new (*green*) adipocytes. However, there is *no net increase* in adipocyte number in this fat depot. The new, smaller adipocytes gradually get larger to accommodate the energy consumed, but the number stays constant. With time, even in energy balance, adipocytes continuously die as part of a normal turnover process as shown in both panels (**a**, **b**). It is also important to note that it is possible that despite the larger size of the adipocytes in panel (**b**), they remain metabolically healthy as they typically occur in lower-body depots of women. In both cases, this can maintain the metabolic health of the adipose tissue

et al. 2010b). However, this group also noted lower differentiation of femoral compared to abdominal sc preadipocytes in vitro (Tchoukalova et al. 2010a), so the mechanisms may not be cell autonomous. The Bouloumie group recently analyzed the proliferative and differentiation potential of human adipose progenitors in abdominal sc and femoral adipose tissue. They found greater number of adipose progenitors, identified by high CD36 expression after flow sorting of CD34+/CD45−/CD31− cells, in the femoral compared to the abdominal subcutaneous depots of nonobese healthy females, but males were not studied (Gao et al. 2017).

CD36 facilitates fatty acid uptake and storage, so as expected, these progenitors also had higher adipogenic potential. Studies of sex and regional differences in this (and other) subpopulation of adipose progenitors are clearly needed.

Studies of Depot Differences in Preadipocyte Recruitment in Humans In Vivo White et al. used doubly labeled water to trace rates of proliferation of preadipocytes and found higher rates in the femoral compared to the abdominal depot of overweight to obese women (White et al. 2016). Men were not studied. A more recent study from this group tested the "expandability hypothesis" (Virtue and Vidal-Puig 2010) by examining the association of in vivo measures of new adipocyte and preadipocyte formation with direct measures of insulin sensitivity and metabolic health. Contrary to their hypothesis, adipocyte formation was positively related to the ratio of visceral to total adipose tissue (which is metabolically deleterious), and adipocyte formation in both depots over 8 weeks of overfeeding was inversely related to insulin sensitivity (White et al. 2017). One possible explanation is that larger adipocytes are more susceptible to cell death and remodeling occurs to replace them. Direct measures of adipocyte metabolic function and inflammation and a time course of these changes are needed to fully understand the complex and dynamic changes in adipose tissue remodeling.

Orchestration of Adipose Hyperplasia, Hypertrophy, and Remodeling in Male and Female Adipose Tissues

Expanding the Expandability Hypothesis

As alluded to above, the "adipose expandability hypothesis" has gained traction as a mechanism that may link the ability of body fat, or specific fat depots, to expand via hyperplasia to systemic metabolic dysfunction (Virtue and Vidal-Puig 2010). There remain key limitations of our knowledge of the "expandability hypothesis" with regard to (1) regional adipose cellularity and metabolic health and (2) cellular mechanisms of depot-specific adipose growth and remodeling. As originally articulated by Danforth in the year 2000 (Danforth 2000):

> "The inability of the adipose organ to expand to accommodate excess calories and that type II diabetes in the centrally obese, in spite of their unlikely phenotype, is a form of lipodystrophy". He further posited "too few adipocytes predisposes to type II diabetes mellitus" and "could explain the lower prevalence of type II diabetes in the generalized and more hypercellular obese than in the centrally obese, who for genetic or environmental reasons have lost the ability to accommodate excess energy by differentiating new adipocytes."

Clearly, the adipose organ can no longer be considered a single entity, so it is necessary to extend the "expandabilty" hypothesis to consider sex and depot dependence of the growth of specific adipose tissues, both visceral and subcutaneous, as demonstrated by the studies on the rates of abdominal and femoral preadipocyte recruitment in adipose tissue of women described in the previous section.

Based on sampling of abdominal subcutaneous adipose tissue, seminal studies by Arner's group using novel methodology assessed the integration of a carbon isotope into DNA (as a result of an environmental exposure during a discrete period). Results indicated that adipocyte turnover occurs but that the rate of turnover (~10% per year, half-life = 10 years) is similar in lean and obese men and women (Spalding et al. 2008). These data imply that adipose, like other tissues, undergoes a normal remodeling process as adipocytes die and are replaced by new ones. As might be predicted, the rate of appearance of new adipocytes in the abdominal sc fat depot is lower in subjects with more hypertrophic abdominal sc adipose tissue, independent of sex, consistent with the hypothesis that excess hypertrophy is driven by an inability to recruit preadipocytes through adipogenesis (Arner et al. 2010). There is only limited knowledge of depot differences in fat depot expansion (i.e., a net increase in adipocyte number) and turnover. Potential sex differences in the cellularity of different depots and as a function of fat distribution remain a significant gap in knowledge. It is possible that the metabolic health of a depot depends on its ability to remodel as well as expand, with or without a net increase in adipocyte number.

Measuring Regional Adipose Cellularity While it is straightforward in animal studies where the mass of a specific depot and its lipid content are easily assessed by dissection, lipid extraction, and weighing, and even possible to combine with changes in depot adipocyte number over time in different groups of animals, and, ideally, lineage tracing with measurements of adipocyte cellularity, it is not possible in humans. Thus, most studies of adipocyte number in humans are based on sampling of only one depot, usually the abdominal (Spalding et al. 2008). However, recent advances in imaging of the adipose tissues, by dual-energy X-ray absorptiometry (DXA) with estimates of visceral vs abdominal subcutaneous depot size, magnetic resonance imaging, or computerized tomography, combined with depot sampling for cellularity have allowed a comparison of the effects of sex and level of obesity on adipose cell number in humans.

Sex Differences in the Cellularity of Specific Human Adipose Depots: Relationships to Metabolic Dysfunction

Abdominal Subcutaneous Studies by Arner's group found that the higher fat mass in obese individuals is related to both higher adipocyte size (as measured in isolated mature adipocytes) and number (based on an estimate of depot size by DXA Andersson et al. 2017). Abdominal sc adipocyte size plateaus as a function of increased abdominal sc fat mass. Adipocyte size was greater in nonobese men than women, and conversely, nonobese women had a higher number of adipocytes compared to men. No differences in either size or number were found between obese men and women, as both groups plateau at the same large size. Nevertheless, within each sex, adipocyte size was associated with measures of insulin resistance, although the slope of the relationship was greater in men. This study also confirmed numerous

prior reports based on estimates of total rather than regional fat to calculate cellularity that fat cell number was higher in obese than nonobese and stable over time in all subjects. Consistent with the connection of abdominal adipocyte size and metabolic abnormalities, a recent clinical study that included men and women but did not assess sex differences, short-term overfeeding led to abdominal sc adipocyte enlargement in overweight/obese insulin-sensitive, but not insulin-resistant, subjects, accompanied by increased visceral and hepatic fat and reduced insulin sensitivity (Mclaughlin et al. 2016). This study did not address potential changes in lower-body fat depots, which are likely to vary by sex and may attenuate the metabolic stress imposed by overfeeding.

Omental As for the abdominal sc, the size of visceral (omental) depot in humans is determined by a combination of adipocyte size and number (Arner et al. 2013), independent of sex when controlled for total body fat or BMI. However, adipocyte size, not number, correlates with insulin resistance (Arner et al. 2013). Tchernof et al. found that in women with more omental adipocyte hypertrophy, the adipogenic capacity of subcutaneous adipocytes is lower (Lessard et al. 2014), suggesting that the inability of the subcutaneous depot for hyperplastic expansion leads to excess hypertrophy of adipocytes in the omental depot, which would be deleterious to metabolic health.

Femoral The relative contributions of hypertrophy and hyperplasia to sex differences in lower-body fat depot expansion and hence metabolic protection in humans remain unclear. Many studies note that femoral/gluteal adipocytes of premenopausal women are larger than those in the abdominal sc depot, suggesting a limitation of adipogenesis or a greater intrinsic capacity of these adipocytes to “safely” store fat. However, few studies have calculated regional adipocyte number (i.e., by measuring fat mass in the abdomen and thigh by imaging methods). In one of the few fairly large studies (188 females, 133 males of varying BMI) that compared adipocyte size and regional fat mass between men and women, Jensen and colleagues found that thigh adipocyte numbers increased significantly with BMI in premenopausal females, but not in men. For any given increase in lower-body fat mass, the increase in femoral adipocyte size was significantly greater in men than in women (Tchoukalova et al. 2008). This result implies that the femoral depot of males may have lower capacity for preadipocyte proliferation or differentiation, but this remains to be demonstrated. As well, possible sex differences in the association of femoral adipocyte size and number and adipogenic capacity to the metabolic activity of the depot and the individual have yet to be systematically studied. A recent small, but intriguing, clinical study (Cox-York et al. 2017) suggested that estrogen treatment increases adipogenesis of femoral but not abdominal APCs in postmenopausal women. These data suggest a mechanism for differences in expansion capacity in pre- and postmenopausal women that merits further investigation.

Methodological Considerations in Estimating Adipocyte Cellularity It should be kept in mind that some methods for assessing adipocyte size, especially in isolated cells or histology sections, may underestimate the total number of smaller

adipocytes (Laforest et al. 2017). The measurement of mature, isolated adipocytes after they are separated by collagenase digestion does not "count" smaller adipocytes (< ~30 μm) that do not float well. The use of electronic (e.g., Coulter counting) measurement of osmium-fixed cells detects greater numbers of small adipocytes such that distributions are often bimodal. The appearance of smaller adipocytes may represent new, insulin-sensitive adipocytes or dysfunctional ones that cannot efficiently store fat (Tchkonia et al. 2010). Dynamic variations in these populations occur over time, so their presence is difficult to interpret in cross-sectional studies (Mclaughlin et al. 2014). This issue may lead to conflicting results in the literature on the association of fat cell size distribution and metabolic health. In addition, osmium, commonly used to fix adipose tissue prior to Coulter counting, fixes all lipid droplets, including the remaining lipid in dead adipocytes within crown-like structures that are in the process of being removed by macrophages. We conclude that it is best to combine multiple approaches to assess adipose cellularity with immunocytochemical measurements of cell senescence and death to gain a better understanding of adipose tissue cellularity in response to aging and diet challenges.

Mechanisms of Adipose Tissue Remodeling and Potential Importance for Depot and Sex Differences in Depot Growth and Metabolism

As evidence accumulates that the capacity of a fat depot to expand via hypertrophy vs hyperplasia is an important determinant of metabolism and health, increased research attention is directed toward developing a mechanistic understanding of how these processes are regulated. Tissue remodeling is a dynamic process, present in all tissues, in which the breakdown of the extracellular matrix (ECM), including collagens, glycoproteins, and proteoglycans, permits progenitor cells to proliferate, migrate, and differentiate, as reviewed by Werb (Bonnans et al. 2014). As applied to adipose tissue, the term remodeling includes changes in the turnover of adipocytes (i.e., the appearance of new adipocytes and their disappearance after death), recruitment of beige progenitors that give rise to cells with multiple lipid droplets and more mitochondria and thermogenic capacity, as well as "transdifferentiation" of different cells types within the tissue (as reviewed by Scherer Sun et al. (2011) and Cinti Giordano et al. (2014)).

Importance of ECM Remodeling in Adipose Tissue Growth and Function Although the molecular details of adipogenesis are fairly well understood in model systems in vitro, it is increasingly appreciated that the local microenvironment within different male and female adipose tissues in vivo, i.e., ECM composition, cross talk among adipocytes, adipose progenitors, fibroblasts, endothelial cells via paracrine interactions, innervation, and blood supply/angiogenesis, is an important determinant of both adipocyte function and progenitor recruitment

(as reviewed by Cowan Pope et al. (2016) and Scherer Sun et al. (2011)). The ECM of adipose tissue includes multiple types of collagens, elastin, laminin, fibronectin that transduce multiple signals to the adipocyte via integrins. The mechanical properties of this matrix are likely to vary with depot, sex, and obesity, and its remodeling is required for adipogenesis. The composition of the ECM, in particular the content of specific collagens secreted by fibroblasts, adipose progenitors, and myofibroblasts, changes its stiffness, which can affect the ability of the adipocyte to expand (Sun et al. 2011). A matrix depleted of collagen VI, for example, allows for large increase in adipocyte size, but maintenance of their insulin sensitivity and endocrine functions. Human studies also link the level of fibrosis, i.e., the accumulation of excess collagens in the matrix, to adipocyte size (Pasarica et al. 2009). Thus, analysis of the ECM composition, how it is remodeled and its effects on adipocyte function and preadipocyte recruitment in male vs female adipose tissues, as a function of nutritional state, has become an important new area of obesity research (Muir et al. 2016; Liu et al. 2016).

Sex differences in the expression of key enzymes that remodel the ECM have been observed in mouse models (Martinez-Santibanez et al. 2015). We recently found that matrix metalloproteinase 3 (MMP3), which is anti-adipogenic, is expressed in mouse adipose progenitors and is significantly higher in the inguinal depot of females than males. HFD-induced obesity downregulated MMP3 protein in both the inguinal and gonadal depots of females but not males (Wu et al. 2017). Furthermore, HFD-induced obesity tended to increase the ratio of a tissue inhibitor of metalloproteinase, TIMP4 to MMP3 (significantly in inguinal), while it decreased the ratio in males. We therefore suggested that a dynamic balance of MMPs and TIMPs modulates the recruitment and differentiation of preadipocytes in a sex-dependent manner. Consistent with this idea, we found that addition of recombinant MMP3 suppressed, and TIMP4 increased, adipogenesis in primary cultures of human APCs, consistent with a role in the balance of these factors, and undoubtedly others, in the adipose tissue expansion (Wu et al. 2017). Undoubtedly these observations are at the tip of the iceberg in understanding the complex processes by which sex-dependent adipose remodeling promotes the metabolic health of the adipose tissues.

The importance of the depot differences in ECM in determining the differentiation of APCs is supported by studies in mice and humans. Adipose progenitors from the epididymal depot of male mice, which differentiate poorly in standard two-dimensional culture, differentiated more when placed in decellularized matrix from the subcutaneous depot (Grandl et al. 2016). Similarly, decellularized ECM of the omental depot of nondiabetic compared to diabetic individuals more efficiently improved adipocyte differentiation and function (Baker et al. 2017).

Mechanisms of Adipocyte Death and Turnover The process whereby "old" senescent or dysfunctional adipocytes are replaced through the recruitment and differentiation of ASCs remains incompletely understood but has been suggested to involve pyroptosis, a proinflammatory programmed cell death (Giordano et al. 2013; Tchkonia et al. 2010). This process is accompanied by recruitment of macrophages, their activation and formation of crown-like structures around dead

adipocytes. Male adipose tissue, especially the gonadal depot, is highly susceptible to HF-induced inflammation (Van Beek et al. 2015; Grove et al. 2010; Wu et al. 2017). HFD induces far less adipocyte death and tissue inflammation with formation of crown-like structures in female compared to male mice. However, mechanisms of sex differences in these processes have not been extensively investigated but may also be related to cell-autonomous sex differences in hematopoietic cells and lymphocytes, which are lower in females than in males (Singer et al. 2015). The relative interplay of cell-autonomous properties of adipose progenitors and immune cells is an important area for future work.

Inflammation and Sex Differences in Adipose Remodeling In the mouse, male gonadal adipose tissue exhibits much higher rates of remodeling as evidenced by the appearance of activated macrophages with higher inflammatory cytokine production and crown-like structures surrounding dead adipocytes. As inflammatory cytokines, specifically TNFα, potently inhibit adipogenesis, the increased adipogenesis observed in HF-fed male gonadal depots is surprising. However, low concentrations of cytokines function as growth factors, and indeed recent evidence from the Scherer lab suggested that inflammation is required for adipogenesis (Wernstedt Asterholm et al. 2014). A mouse engineered to express a dominant negative TNF in adipose tissue had lower adipogenesis, more crown-like structures, and higher fibrosis after long-term HFD (22 weeks). These results suggest that inflammation, in particular TNFα in this study, is critically important for the growth and remodeling of the ECM and hyperplastic adipose tissue expansion. Sex differences in adipose inflammation are well documented (Grove et al. 2010; Wu et al. 2017).

Physiological Control of Adipose Metabolism and Remodeling The sympathetic innervation of adipose tissue is mainly perivascular and thereby controls blood flow to the tissue but also includes parenchymal nerves that contain norepinephrine as well as neuropeptide Y (NPY). Innervation density increases with prolonged fasting in the retroperitoneal but not epididymal depot of male rats (Giordano et al. 2005). A sex difference was detected by Kim et al. (Kim et al. 2016) who found higher sympathetic innervation of female compared to male gonadal, but not inguinal, adipose tissue of mice. β3-adrenergic signaling increased browning of female gonadal fat but had no effect in male gonadal fat, and this difference was abolished after induction of ovarian failure. The treatment was equally effective in the inguinal depot of both sexes (Kim et al. 2016). Thus sex differences in the "beiging" of adipose tissue from white to brown are at least partly under neural control. No studies to date have specifically examined sex differences in cold-induced browning, but Mahdaviani et al. recently noted that deletion of mitofusin 2, which is required for mitochondrial fusion and adaptation to cold-induced thermogenesis in brown adipose tissue, resulted in higher efficiency of ATP production, higher FA oxidation, and protection against HFD-induced obesity in female but not male mice (Mahdaviani et al. 2017).

Angiogenesis also plays a critical role in adipose metabolism and remodeling. If the vascularity of the adipose tissue does not increase with adipocyte size, local hypoxia can result, as reviewed recently (Crewe et al. 2017). Possible sex differences

in the angiogenic potential of adipose endothelial cells and production of proangiogenic factors such as VEGF require further research attention.

Are Sex Differences in APCs Cell Autonomous or Driven by the Depot Microenvironment? Local factors, rather than intrinsic depot or sex differences in APCs, may drive their proliferation and differentiation. Jeffery et al. found that transplant of male inguinal APCs, which do not proliferate as fast as male gonadal APCs, into female inguinal depot increased their proliferation. Thus, like endogenous female APCs, proliferation was similar in both depots (Jeffery et al. 2015). Further, placing male inguinal preadipocytes into the gonadal depot (of males) leads to higher proliferation and differentiation than observed in the context of their depot of origin. Thus, the low rates of adipogenesis in gonadal (epididymal) APCs and the better adipogenesis of male inguinal adipocytes that is observed in standard culture conditions in vitro do not predict their in vivo behavior (Macotela et al. 2012). Studies from the Zhang lab showed that the size achieved by transplanted APCs was significantly affected by the sex of the donor mouse in a leptin receptor-dependent manner, such that female donor cells were significantly smaller and depended on the presence of the leptin receptor (Guo et al. 2009). Identification of the intrinsic and local factors in the microenvironment of each fat depot that influence sex-dependent adipogenesis will be an important topic for future study.

Few studies have directly compared the adipogenic capacity of male and female adipose progenitors. Intriguingly, Schlezinger found that bone marrow-derived stem cells from females had higher adipogenic capacity when activated by rosiglitazone, a PPARγ agonist, independent of ovarian status (Bragdon et al. 2015), but no studies have systematically compared the in vitro adipogenic capacity of adipose-derived stem cells. Some early studies detected depot differences in the adipogenesis of female gonadal (parametrial) and perirenal preadipocytes of ovariectomized rats (Lacasa et al. 2001).

Sex Steroids May Play a Role in Determining Adipose Tissue Growth and Remodeling Jeffery et al. found that ovariectomy inhibited the female-specific effect on the proliferation of adipose progenitors as determined by BrdU labeling in C57BL/6 mice. Administration of estrogen to males increased HFD-induced APC proliferation in inguinal subcutaneous adipose tissue so that it was equal to that in gonadal, i.e., the "female" pattern (Jeffery et al. 2016). Adipose inflammation and crown-like structures were not assessed in this study. However, Grove et al. found that ovariectomy barely increased inflammation so that the number of crown-like structures remained far below levels in males (Grove et al. 2010). Future studies should evaluate the ovariectomy effects on measures of inflammation over the time course required for the recruitment and differentiation of adipose progenitors.

Intrinsic Differences in Adipose Tissue Revealed by Transplant Studies A number of recent studies have attempted to address the question of whether depot differences in adipose tissue are cell autonomous. These have been extensively reviewed (Tran and Kahn 2010). In brief, grafting subcutaneous adipose tissue in the abdominal cavity, but not subcutaneously, improved glucose and insulin

tolerance, whereas gonadal fat transplants have a deleterious or no effect. Published studies to date have only examined males. Our preliminary studies show that transplantation of female compared to male inguinal sc fat into the subcutaneous compartment of male recipients improved glucose tolerance; the mechanisms involved are under investigation (Wu, Lee, Fried, unpublished observation).

Remodeling of Adipose Tissue via Transdifferentiation of White Adipocytes Cold adaptation causes the appearance of beige adipocytes, which have more numerous mitochondria, multiple lipid droplets, and thermogenic properties of brown adipocytes. These can be derived from "beige" precursors, or as some evidence indicates, functionally similar "brite" adipocytes may arise by a process of "transdifferentiation." Cinti has added "more color" to our understanding of adipocyte phenotypes and remodeling with the observation that during pregnancy and lactation, the inguinal sc depot (the mammary depot in mice) undergoes a profound remodeling. So-called pink adipocytes within this depot "transdifferentiate" into epithelial cells that support lactation (Giordano et al. 2014). Excellent reviews of these topics are available (Lin and Farmer 2016; Ramseyer and Granneman 2016; Sanchez-Gurmaches and Guertin 2014; Sanchez-Gurmaches et al. 2016).

Rodents as Models for Sex Differences in Human Adipose Biology

Several differences between human and mouse models should be kept in mind while investigating adipose sex differences (Wu et al. 2017; Grove et al. 2010; Medrikova et al. 2012). Women have higher body fat percentage than men, but similarly aged female mice are leaner than males, although the magnitude of this difference diminishes with age. The inguinal fat depot of mice is the largest subcutaneous depot and often considered analogous to the gluteal-femoral sc depot of humans. However, adipocytes in this depot are smaller than those in other depots such as gonadal (parametrial in females, epididymal in males) in both sexes, while in humans, gluteal-femoral adipocytes are larger. Women have more fat in the gluteal-femoral depot compared to men, while in mice, the inguinal subcutaneous depot is a similar percent of total body fat in both sexes and, by mass, is lower in females (Grove et al. 2010; Wu et al. 2017; Palmer and Clegg 2015). Inguinal depots of female rodents include mammary glands, so these may be more analogous to breast adipose tissue. Additionally, human gluteal-femoral adipocytes express antilipolytic α2-adrenergic receptors, whereas these are not expressed in any depot of mice. Gonadal depots (parametrial and epididymal) do not exist in humans. Although the epididymal depot of males is often described as a "visceral" depot, it is not in the strict sense in that it does not drain portally. On the other hand, like the human omental depot, it lies over the stomach within the peritoneal cavity and thereby may have a special relationship with the gut. Further, the transcriptome profiles of epididymal fat are more similar to visceral than subcutaneous of humans (Gesta et al. 2006). Most notably, omental fat in humans and epididymal in male mice become more inflamed in obesity and

develop more crown-like structures. Mesenteric adipose tissue in rodents and humans appears similar anatomically in that adipocytes are in close association with the intestine and the depot drains portally. Although these similarities and differences are important to keep in mind, rodents have proven a useful model to analyze basic aspects of human adipose tissue growth and development. Furthermore, with respect to metabolism, the insulin sensitivity of mouse and rat adipocytes from females is higher than in males, similar to the sex differences in abdominal adipocytes from women and men (Macotela et al. 2009; Guerre-Millo et al. 1985; Foley et al. 1984).

Summary and Conclusion

Studies of mice and humans indicate substantial sex differences in adipose tissue development, growth, and metabolic capacities that determine body shape and metabolic health, as well as in the mechanisms involved. Intriguingly, the local microenvironment within fat depots, as well as cell-autonomous properties, may influence the adipogenic differentiation capacity of adipose progenitors in a sex-dependent manner. However, the fundamental mechanisms that drive these sex differences are only beginning to be understood. Although sex differences in adipose metabolism in men and women with upper- vs lower-body fat distributions are fairly well studied, there are very few studies of adipose tissue growth and remodeling in men vs women with defined fat distribution. Given the strong influence of adipose tissue distribution and biological sex on multiple aspects of metabolic health and some basic differences in the physiology of mouse vs human adipose tissues, there is a strong rationale for well-powered studies of male and female human adipose tissues at the genomic and functional levels in phenotypically well-characterized nonobese and obese men and women.

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Men Are from Mars, Women Are from Venus: Sex Differences in Insulin Action and Secretion

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Abstract Sex difference plays a substantial role in the regulation of glucose metabolism in healthy glucose-tolerant humans. The factors which may contribute to the sex-related differences in glucose metabolism include differences in lifestyle (diet and exercise), sex hormones, and body composition. Several epidemiological and observational studies have noted that impaired glucose tolerance is more common in women than men. Some of these studies have attributed this to differences in body composition, while others have attributed impaired insulin sensitivity as a cause of impaired glucose tolerance in women. We studied postprandial glucose metabolism in 120 men and 90 women after ingestion of a mixed meal. Rates of meal glucose appearance, endogenous glucose production, and glucose disappearance were calculated using a novel triple-tracer isotope dilution method. Insulin action and secretion were calculated using validated physiological models. While rate of meal glucose appearance was higher in women than men, rates of glucose disappearance were higher in elderly women than elderly men while young women had lower rates of glucose disappearance than young men. Hence, sex has an impact on postprandial glucose metabolism, and sex differences in carbohydrate metabolism may have important implications for approaches to prevent and manage diabetes in an individual.

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Introduction

The prevalence of glucose intolerance and type 2 diabetes mellitus (T2D) is rapidly increasing worldwide, and the mechanisms responsible for this remain an area of active investigation.

In health, a narrow range of plasma glucose concentrations are maintained due to regularized release into and disposal from the circulation to meet the supply and demand of glucose. Apart from insulin which is the major determining factor of glucose tolerance, there are also other mechanisms such as glucose effectiveness (the ability of glucose per se to enhance glucose disposal and suppress hepatic glucose production) and a number of hormonal factors which contribute to maintain plasma glucose homeostasis. We and others have demonstrated that glucose tolerance decreases with age and impairment in insulin secretion and insulin action contributes to worsening glucose tolerance of aging (Basu et al. 2003a; Ryan 2000; Gomyo et al. 2004; Tessari 2000a, b; Bonadonna et al. 1994; Jackson et al. 1988). Sex-related differences in glucose regulation have been demonstrated (Basu et al. 2006; Dunstan et al. 2002; Williams et al. 2003; DECODE Study Group 2003; Sicree et al. 2008; Qiao et al. 2003; Morita and Ishigaki 2015; Ryan 2000b; Perseghin et al. 2001; Borissova et al. 2005; Blaak 2005; Bjorntorp 1997). Studies have shown higher prevalence of impaired glucose tolerance in women and impaired fasting glucose in men (Basu et al. 2006; Dunstan et al. 2002; Williams et al. 2003; DECODE Study Group 2003; Sicree et al. 2008; Qiao et al. 2003). The underlying mechanisms for these sex-related differences in postprandial glucose concentrations remain under active investigation. Several factors such as differences in lifestyle including diet and exercise (Morita and Ishigaki 2015; Ryan 2000b), differences in sex hormones (Morita and Ishigaki 2015; Ryan 2000b; Perseghin et al. 2001; Borissova et al. 2005), and differences in body composition (Blaak 2005; Bjorntorp 1997) may contribute to sex-related differences in glucose tolerance that have been reported.

Effect of Sex on Postprandial Glucose Metabolism

Postprandial glucose metabolism is regulated by pattern of insulin secretion, hepatic and peripheral insulin action, and glucose effectiveness. Postprandial glucose concentrations reflect a net balance between the amount of glucose entering (rate of appearance: Ra) and leaving (rate of disappearance: Rd) the circulation. Ra in turn is largely dependent on the rate of appearance of meal carbohydrate (MRa) and, to a lesser extent, rate of endogenous glucose production (EGP), while Rd reflects the rate of whole-body glucose uptake. Following meal ingestion, blood glucose concentrations rise for as long as Ra exceeds Rd and start to fall when Rd exceeds Ra. Many studies in the past have evaluated insulin action either with the IVGTT or insulin tolerance test (Basu et al. 2003a; Muller et al. 1996; Kohrt et al. 1993;

Lyssenko et al. 2005; Goodpaster et al. 2003; Kanaya et al. 2005; Utzschneider et al. 2004; Ferrannini et al. 1996; Boden et al. 1993; DeFronzo 1979; Coon et al. 1992; Iozzo et al. 1999; Chang and Halter 2003; Chen et al. 1985; Rowe et al. 1983), both of which are performed by nonphysiological methods. Some have used fasting plasma insulin concentrations as a surrogate for basal insulin secretion which further introduces uncertainty because several factors such as hepatic insulin extraction, degree of obesity, insulin resistance, etc. modulate insulin secretion and action. It is possible to estimate these parameters of postprandial carbohydrate metabolism using the isotope dilution technique. Similarly, numerous studies (Ferrannini et al. 1988; Fe'ry and Balasse 1994; Firth et al. 1986; Livesey et al. 1998; Pehling et al. 1984; Steele et al. 1968) have used the dual-tracer isotope dilution approach where a glucose tracer was infused intravenously to measure the rate of appearance of another glucose tracer mixed with the glucose contained in the mixed meal. However, to improve the measurement precision by minimizing nonsteady-state errors in calculation of postprandial glucose turnover, a third glucose tracer was infused intravenously to mimic the anticipated rate of appearance of meal glucose (Basu et al. 2003b). This triple-tracer approach is currently the gold standard for estimation of postprandial glucose turnover in nondiabetic humans and has been validated in both type 1 and type 2 diabetes (Basu et al. 2003b, 2009; Saad et al. 2012).

To determine postprandial carbohydrate turnover using the triple-tracer technique, a series of experiments were undertaken in 120 men [90 elderly (68.6 ± 0.6 years) and 30 young (23.5 ± 0.6 years)] and 90 women [60 elderly (70.3 ± 0.8 years) and 30 young (22.3 ± 0.6 years)] to determine rates of meal appearance, EGP, and glucose disposal after ingestion of a mixed meal (Basu et al. 2003a, b). In this triple-tracer approach, subjects ingested a mixed meal (45% CHO, 40% fat, and 15% protein) containing 75 g glucose labeled with [1-^{13}C]glucose, while [6-^{3}H]glucose and [6,6-$^{2}H_2$]glucose were infused intravenously in patterns that minimized the change in the plasma ratios of [6-^{3}H]glucose to [1-^{13}C]glucose and of [6,6-$^{2}H_2$]glucose to endogenous glucose, respectively. Rates of meal appearance and EGP measured with this approach were essentially model independent, since nonsteady-state error was minimized by this approach. Net insulin action (SI) and the effects of insulin on glucose disposal (SI*) were measured with the unlabeled and labeled oral "minimal" models (Basu et al. 2003a, b, 2009; Saad et al. 2012; Caumo et al. 2000; Dalla Man et al. 2004), respectively, and insulin secretion and clearance measured with the C-peptide minimal model (Dalla Man et al. 2002, 2005a, b; Breda et al. 2001; Toffolo et al. 1995; Toffolo et al. 2006).

Fasting plasma glucose concentrations were lower in women than men regardless of age, but after meal ingestion, postprandial glucose concentrations were higher in young women compared to young men, whereas postprandial glucose concentrations did not differ between elderly women and elderly men. Fasting as well as post-meal insulin, C-peptide, and glucagon concentrations did not differ between elderly men and elderly women. On the other hand, postprandial integrated insulin and C-peptide concentrations were higher in young women than young men despite comparable concentrations of these hormones during fasting state suggesting

Table 1 Effects of sex on glucose metabolism, insulin action, insulin secretion, disposition index, glucose effectiveness, and hepatic insulin extraction after ingestion of a mixed meal

	Elderly women vs. elderly men	Young women vs. young men
Meal appearance	↑	↑
Glucose production	↔	↔
Glucose disappearance	↑	↔
Insulin action	↔	↓
Insulin secretion	↔	↔
Glucose effectiveness	↑	↑
Disposition index	↑	↓
Hepatic insulin extraction	↔	↔

that young women possibly had impaired insulin action compared to men of corresponding age.

Rates of EGP were higher in women than men regardless of age in the fasting state, but following meal ingestion EGP was rapidly suppressed in women and was at rates comparable to men in both age groups. Rates of meal appearance were higher in women than men in both age groups. Postprandial glucose disposal was higher in elderly women than elderly men. On the contrary, postprandial glucose disposal was lower in young women compared to young men suggesting impaired insulin action in young women compared to young men (Table 1).

Effect of Sex on Model Indices

Insulin action and secretion were calculated using the validated physiological models. While net insulin sensitivity (S_I) was numerically lower in women compared to men regardless of age, effect of insulin on glucose disposal (S_I*) was significantly lower in young women than young men, but SI* did not differ between elderly men and women (Figs. 1 and 2).

All indices of insulin secretion static (Phi_{static},), dynamic ($Phi_{dynamic}$), and total (Phi_{total}) were similar in women and men of both age groups (Figs. 3 and 4). Hepatic insulin extraction did not differ between women and men of both age groups although it was higher in elderly participants of both sexes compared to their younger counterparts (Fig. 5).

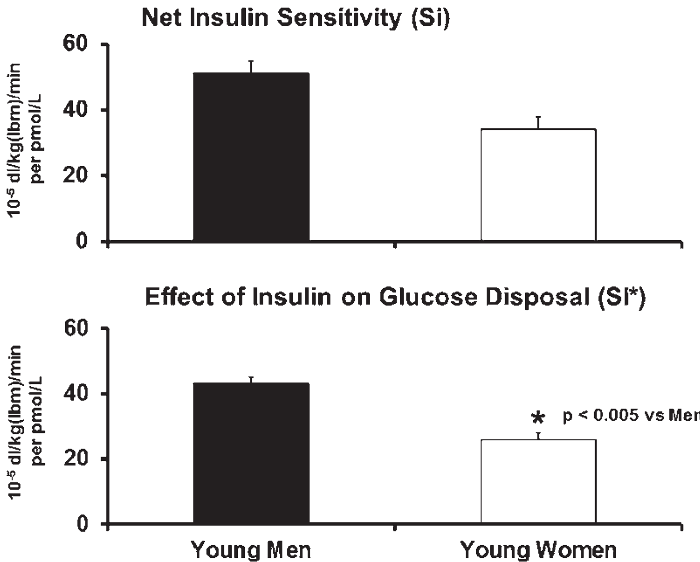

Fig. 1 Insulin action (S_i and S_i*) observed in elderly men and women and young men and women after ingestion of a mixed meal. *$P < 0.01$ vs. young men

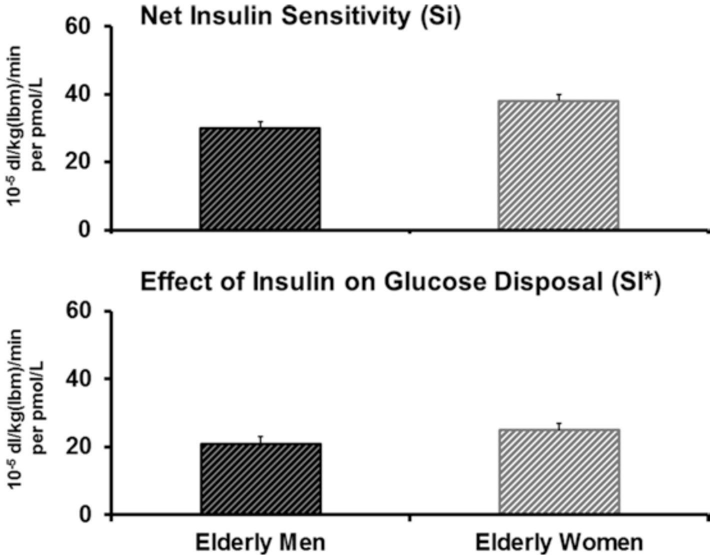

Fig. 2 Insulin action (S_i and S_i*) observed in elderly men and women and young men and women after ingestion of a mixed meal. *$P < 0.01$ vs. young men

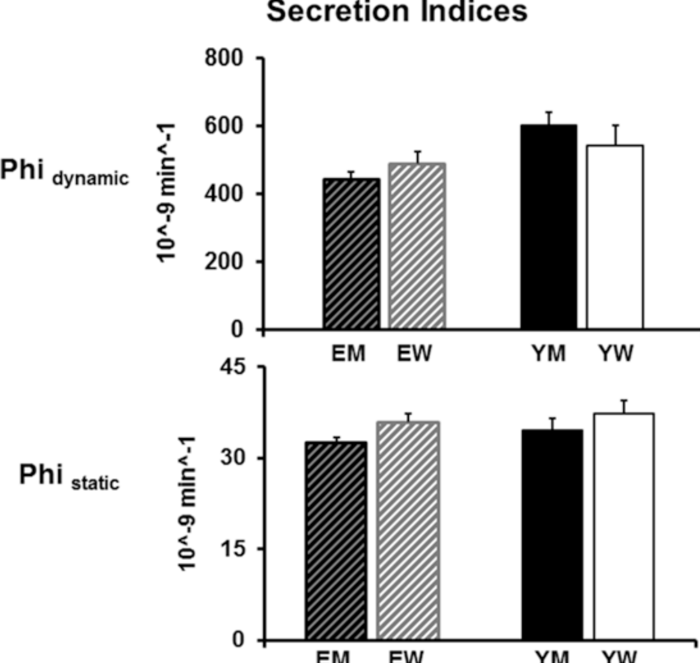

Fig. 3 Indices of insulin secretion ($Phi_{dynamic}$, Phi_{static}, Phi_{total}) observed in elderly men and women and young men and women after ingestion of a mixed meal. *$P < 0.001$ vs. elderly men; #$P < 0.05$ vs. young men

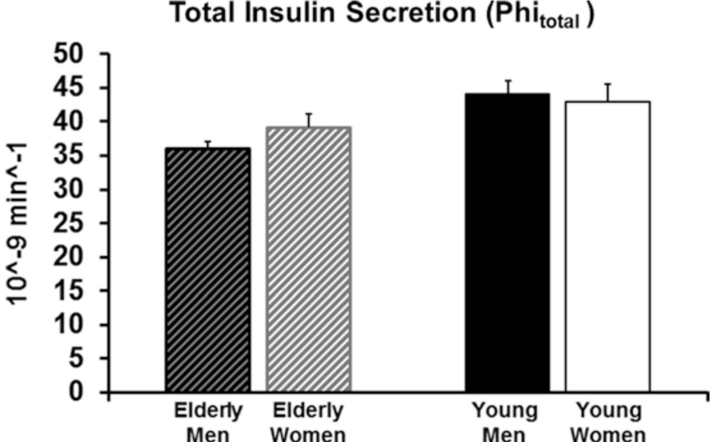

Fig. 4 Indices of insulin secretion ($Phi_{dynamic}$, Phi_{static}, Phi_{total}) observed in elderly men and women and young men and women after ingestion of a mixed meal. *$P < 0.001$ vs. elderly men; #$P < 0.05$ vs. young men

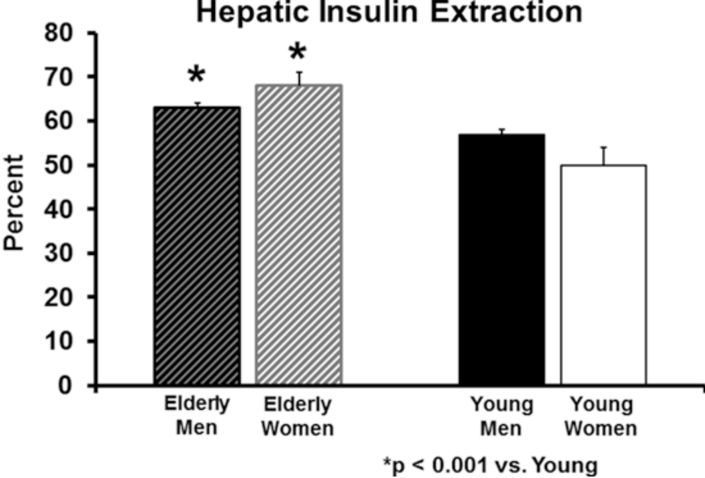

Fig. 5 Disposition index observed in elderly men and women and young men and women after ingestion of a mixed meal. *$P < 0.001$ vs. elderly men; $^{\#}P < 0.05$ vs. young men

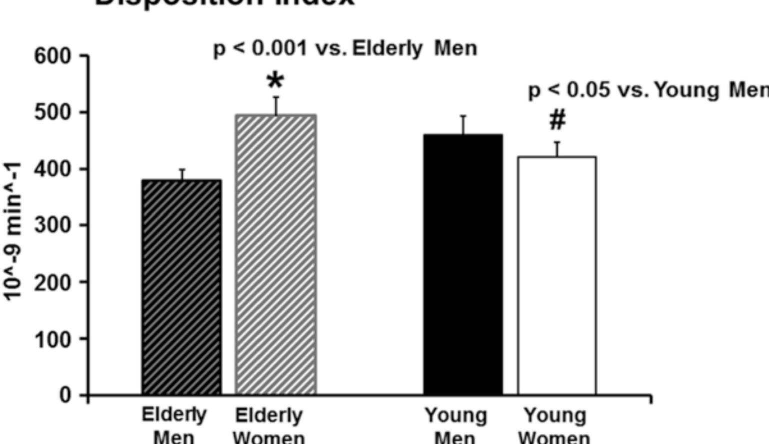

Fig. 6 Hepatic insulin extraction observed in elderly men and women and young men and women after ingestion of a mixed meal. *$P < 0.001$ vs. young men and women

Disposition index (DI) an index of beta cell function appropriate for the degree of insulin resistance is the product of insulin sensitivity and insulin secretion ($S_I \times Phi_{total}$) calculated using the physiological models. DI was significantly higher in elderly women than men suggesting higher insulin secretion in elderly women for a given level of insulin action which enables them to compensate for higher rates of meal appearance. DI was lower in young women compared to young men suggesting that insulin secretion is not enough for a given level of insulin action in young women (Fig. 6).

The study concluded that sex impact on insulin secretion, insulin action, hepatic insulin extraction, and glucose effectiveness results in substantial differences in the regulation of postprandial glucose metabolism in elderly as well as young men and women.

Effect of Sex on Glucose Effectiveness

Net glucose effectiveness (GE), the ability of glucose to enhance its own disposal and suppress its own production in the liver, was comparable between women and men in both age groups. The effect of glucose on its own disposal (GE*) was significantly higher in women than men which suggests that higher GE* compensates for lower insulin action observed in young women (Figs. 7 and 8).

Summary

The data from these studies indicate that postprandial glucose regulation differs in women and men regardless of age. Although postprandial glucose concentrations were comparable in elderly participants of both sexes, the rate of meal appearance

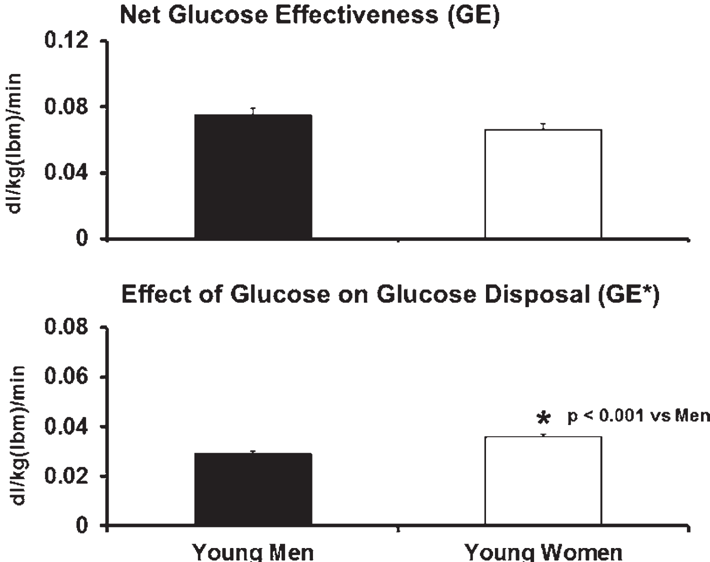

Fig. 7 Indices of glucose effectiveness (GE and GE*) observed in elderly men and women and young men and women after ingestion of a mixed meal. *$P < 0.001$ vs. elderly men; #$P < 0.001$ vs. young men

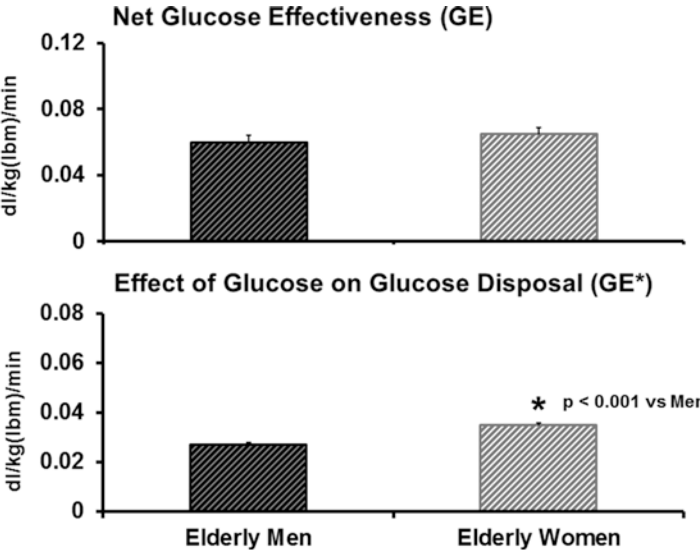

Fig. 8 Indices of glucose effectiveness (GE and GE*) observed in elderly men and women and young men and women after ingestion of a mixed meal. *$P < 0.001$ vs. elderly men; $^{\#}P < 0.001$ vs. young men

and postprandial rates of glucose disposal were significantly higher in elderly women than men suggesting that any impairment in glucose disposal may lead to glucose intolerance in elderly women. Similarly, in young participants of both sexes, postprandial glucose concentrations were comparable, but rates of meal appearance and ability of glucose to stimulate its own disposal were substantially higher in women than men which compensate for reduced ability of insulin to stimulate postprandial glucose disposal in young women. Hence, due to differences in regulation of postprandial glucose metabolism, therapeutic approaches for managing postprandial hyperglycemia in men and women will also differ. Further studies are needed to evaluate sex differences in management of fasting and postprandial hyperglycemia in people with diabetes.

Conclusion

Regulation of postprandial glucose metabolism differs between men and women. Hence, sex difference in carbohydrate metabolism may have important implications for strategies to prevent and manage type 2 diabetes in humans.

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The Role of Sex and Sex Hormones in Regulating Obesity-Induced Inflammation

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Abstract Metabolic and non-metabolic complications due to obesity are becoming more prevalent, yet our understanding of the mechanisms driving these is not. This is due to individual risk factor variability making it difficult to predict disease outcomes such as diabetes and insulin resistance. Gender is a critical factor in obesity outcomes with women having more adiposity but reduced metabolic complications compared to men. The role of immune system activation during obesity is an emerging field that links adiposity to metabolic syndrome. Furthermore, evidence from animal models suggests that sex differences exist in immune responses and, therefore, could be a possible mechanism leading to sex differences in metabolic disease. While there is still much to learn in the area of sex-differences research, this chapter will review the current knowledge and literature detailing the role of sex and sex hormones on adiposity and metabolically induced inflammation in obesity.

Sex Differences and Metabolic Disease

Obesity is observed in an alarmingly high proportion of the global population and affects all demographics (Nagareddy et al. 2014). An individual's response to overnutrition, however, is highly variable and may depend on several factors including sex, age, and ethnicity (Demerath et al. 2007; Karastergiou et al. 2012). Significant metabolic and phenotypic differences exist between sexes in obesogenic environments that likely contribute to differences in the metabolic responses to a high-fat diet (HFD). While women have higher obesity rates than men, men are more likely to develop obesity-associated comorbidities (Nagareddy et al. 2014; Meyer et al. 2006; Onat et al. 2016), which are partly due to the difference in adipose tissue distribution between the sexes (section "Sex Differences in Body Fat Distribution"). Androgens and estrogens are thought to play a key role in these sex differences (Alexandra Kautzky-Willer and Pacini 2016; Palmer and Clegg 2015;

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Mauvais-Jarvis 2011) and have been implicated in playing a role in lipolysis, muscle metabolism, and satiety (De Pergola 2000; Karastergiou et al. 2012; Krotkiewski et al. 1983; Ribas et al. 2016; Wade et al. 1985). After menopause, females exhibit an elevated risk of developing metabolic disorders due to fluctuating levels of circulating androgens and estrogens (Arnetz et al. 2014; Onat et al. 2016; Romero-Aleshire et al. 2009; Pfeilschifter et al. 2002). Given this phenomenon, menopausal hormone therapy has been used clinically to address the development of metabolic disorders in obese individuals but has been met with little success in the metabolic improvement (Manson et al. 2013; Lovre et al. 2016). It is evident that sex hormones play a role in the onset of metabolic disorders, but their role in the development of these disorders remains unclear.

One active area of investigation is the study of immune system activation during obesity to better understand the link between adiposity and sex differences in metabolic disease. While there is still much to learn in this area of sex difference research, in this chapter we will review the current knowledge and literature on the role of sex and sex hormones on adiposity and metabolically induced inflammation in obesity.

Sex Differences in Body Fat Distribution

Much of the work on adipose tissue responses to obesity has been conducted in male animal models because they gain weight more readily. During high-fat diet exposure, adiposity increases systemically, but there is a critical distribution of subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) fat stores which is modulated by several factors. Understanding this distribution is clinically relevant given that body composition plays a significant role in disease predisposition. Sex steroid hormones modulate and influence adipose tissue distribution between the genders, but many of the cellular and molecular mechanisms behind this regulation of adiposity remain to be fully understood. Women, overall, have higher adiposity compared to men, yet at the same body mass index (BMI), men have a higher incidence of metabolic disease (Jackson et al. 2002; Onat et al. 2016; Meyer et al. 2011).

Typically, in men, central/abdominal fat deposition correlates with an increased susceptibility for metabolic complications (Meyer et al. 2006; Francesca Amati et al. 2012; Smith et al. 2001; Michaud et al. 2012; Kissebah et al. 1982; Alexandra Kautzky-Willer and Pacini 2016), while premenopausal women show enhanced lower and peripheral fat deposition with a lower incidence of metabolic disorders. During menopause, estrogen levels decline, and the proportion of testosterone in women is higher. This leads to increased visceral adiposity and further demonstrates the role of sex hormones on body fat distribution (Smith et al. 2001; Meyer et al. 2011; Carr 2003).

Sex hormones also play a specific role in depot patterns of adipogenesis (Jeffery et al. 2016), with males preferentially expanding adipocytes in visceral gonadal white adipose tissue (GWAT) over subcutaneous inguinal white adipose tissue

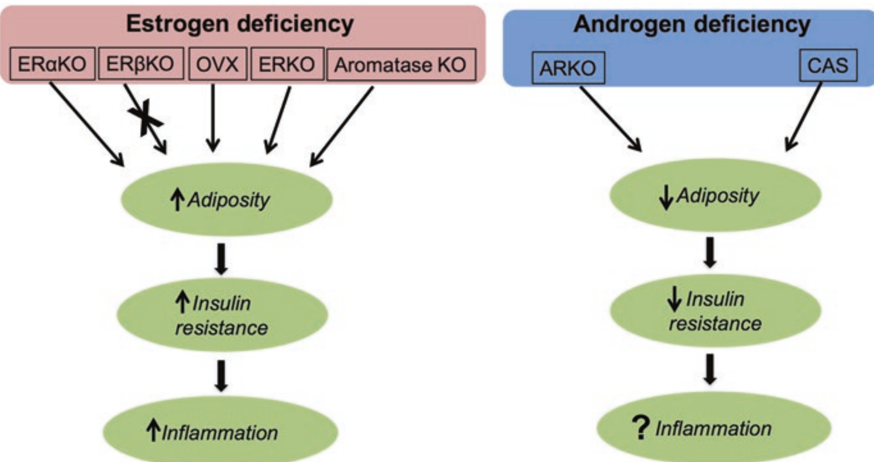

Fig. 1 Summary of estrogen and androgen deficiency models studied in the development of adiposity, insulin resistance, and inflammation in obesity. *Arrows* indicate ↑ increase and ↓ decrease; **X** indicates no change; **?** indicates inconclusive evidence

(IWAT). Females, however, are better able to expand adipocytes in both GWAT and IWAT (Bloor and Symonds 2014). While estrogens are produced primarily in the ovaries and testes after aromatization of testosterone, local estrogens may impact adipocyte function and are increased in proportion to total body adiposity (Schneider et al. 1979; Tchernof et al. 1995). During menopause, estrogen levels decline, and the proportion of testosterone in women is higher, leading to loss of SAT and a gain in VAT, the depot associated with increased inflammation and systemic insulin resistance (Smith et al. 2001; Meyer et al. 2011; Carr 2003).

One possible mechanism for sex hormones would be the regulation of key proteins in adipose tissue through steroid receptors. Not only are sex hormone receptors – estrogen receptor (ER) and androgen receptor (AR) – present in the adipose tissue, but adipose itself has a critical role in the production and metabolism of sex hormones (Mayes and Watson 2004; Wade et al. 1985). The disparity in fat distribution is therefore attributed to effects of differential sex hormone profiles of estrogens and testosterone (Michaud et al. 2012) and interactions within the adipose tissue system (Mayes and Watson 2004; Wade et al. 1985). Rodent model studies of estrogen deficiencies (ovariectomy, aromatase knockout *(KO)*, estrogen receptor (ER) – *ERαKO*, *ERβKO*, and double *ERα/ERβKO* mice) and androgen deficiencies (castration and androgen receptor knockout *(ARKO)*) as well as clinical studies in women with PCOS, postmenopausal women, and aromatase-deficient men (Morishima et al. 1995; Carani et al. 1997) have demonstrated the protective function of estrogens against metabolic syndrome by maintaining insulin sensitivity and adiposity during obesity (Fig. 1) (Simpson and Jones 2007; Jones et al. 2000; Wade et al. 1985; Bruun et al. 2003; Dubois et al. 2016; Rubinow et al. 2015). Elucidating the mechanisms by which men and women store fat is critical to the understanding

of obesity and metabolic syndrome. This area has been discussed elaborately in chapter “Cellular Mechanisms Driving Sex Differences in Adipose Tissue Biology and Body Shape in Humans and Mouse Models.”

Obesity-Induced Meta-inflammation

Obesity-Induced Meta-inflammation in Males

Obesity is known to induce a state of chronic, low-grade inflammation referred to as meta-inflammation. An overwhelming majority of these studies have been performed in males due to an exacerbated inflammatory response when compared with females; however, obesity-induced meta-inflammation in females is discussed in greater detail in “Sex Differences in Meta-inflammation.” In males, this inflammatory induction has been implicated in the development of metabolic syndrome and its associated pathophysiological consequences, such as insulin resistance, cardiovascular disease (CVD), and many non-metabolic obesity-related sequelae (Lumeng and Saltiel 2011). The link between obesity and a pro-inflammatory state has largely been related to adipose tissue inflammation, but the mechanisms remain largely elusive. Despite this, it is evident that meta-inflammation is linked to the onset of obesity-related metabolic and non-metabolic disorders through activation of leukocytes, particularly of the myeloid lineage, in both human and male mouse models (Kullo et al. 2002; Nagareddy et al. 2014; Singer et al. 2014). These leukocyte subtypes include neutrophils, macrophages, and monocytes that are significantly correlated with adiposity. Clinical studies have shown that they are also associated with increased disease risk (Elgazar-Carmon et al. 2008; Rull et al. 2010). These inflammatory leukocytes increase in number with obesity. Additionally, they undergo functional differences and several pro-inflammatory cytokines such as interleukin-6, interleukin-1β, and tumor necrosis factor-α (IL-6, IL-1β, and TNF-α, respectively), and chemokines such as monocyte chemoattractant protein-1 (MCP-1/CCL2) and its receptor, CCR2, are found to be elevated in obese individuals (Morris et al. 2011; Sartipy and Loskutoff 2003). These signals originate in the VAT and have systemic effects. Adipocytes themselves produce many of these signals (Pellegrinelli et al. 2014; Hu et al. 1996) but to a lower extent compared to the chemokines and cytokines produced from the adipose tissue macrophage (ATM) (Weisberg et al. 2003; Makki et al. 2013).

During a state of high-fat diet exposure, gonadal white adipocytes increase in size and number, leading to expansion of the visceral WAT depots and apoptosis of adipocytes (Sorisky et al. 2000; Jo et al. 2009). During this process ATMs accumulate specifically in the GWAT depot, and as a result, systemic inflammation is increased (Lumeng et al. 2007, 2008; Weisberg et al. 2006). Inhibitors of pro-inflammatory cytokines and chemokines have been shown to improve insulin sensitivity and link ATMs to metabolic syndrome (Weisberg et al. 2006; Winer et al. 2009).

Resident and Recruited Adipose Tissue Macrophages with Obesity in Males

One well-documented inflammatory phenotype resulting from an obesogenic environment is the accumulation of pro-inflammatory, classically activated, M1/CD11c^{+} ATMs in the VAT. This activated ATM population is expanded in male animal models (Lumeng et al. 2007) and clinical studies in both sexes (Wentworth et al. 2010; Tordjman et al. 2008) and strongly correlates with insulin resistance. In a normal homeostatic state, resident ATMs are the predominant macrophage subset present in the adipose tissue and function to maintain homeostasis. These cells represent between 10% and 15% of all cells in the stromal vascular fraction (SVF) in a lean state and are distinguished by a CD64^{+}/CD11c^{-} phenotype (Lumeng et al. 2007; Cho et al. 2016). During HFD exposure there is an early shift in macrophages with initial proliferation of resident ATMs (Lee et al. 2011). After a threshold of obesity, there is an expansion of the pro-inflammatory CD11c^{+} ATM population that represents between 45% and 60% of the SVF in adipose tissue (Weisberg et al. 2003). However, this work has predominantly been investigated in male animal models.

In these male animal models, Ly6c^{hi} /CCR2^{+} monocytes increase with obesity and are recruited to the adipose tissue where they develop into CD11c^{+} ATMs (Westcott et al. 2009; Weisberg et al. 2006) (Fig. 2). Blockade of these monocytes reduces the number of CD11c^{+} ATMs and improves insulin sensitivity. Once these Ly6c^{hi} monocytes reach the adipose tissue, they are driven to differentiate into CD11c^{+} macrophages and are often found to congregate in areas surrounding dead and dying adipocytes, referred to as crown-like structures (CLS) (Fig. 2c) (Westcott et al. 2009). Chemokine signals, such as MCP-1, drive monocytes to traffic into adipose tissue. In addition, these signals cause the expansion of myeloid cell differentiation from hematopoietic stem cells (HSC) (Nagareddy et al. 2014; Singer et al. 2014). Several studies have shown that the stem and progenitor cells within the bone marrow (BM) compartment remain altered after chronic HFD exposure. In vitro, HFD BM has pro-inflammatory properties, mirroring increased inflammation in in vivo studies performed with obese mouse bone marrow transplant (BMT) (Singer et al. 2014) and weight loss investigations (Zamarron et al. 2017). Specifically, HSCs are primed to produce macrophages and neutrophils after HFD bone marrow is used in BMTs. In these BMTs, HFD recipient mice have more CD11c^{+} ATMs and impaired insulin resistance compared to control, normal diet (ND) recipient mice (Nagareddy et al. 2014; Singer et al. 2014). Similarly, hyperglycemia has been shown to prime the progenitor compartment to produce the same activated macrophages. While initially this macrophage profiling was performed in adipose tissue, it is now clear from human and mouse obesity studies that myeloid cells (neutrophils and macrophages) expand in most other metabolic tissues such as the pancreas, brain, muscle, and liver. Within these tissues these myeloid cells contribute to organ dysfunction (Lawrence et al. 2012; Kanda et al. 2006; Morris 2015). Therefore, it is reasonable to hypothesize that differences in metabolic inflammation may contribute to differential risk for metabolic disease, especially in males.

Inflammatory Changes in Visceral Adipose Tissue with Obesity	
Adipocyte size	*Increased in both sexes (Female>Male)*
Myeloid Precursors	*Increased in males*
LY6C^hi Monocytes	*Increased in males*
CD11c+ ATMs	*Increased in males*
CD11c- ATMs	*Increased in both sexes (Female>Male)*
Pro-Inflammatory Cytokines	*Increased production in males*
Chemokines	*Increased production in males*

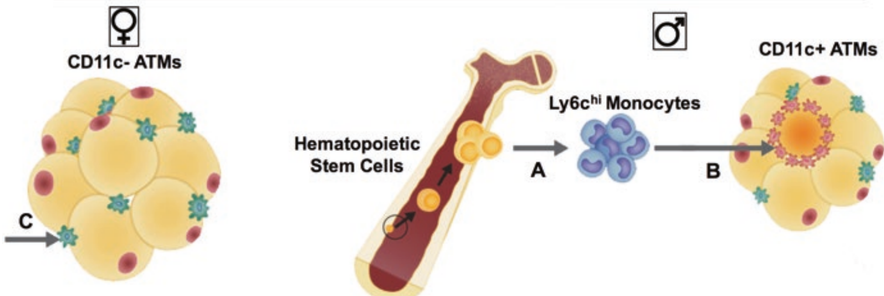

Fig. 2 Male and female models of obesity indicate increases in male myeloid progenitors, myeloid cells, and pro-inflammatory cytokines when compared with females (table). During obesity there are significant sex differences in obesity-induced inflammation. In males, hematopoietic stem cells develop into myeloid precursors and monocytes (**a**), these monocytes are recruited into adipose tissue as $CD11c^+$ adipose tissue macrophages in crown-like structures around dying adipocytes (**b**), while in females there is an increase in only the $CD11c^-$ adipose tissue macrophage population (**c**)

There has been a lot of work done on understanding the mechanistic link between inflammation, obesity, and insulin resistance, yet the majority of this work has been conducted in male animal models. Sex-dependent changes in adipose tissue structure and composition may also account for these differential responses to obesity (Finkelstein et al. 2013). The major cell types in adipose tissue are adipocytes, leukocytes, and adipocyte progenitors. Sex hormone receptors and sex-specific functional differences have been identified in all of these cell types in adipose tissue but not well described for the leukocyte changes. Adipose tissue expansion and metabolic disease have many differences between sexes, so it is important to assess this meta-inflammation in females and males.

Sex Differences in Meta-inflammation

Sex Differences in Innate Immunity

Taking an immune system-focused perspective, men and women have several differences in innate immunity and hematopoiesis (Mierzejewska et al. 2015). This is demonstrated by the higher prevalence of autoimmune conditions in women and the lower lipopolysaccharide (LPS) responsiveness in female leukocytes (Rettew et al. 2009; Imahara et al. 2005; Marriott et al. 2006). These dampened responses are specifically related to lower expression of toll-like receptor 4 (TLR4) (Marriott et al. 2006) and lower cytokine production. This is clinically demonstrated by a reduced sepsis rate among women (Marriott et al. 2006). Peripheral blood mononuclear cells (PBMCs) from men produce more pro-inflammatory TNF-α and less protective IL-10 than PBMCs from women following LPS stimulation (Asai et al. 2001). Peritoneal macrophages from male mice express higher levels of TLR4 and produce more CXC-chemokine ligand 10 (CXCL10) following LPS stimulation than macrophages from females (Marriott et al. 2006). Neutrophils from men also express higher levels of TLR4 and produce more TNF-α than female neutrophils both constitutively and following activation with LPS (Aomatsu et al. 2013). Beyond LPS models, this dimorphism has been demonstrated in viral assays demonstrating that PBMCs from men result in higher TLR9 activation and increased IL-10 production which is positively correlated with androgen concentrations in males (Torcia et al. 2012).

The monocyte and neutrophil pools themselves are larger and traffic more readily in males compared to females (Kay et al. 2015). This is then altered after menopause when women have an increased production of pro-inflammatory cytokines, regardless of stimulation (Pfeilschifter et al. 2002). Consistent with a role for sex hormones, 17-β estradiol-treated macrophages have a reduced LPS response through controlling nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) intracellular localization (Ghisletti et al. 2005). 17-beta estradiol has even been shown to exert an anti-inflammatory effect on adipocytes directly through reduced production of chemokines and NF-κB-mediated cytokines (Mu et al. 2016). However, chronic estradiol administration in vivo to ovariectomized mice enhances pro-inflammatory cytokine production of IL-1β, IL-6, and TNF-α (Calippe et al. 2008, 2010).

In addition to the role of sex hormones influencing leukocyte biology, genes on sex chromosomes are also thought to play a role in sex differences in immune function. With women expressing two X chromosomes, it is important to recognize that there are some X-linked genes that play a role in inflammatory responses especially those influencing the NF-κB pathway, apoptosis, phagocytosis, and the TLR cascade (Spolarics 2007). This is thought to contribute to the different cellular responses in females. Women are also known to have a higher rate of autoimmune conditions linked to X-chromosome genes leading to loss of self-tolerance (Libert et al. 2010). This has been studied through the use of transgenic mice to permit distinction

between traits due to the XX or XY complement compared to gonadal sex. In these mice, it is clear that the XX complement increases the risk for both lupus and experimental autoimmune encephalomyelitis (Smith-Bouvier et al. 2008; Teuscher et al. 2006). However, the effects of these genes on macrophage development and monocyte production have yet to be determined.

Sex Differences in Metabolically Induced Inflammation

One area where there is a clear divergent response to obesity in men and women is adipose tissue function, which is likely related to the strong differences in disease outcomes. While women have a higher percentage of body fat than men, they have a lower prevalence of diabetes (Kuhl et al. 2005). For any given combination of risk factors, men with metabolic syndrome have double the risk of heart attack and stroke as women (Goff et al. 2014). Metabolically induced inflammation is tightly linked to these diseases and adipose tissue function; assessing sex differences in meta-inflammation is an important area of investigation to explain these clinical outcomes.

In clinical studies focused on obesity, adipose tissue TNF-α concentrations are correlated with obesity and insulin resistance in patients with and without T2DM (Hotamisligil and Spiegelman 1994; Kern et al. 2001). While women have more adiposity, they have reduced metabolic disease with less overall inflammation. It is important to note that while they have reduced inflammation when compared to obese men, there is still an association with similar pathways of inflammation as is seen in men. In obese women, TNF-α mRNA expression in adipose tissue is correlated with increases in adipocyte lipolysis, fasting plasma glucose, insulin, and triacylglycerol concentrations (Zhang et al. 2002; Hotamisligil et al. 1993), as in men but to a lower extent.

In animal models, male mice gain more weight when challenged to the same duration of HFD exposure as their female counterparts (Grove et al. 2010; Hong et al. 2009). In males, the GWAT specifically shows an increased accumulation of ATMs, while females are protected from this infiltration (Singer et al. 2015). Full profiling of this adipose depot in both clinical and animal studies demonstrates this sex difference in inflammation and insulin resistance. As a result, the metabolic "protection" observed in female mice is at least in part due to inflammatory-induced metabolic impairments. The ATMs that accumulate in male animals with HFD are specifically more inflammatory with a $CD11c^{+}$ profile, exacerbated pro-inflammatory cytokine production, and further contribute to insulin resistance in male mice, compared to female mice, even when weight matched (Singer et al. 2015; Estrany et al. 2013; Stubbins et al. 2012).

Investigations into what drives this inflammatory difference in male and female murine models of obesity offer interesting insights into the origins of these ATM changes (Fig. 2). Evaluation of monocytes and hematopoietic progenitors in diet-induced obesity models shows that females do not have an expansion of the myeloid

progenitors with HFD. Ex vivo studies of BM from mice stimulated with LPS or palmitic acid also showed lower pro-inflammatory cytokine expression levels (Singer et al. 2015). In competitive BMT experiments, where recipients received both male and female bone marrow in a 1:1 ratio, male BM cells showed enhanced production of CD11c^{+} ATMs in response to HFD. This ATM induction was observable irrespective of the recipient animal's sex and suggests that there is a cell-intrinsic difference in hematopoietic responses to obesity between the sexes, wherein the HSC progenitor population is permanently primed after HFD exposure (Singer et al. 2015). Overall, it appears that female mice are protected from HFD-induced reprogramming of HSCs, ATM accumulation, and insulin resistance, similar to premenopausal women with obesity (Cannon et al. 2014; Folsom et al. 2009). These results demonstrate that male leukocytes have cell autonomous differences from female leukocytes in their response to obesity.

During obesity, there are lymphoid and myeloid changes that occur as a result of direct T-cell activation. In HFD-fed male mice, large numbers of CD8^{+} effector T cells infiltrate the GWAT, whereas CD4^{+} helper and FoxP3^{+} regulatory T cells (T reg) are diminished. In contrast, the anti-inflammatory T reg cell population was found to be increased in the adipose tissue of obese female mice, likely adding to the immune-associated protective metabolic effect (Nishimura et al. 2009; Pettersson US 2012).

Sex differences in meta-inflammation have also been demonstrated in other metabolic tissues during obesity (Ballestri et al. 2017). Estrogens directly suppress liver IL-6 production (Naugler et al. 2007) and myocardial cytokine production (Wang et al. 2005) and influence microbiome inflammatory responses (Markle et al. 2014). In addition, obesity induces inflammation in tissues such as the breast where it has been shown to play a significant role in increasing breast cancer risk (Simpson and Brown 2013).

Role of Sex Hormones in Obesity-Induced Inflammation

Estrogens in Obesity-Induced Inflammation

While sex hormones play a critical role in body fat distribution and adipocyte function, there is still a limited understanding of the role of sex hormones on meta-inflammation. Most research on understanding sex differences has focused on a putative, protective role of estrogens, improving insulin resistance and dampening inflammation in obesity (Gilliver 2010; Vieira Potter et al. 2012; Mauvais-Jarvis et al. 2013). Given the association between increased pro-inflammatory markers and obesity-related complications such as insulin resistance, the association between estrogens and decreased inflammation and decreased cytokines likely plays a role in sex and gender differences in insulin resistance (Steinberg 2007).

In clinical studies, decreased estrogen production during menopause is associated with increased cytokine levels (TNF-α, IL-1β, and IL-6). These levels are substantially lowered in women receiving hormone therapy (Pfeilschifter et al. 2002). Estrogens have generally been reported to have anti-inflammatory properties that contribute to cardiovascular protection, especially in menopausal women through the upregulation of endothelium-derived nitric oxide (NO) (Chen et al. 1999; Sumi and Ignarro 2003; Dantas and Sandberg 2005).

Mechanistic investigations in animal models have manipulated female sex hormones primarily through the use of ovariectomy. In ovariectomized mice, circulating levels of TNF-α are found to be up to sevenfold higher compared with E2-treated ovariectomized animals or those with endogenous estrogen production (Arenas 2005). These elevations in TNF-α are associated with impaired vascular function due to decreased NO levels and impaired metabolism. Ovariectomized animals placed on a HFD have also been found to have an increase in pro-inflammatory cytokines such as IL-6 (Ludgero-Correia et al. 2012), as well as CD11c+ gene expression and ATMs with resultant insulin resistance (Vieira Potter et al. 2012). Within the adipose tissue, profiling of male, female, and ovariectomized mouse gene expression demonstrates that males have a greater inflammatory response than ovariectomized females (Grove et al. 2010). While there may be several important regulators, only a few mechanisms have been investigated at this point. MCP-1 production from obese ovariectomized animals (Kim 2013) and a role for TNF receptor superfamily member 14 (TNFRSF14) in enhancing reactive oxygen species (ROS) and adipose tissue inflammation are several candidates (Choi et al. 2014).

While HFD-fed ovariectomized mice have impaired insulin signaling associated with rapid recruitment of macrophages and elevated macrophage infiltration, estradiol supplementation improves insulin sensitivity and reduces inflammation (Vieira Potter et al. 2012; Stubbins et al. 2012; Shen et al. 2014). Specifically, administration of estrogens to ovariectomized female mice significantly reduced expression of IL-6, TNF-α, and CD68 (Yonezawa 2012). Estradiol treatment in male animals has also demonstrated a reduction in age-induced inflammation (Stout et al. 2017).

Contrary to these studies, some groups have found that supplementation of estradiol in ovariectomized mice can lead to enhanced inflammatory cytokines even in the context of improved insulin sensitivity. Yet, marrow chimeras from *ERαKO* mice demonstrate protection from diet-induced inflammation (Riant et al. 2009). This would suggest that estrogens can have effects on inflammation that are independent of their effects on metabolism.

ERα whole body KO mice have been found to have insulin resistance and enhanced visceral adipose tissue inflammation (Davis et al. 2013; Ribas et al. 2010). Adipocyte-specific ERαKO mice (AdipoERα) have enhanced fibrosis and also elevated inflammation with TNF-α and TLR4 gene expression, only in males (Davis et al. 2013). In the absence of both ERα and ERβ, when *AdipoERα* mice were bred into the *ERβKO* background, TNF-α and TLR4 were elevated in both sexes suggesting a protective role for ERβ in the absence of ERα on inflammation. These data suggest that adipose tissue and adipocyte ERα and ERβ protect against adiposity

and inflammation in both males and females. This has been partially related to ERα-specific regulation of prolyl hydroxylase 3 (PHD3) leading to expression of hypoxia-inducible factor 1-α (HIF1α) which then promotes an inflammatory and fibrotic adipose tissue environment (Kim et al. 2014). Consistent with these findings, males overexpressing the aromatase enzyme have higher estradiol levels, are more insulin sensitive, and have a reduction in adipose tissue macrophages (Ohlsson et al. 2017).

Furthermore, myeloid-specific ERα deletion has been shown to impair glucose tolerance, enhance insulin resistance, and lead to increased atherosclerosis when placed in an LDL receptor KO animal (Ribas 2011). Mice reconstituted with ERα myeloid KO marrow had increased visceral adiposity and increased adipose tissue inflammation. Additionally, these cells have enhanced cytokine production when stimulated with palmitate compared to wild-type animals (Ribas 2011). These studies overall emphasize the role of ER signaling in myeloid-specific cells, separately from the adipocytes, in impacting adiposity and tissue inflammation (Fig. 1).

Role of Androgens in Obesity-Induced Inflammation

Women with PCOS have excess androgens and are at a much higher risk for metabolic syndrome (insulin resistance, β-cell dysfunction, and adipose tissue inflammation) (Spritzer et al. 2015; Marino et al. 2012; Chazenbalk et al. 2012). This is consistent with the phenotype seen in males and has been recently investigated as a possible cause of male-enhanced metabolic inflammation. Hypogonadal men are known to be at an increased risk for diabetes likely due to alterations in both lean and fat mass as described above and androgen deficiency-induced B-cell dysfunction (Navarro et al. 2016). Clinical evidence has shown that women with PCOS have more $CD11c^{+}$ ATMs (Huang et al. 2013). Similarly, *ARKO* myeloid cells in *LDLKO* mice have improved atherosclerosis, suggesting a harmful role of macrophage-specific AR in cardiovascular disease (Huang et al. 2014).

The role of androgens in obesity-induced inflammation is unclear with no direct human studies on the topic of the role of androgens on immune cells specifically. Activation of macrophages by testosterone impairs wound healing (Gilliver 2010; Gilliver et al. 2007), and in castrated mice gene pathways for innate immunity are downregulated (Cernetich et al. 2006), linking androgens to immune system activation.

The two main models that have been used to investigate this in obesity have been castration and *ARKO* models. The literature of androgens on metabolism and inflammation is quite contradictory. While androgens may improve β-cell insulin production (Mauvais-Jarvis 2011; Navarro et al. 2016) and muscle and liver insulin sensitivity (Navarro et al. 2015), the role in adipose tissue remains unclear. Castration has been demonstrated to have a variety of effects on metabolism, but specific studies evaluating inflammation have demonstrated that while adiposity is increased in castrated HFD mice, inflammatory cytokine expression profiles are consistent with

ND-fed castrated mice (Floryk et al. 2011). In *ARKO* mice, there is minimal change in inflammation (Rubinow et al. 2015; Dubois et al. 2016). Likewise, *ARKO* female mice show an increased degree of atherosclerosis (Fagman et al. 2015). Furthermore, the AR agonist dihydrotestosterone (DHT) reduces atherosclerosis, subcutaneous fat mass, and cholesterol levels in ovariectomized mice, suggesting the protective role of androgens against diet-induced atherosclerosis in female mice.

Hematopoietic AR deficiency increases visceral adiposity, but it does so with reduced liver macrophages as well as lower circulating IL-6, suggesting a metabolic contribution of hematopoietic androgen signaling to obesity (Rubinow et al. 2015). The effect of androgens on metabolic inflammation may also play an early role in development given that models mimicking PCOS have demonstrated that offspring of mothers exposed to androgens have increased insulin resistance, adiposity (Roland and Moenter 2011), and increased inflammation (Daan et al. 2016).

Summary

Obesity is a growing epidemic with enormous public health costs. There is a need to delineate sex-related differences in obesity-induced disorders in order to increase the clinical efficacy of treatment in both sexes. Studies support the hypothesis that there is sexual dimorphism in adipose tissue function, with evidence for a plausible role of sex hormones, especially estrogens, in adipose function, yet the mechanisms that control the regulation of fat distribution in both males and females are poorly understood. Many questions remain unanswered in our understanding of androgen metabolism in adipose tissue. The direct depot- and sex-specific effects of androgens on adipose tissue insulin sensitivity remain to be elucidated. Translation of in vivo and in vitro research findings is crucial to defining mechanisms of insulin resistance in women with PCOS. Although rodent models of estrogen and androgen deficiency have provided insight into understanding the role of sex hormones in metabolically induced inflammation, there are still differences in inflammation between rodents and humans. Substantial efforts are required to achieve clinical application in human subjects. Much remains to be learned about the factors from the microenvironment that influence adipogenesis in adipose tissue depots in both sexes and how they subsequently contribute to regional fat distribution and metabolic health. Further investigations into the role of sex hormones in eliciting a differential inflammatory response are required to treat complications such as autoimmunity. Understanding the regulation of adipogenesis and macrophage recruitment and proliferation could offer a new way to influence adipose tissue function, leading to new treatments for obesity, inflammation, and type 2 diabetes.

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Sex Differences in Leptin Control of Cardiovascular Function in Health and Metabolic Diseases

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Abstract Leptin, the adipocyte-derived hormone identified in 1994 for its major role in the control of satiety and body weight regulation, is an adipokine secreted in a sex-specific manner. Although it has clearly been established that females secrete three to four times more leptin than males and that this sexual dimorphism in leptin secretion is exacerbated with overweight and obesity, the origin and the physiological consequences of this sexual dimorphism remain ill-defined. The adipose tissue is the major site of leptin secretion; however, leptin receptors are ubiquitously expressed, conferring to leptin, and indirectly to the adipose tissue, a potential role in the control of numerous physiological functions. Besides its major role in the control of food intake and energy expenditure, leptin has been shown to contribute to the control of immune, bone, reproductive, and cardiovascular functions. The goal of the present chapter is to review and discuss the current knowledge on the contribution of leptin to the control of cardiovascular function while focusing on the impact of the sexual dimorphism in leptin secretion and of the pathological increases in leptin levels induced by overweight and obesity.

Abbreviations

ANS	Autonomous nervous system
ARC	Arcuate nucleus
BMI	Body mass index
BP	Blood pressure
Db	Gene coding for leptin receptor
ECG	Electrocardiogram
HF	High frequency
LF	Low frequency

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L-NAME	*N*ω-nitro-l-arginine methyl ester
MR	Mineralocorticoid receptor
MSNA	Muscular sympathetic nerve activity
NO	Nitric oxide
Ob	Gene coding for leptin
Ptp1b	Protein tyrosine phosphatase 1b
SNA	Sympathetic nerve activity

Introduction

Historical View

In the early 1970s, *Douglas Coleman*, at the Jackson Laboratory, revolutionized the field of obesity by providing the first clear evidence that obesity, a disease initially thought to result from behavioral and social disorders ("gluttony and sloth"), has a biological origin and involves a blood-borne factor that acts to decrease body weight. Using elegant parabiosis studies with the newly discovered obese *ob* and diabetic *db* mice, *Coleman* demonstrated that obesity in *ob* mice involves the lack of a circulating satiety factor, whereas obesity in *db* mice was caused by an insensitivity to this same substance (Coleman 1973; Coleman and Hummel 1973). After 8 years of laborious work using positional cloning, the group of *Jeffrey Friedman* at the Rockefeller University identified the mutated gene responsible for obesity in the *ob* mouse strain two decades later (Zhang et al. 1994) and reported that administration of the recombinant *ob* peptide ameliorated the *ob* phenotype (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). Based on the observation that the *ob* peptide reduces body weight, *Friedman* named the product of the *ob* gene "leptin" from the Greek *leptos* for "thin." Research rapidly appeared depicting that leptin is a satiety signal released from the adipose tissue that acts in the central nervous system to complete the feedback loop regulating appetite and energy expenditure. This discovery represented a massive step forward in the understanding of obesity.

Sexual Dimorphism in Leptin Secretion

Cloning of the mouse *ob* gene and its human homologue (Zhang et al. 1994) stimulated considerable interest in the role of the adipose tissue as an endocrine organ and led to numerous studies investigating the correlation between body mass index (BMI), adipose tissue mass, and leptin levels. These studies identified that the human adipose tissue produces a nonmutated form of the *ob* gene and that serum leptin levels are closely associated with body fat mass (Considine et al. 1996;

Hickey et al. 1996). Interestingly these studies led to the discovery of a striking sexual dimorphism in leptin production (Considine et al. 1996; Frederich et al. 1995; Hickey et al. 1996; Maffei et al. 1995; Saad et al. 1997). It was shown that women and female rodents secrete two to four times more leptin than their male counterparts. This difference in fasting baseline plasma leptin levels was observed across a broad spectrum of ages, BMIs, and body fat compositions in both rodents (Frederich et al. 1995; Maffei et al. 1995) and humans (Maffei et al. 1995; Considine et al. 1996; Hickey et al. 1996; Saad et al. 1997; Schwartz et al. 1996; Haupt et al. 2005; Gellstrom et al. 2000; Licinio et al. 1998; Guerra et al. 2008).

Despite two decades of research and numerous studies, the origin of the sexual dimorphism in leptin levels remains a subject of debate. Initial studies conducted in relatively small populations observed that correction of leptin levels by the percentage of adipose mass abolished the sex difference in leptin levels. Based on these observations, the sexual dimorphism in leptin levels was attributed to the greater body fat content of women (Considine et al. 1996; Maffei et al. 1995). However, subsequent studies conducted with larger populations rapidly challenged this concept and reported a persistence of the sexual dimorphism in leptin levels after correction for body mass or percentage of body fat (Saad et al. 1997; Frederich et al. 1995). While investigating the origin of the sexual dimorphism, *Lönnqvist* et al. evoked an intrinsic genomic difference as lean and obese women exhibit a higher adipocyte expression of the *ob* gene than men (Lonnqvist et al. 1995). *Havel et al.* investigated the contribution of female sex steroid hormones and reported similar absolute and adiposity-corrected plasma leptin levels in premenopausal women, untreated postmenopausal women (without menses for 1 year, post-bilateral ovariectomy, or with follicle-stimulating hormone levels superior to 50 mlU/ml), and postmenopausal women receiving hormone replacement. This suggested that absolute and adiposity-corrected plasma leptin levels are independent of age, reproductive status, and hormone replacement in women (Havel et al. 1996). Additional experiments conducted in obese women further minimized the contribution of hormonal status by reporting that neither absolute nor adiposity-corrected plasma leptin concentrations were different in untreated overweight postmenopausal women and overweight postmenopausal women with hormone replacement (Havel et al. 1996). In light of these results, neither female sex steroids, reproductive hormonal status, nor higher adiposity appears likely to be the cause of the sexual dimorphism in circulating leptin levels (Douchi et al. 2002; Havel et al. 1996).

While the aforementioned studies tend to minimize the contribution of total adiposity and female sex steroids to the control of leptin levels, several lines of evidence involve male sex steroids. Indeed, while positive associations between adiposity-adjusted leptin levels and total and bioavailable testosterone have been reported (Thomas et al. 2000; Paolisso et al. 1998), testosterone substitution has been shown to normalize elevated serum leptin levels in pubertal children (Roemmich et al. 1998) and hypogonadal men (Jockenhovel et al. 1997; Behre et al. 1997). The strongest body of evidence supporting a role for testosterone in the control of leptin levels is, however, provided by studies conducted in transgenders. It has indeed been reported that male-to-female transition with estrogen and

antiandrogen treatment increased body weight, body fat, and leptin levels, while testosterone administration in female-to-male transsexuals resulted in a decreased subcutaneous adipose mass and a reduction in plasma leptin levels (Elbers et al. 1997). A potential mechanism whereby testosterone appears to regulate leptin levels is by controlling body fat composition and distribution. Besides promoting lean mass expansion, a major role of testosterone consists in repressing subcutaneous adipose tissue growth (Elbers et al. 1997; Frederiksen et al. 2012). This leads to a lesser subcutaneous adipose mass in men and a more developed in women. Due to a higher *ob* gene expression, the subcutaneous adipose tissue produces more leptin than the visceral (Lonnqvist et al. 1995; Masuzaki et al. 1995; Ostlund et al. 1996). This difference in leptin production between adipose tissues is even exaggerated in females. Indeed, leptin mRNA expression is greater in abdominal subcutaneous fat than in abdominal visceral fat, and the ratio of subcutaneous to visceral fat leptin (*ob*) mRNA expression is 3.6-fold higher in women than in men (Van Harmelen et al. 1998; Montague et al. 1997). Therefore, it is believed that the origin of the sexual dimorphism in circulating leptin levels resides in the presence of testosterone, which represses leptin secretion via reducing subcutaneous adipose mass and potentially *ob* gene expression. Arguments to support this concept were provided by demonstrating that leptin levels correlate to hip circumference in women (Bennett et al. 1997) and that adjustment of serum leptin concentration for differences in relative body composition (fat mass and fat-free mass), body fat distribution (subcutaneous and intra-abdominal adipose tissue) (Nagy et al. 1997), and testosterone (Martin et al. 2002) abrogates the sexual dimorphism in circulating leptin levels.

Physiological Role of Leptin: Control of Cardiovascular Function

Based on the initial discovery that leptin is a satiety factor secreted by the adipose tissue and informing the brain of the status of the energy store, the interest in leptin focused initially on appetite, metabolism, and adiposity. However, evidence was rapidly provided that this humoral substance exerts a number of extrametabolic effects. The long signaling form of the leptin receptor, initially thought to be expressed in the hypothalamus only, appeared to be ubiquitously expressed, and leptin has been reported to intervene in the control of immune, reproductive, bone, and cardiovascular functions. The present chapter will focus on the role of leptin in the control of the cardiovascular function only.

Leptin and Sympathetic Control of Blood Pressure

Early studies investigating the role of leptin in body weight control reported that leptin-induced weight loss could not be completely attributed to decreases in food intake (Halaas et al. 1995) and reported that deficiency in leptin or leptin receptor,

in *ob* and *db* mice, respectively, led to a defective thermogenesis (Halaas et al. 1995). In the light of these reports, it had been hypothesized that leptin regulation of fat mass involves activation of the sympathetic nervous system to increase thermogenesis and energy expenditure in brown adipose tissue. This hypothesis had been substantiated by experiments by *Collins et al.* demonstrating that leptin increases norepinephrine turnover in intrascapular brown adipose tissue, suggesting increased sympathetic outflow to this thermogenic organ (Collins et al. 1996). Interested in determining whether leptin selectively increases sympathetic activity toward thermogenic organs, the group of *William Haynes* measured sympathetic response to leptin in nerves innervating the brown adipose tissue but also the kidneys, hindlimbs, and the adrenal glands. Against all expectations, an increased sympathetic nerve activity (SNA) in response to leptin was reported in both thermogenic and cardiovascular organs. It was also shown that mutation in the *db* gene in the obese Zucker rat abolished leptin-mediated increases in SNA (Haynes et al. 1997a). These data provided the first evidence suggesting that leptin could contribute to cardiovascular control as well as thermogenesis via mechanisms requiring intact leptin receptors.

Leptin Control of Blood Pressure in Male Animals

Early evidence demonstrating that leptin intervenes in the control of cardiovascular function, notably blood pressure (BP) regulation, was provided by the group of *John Hall* who demonstrated that rising leptin levels via chronic intravenous or intra-arterial infusion of leptin in healthy male Sprague-Dawley rats elevated BP and heart rate, despite decreasing body weight, a condition which would characteristically be associated with a reduction in BP (Shek et al. 1998). In subsequent studies, *Correia et al.* reported that chronic intracerebroventricular injection of leptin reproduced the BP and heart rate effects of systemic leptin infusion, an observation that suggested a central origin for the effects of leptin on cardiovascular regulation and the contribution of the autonomic nervous system (ANS) (Correia et al. 2001). The contribution of the ANS, notably of the sympathetic branch of the ANS, was established through the demonstration that α- and β-adrenergic receptor blockade prevented leptin-mediated increases in BP and heart rate (Carlyle et al. 2002). Consistent with the decreases in BP induced by adrenergic receptor blockade, elevated plasma catecholamine (Satoh et al. 1999), increased renal and lumbar SNA, and decreased skeletal and splanchnic vascular conductances accompanied BP elevation induced by acute intracerebroventricular leptin injection in male Wistar rats (Dunbar et al. 1997). Access to genetically engineered lean mouse models that either overexpress leptin (Aizawa-Abe et al. 2000), are hypersensitive to leptin (Belin De Chantemele et al. 2009), or target a specific protein of the leptin signaling pathway (genome-wide or in specific neurons) allowed further confirmation of the interaction between leptin, sympathetic activity, and BP but also facilitated the

identification of the hypothalamic nuclei and signaling pathways involved in leptin control of sympathetic tone, notably renal SNA (Hall et al. 2015; Harlan and Rahmouni 2013; Simonds et al. 2014). Consistent with the observation that arcuate nucleus (ARC) neurons send projections to sympathetic preganglionic neurons in the intermediolateral nucleus, it was demonstrated that injections of leptin into the ARC increased renal and brown adipose tissue SNA (Rahmouni and Morgan 2007), while lesions of the arcuate nucleus prevented increased brown adipose tissue SNA when leptin was infused (Mark 2013). Furthermore, leptin receptor deletion in the ARC attenuated increases in renal SNA and brown adipose tissue SNA evoked by leptin (Harlan et al. 2011a). These studies suggest that the ARC is an important site for the effects of leptin on SNA. In fact, deletion of leptin receptor only in proopiomelanocortin neurons, a major type of neuron in the ARC, prevents increased BP during chronic hyperleptinemia (do Carmo et al. 2011). Collectively, these studies demonstrate that leptin contributes to BP and heart rate control via central activation of the ANS in lean healthy male animals. However, it is critical to recognize that, at the exception of our study in leptin-sensitized animals where hypertension was observed without exogenous leptin administration (Belin de Chantemele et al. 2009), the link between leptin, SNA, BP, and heart rate has essentially been established with supraphysiological doses of leptin often a thousandfold higher than the physiological concentration. While the mechanisms identified certainly contribute to the cardiovascular phenotype of hyperleptinemic male animals (discussed below), they might play a minimal role in the control of cardiovascular function in physiological conditions.

Leptin Control of Blood Pressure in Female Animals

Although compelling evidence indicates that leptin is secreted in a sex-specific manner (Frederich et al. 1995; Maffei et al. 1995), only a very few studies investigated the interaction between leptin, SNA, and BP in female animals. A first study by *Shi et al.* reported that acute intracerebroventricular leptin infusion increased lumbar and renal SNA and elevated heart rate in female rats (Shi and Brooks 2015). The effects of leptin on SNA and heart rate were however observed in proestrus and estrogen-treated ovariectomized rats only. Neither ovariectomized female rats nor rats in the diestrus phase of their menstrual cycle exhibited increases in SNA in response to leptin, suggesting that leptin-mediated increases in SNA require the presence of estrogen. Consistent with the acute studies conducted in male animals (Haynes et al. 1997a), estrogen-treated ovariectomized female rats and female rats in the proestrus phase of their cycle did not exhibit increases in BP in response to leptin, despite presenting increases in splanchnic, lumbar, and renal SNA (Shi and Brooks 2015). This may suggest that leptin concomitantly activated depressor mechanisms compensating for the increase in sympathetic activity. In the present study, leptin was infused intracerebroventricularly. Therefore, this would imply that

leptin activates pressor and depressor mechanisms concomitantly via a central action. Compelling evidence indicates that estrogen is a potent inhibitor of the sympathetic nervous system (Hay 2016) and that women, despite exhibiting two to four times more leptin than men (Schwartz et al. 1996; Considine et al. 1996), have lower SNA than men until menopause (Matsukawa et al. 1998). Therefore, the data by *Shi et al.* appear counterintuitive and may lack in physiological significance. The omission of the sexual dimorphism in plasma and cerebrospinal fluid levels of leptin and the use of a single supraphysiological dose of leptin infused intracerebroventricularly further raise concerns regarding the physiological relevance of the results of the aforementioned study.

More insights into the role of leptin in the control of sympathetic tone and arterial pressure in females have been provided by a recent study by our group investigating the cardiovascular phenotype of mice rendered hypersensitive to leptin via the deletion of protein tyrosine phosphatase 1b, a molecular brake on leptin signaling (Zabolotny et al. 2002). Hypersensitivity to leptin raised BP in a leptin-dependent manner in both male and female animals, despite slightly reducing circulating leptin levels in both sexes of animals. However, leptin sensitization increased sympathetic activity and, more precisely, neurogenic control of BP in male animals only (Belin de Chantemele et al. 2009). Consistent with the human literature indicating that women have a lower central sympathetic neural output to the periphery than males (Hogarth et al. 2007; Matsukawa et al. 1998) despite higher leptin levels (Considine et al. 1996), female leptin-sensitized mice exhibited reduced neurogenic control of BP, reflected by a reduced BP response to ganglionic blockade and lower plasma catecholamine levels (Huby et al. 2016). These data dissociating leptin from sympathetic control in females suggest for the first time that leptin might intervene in the control of cardiovascular function, notably BP via sex-specific mechanisms (Huby et al. 2016).

Leptin Control of Blood Pressure and Sympathetic Tone in Men and Women

The rodent literature investigating the contribution of leptin to sympathetic activity and BP control is quite extensive and collectively suggests that leptin intervenes in the control of BP via regulating sympathetic tone in males. A few studies have investigated the contribution of leptin to sympathetic and BP control in healthy humans only and failed to produce consistent data. The complexity of the measurements as well as the heterogeneity of the populations studied and techniques used are likely the sources of the conflicting results.

The group of *Eric Ravussin* was the first to assess the relationship between leptin and sympathetic activity in humans. Based on the observation that restoration of leptin levels in *ob* mice restored thermogenesis via restoration of sympathetic outflow (Pelleymounter et al. 1995), this group investigated the relationship between fat mass, fasting leptin levels, and basal muscle sympathetic nerve activity (MSNA)

in healthy young adult men. A linear relationship between leptin and MSNA was reported (Snitker et al. 1997). However, a major limitation to this study was the lack of adjustment of leptin levels for body mass index (BMI), suggesting that the relationship between leptin and MSNA could have been secondary to a similar relationship between body fat and MSNA (Scherrer et al. 1994). To prevent any potential interference from BMI, Machleidt et al. measured MSNA response to a supraphysiological dose of leptin in 12 healthy young male subjects matched for BMI. A positive correlation between leptin and MSNA was identified, which further supports a role for leptin in the control of sympathetic in healthy young male subjects (Machleidt et al. 2013). Subsequent studies focused on sex differences. While studying a population of 88 healthy young adult men and women, *Narkiewicz et al.* reported a positive relationship between leptin and heart rate in men but no correlation between leptin and MSNA or BP (Narkiewicz et al. 2001). They showed that this relationship is independent of BMI, waist-to-hip ratio, and percentage of body fat. No relationship between leptin and MSNA or heart rate was reported in young adult women (Narkiewicz et al. 2001). Although in contradiction with the studies by *Snitker* and *Machleidt*, the study by *Narkiewicz* was the first to suggest a potential sex-specific relationship between leptin and autonomic control of the cardiac function. This sex-specific interaction is intriguing and its origin not fully explained. Due to the lower leptin levels and the absence of the inhibitory effects of estrogen, it has been speculated that men are likely more sensitive to leptin and its excitatory effects than women (Hinojosa-Laborde et al. 1999). Several years later, *Flanagan et al.* used power spectral analysis of heart rate variability, an indirect assessment of cardiac autonomic balance, to investigate any potential relationship between leptin and autonomic control of cardiac function. An association between increased fasting leptin levels and increasing heart rate was reported in women. This relationship, specific to women, was not altered by stepwise linear regression adjustment for BMI and other measures of body fat (waist-to-hip ratio, each skinfold measure, and the sum of skinfolds) (Flanagan et al. 2007). Leptin correlated positively with the low-frequency (LF) power and negatively with the high- to low-frequency (HF/LF) ratio of the power spectral analysis, suggesting that leptin is associated with increased cardiac sympathetic and decreased cardiac vagal tone in women. However, no association was seen between leptin and BP (Flanagan et al. 2007). These results are in direct opposition to the results by *Narkiewicz* reporting an association between leptin and heart rate in men (Narkiewicz et al. 2001). The different methods (direct MSNA in the *Narkiewicz's* study versus indirect spectral analysis), the types of sympathetic activity recorded (peripheral skeletal muscle versus cardiac), as well as the conditions of recording (well-controlled resting conditions in the *Narkiewicz's* study versus 15 min ECG recording in the *Flanagan's* study) likely contribute to the different results but also highlight the need for additional and standardized studies investigating the relationship between leptin, sympathetic activity, and BP in men and women. A safe interpretation of these conflicting results is that, in physiological conditions, leptin only minimally contributes to sympathetic tone and BP control in both men and women. Evidence to support this marginal action of leptin in healthy patients is the demonstration that elevation of leptin levels over a 6-day period

negligibly altered cardiac autonomic activity in male subjects (Mackintosh and Hirsch 2001).

Leptin Control of Renal Function

Compelling evidence from the rodent literature indicates that, although acute leptin increases renal, adrenal, and splanchnic SNA, it has minimal effects on BP (Dunbar et al. 1997), suggesting that leptin-mediated sympatho-activation is balanced by depressor mechanisms. Leptin-mediated natriuresis has been identified as a potential depressor mechanism. The kidneys have been reported to express specific leptin binding sites in the renal medulla (Serradeil-le Gal et al. 1997) indicating that leptin may exert direct functional effects in this organ (Emilsson et al. 1997; Serradeil-le Gal et al. 1997) and supporting a potential role for this hormone in the regulation of sodium-volume balance. Evidence in support of the functional role of leptin receptors was provided by reporting that acute leptin infusion increased diuresis and natriuresis, in male animals (Serradeil-le Gal et al. 1997; Haynes et al. 1997b; Jackson and Li 1997; Villarreal et al. 1998), while leptin blockade with a polyclonal antibody reduced urinary sodium excretion and urinary flow (Villarreal et al. 2006). These natriuretic effects of acute leptin infusion have been observed with either intraperitoneal (Beltowski et al. 2002a), intravenous (Villarreal et al. 1998), or intrarenal artery (Jackson and Li 1997) infusion, with no change in either creatinine clearance, activation of the renin-aldosterone axis, glomerular filtration rate, or potassium excretion. Therefore, it has been speculated that the natriuretic effects of leptin result from inhibiting tubular sodium reabsorption (Villarreal et al. 1998, 2000; Jackson and Li 1997). Blockade of nitric oxide (NO) production with the unspecific nitric oxide (NO) synthase inhibitor, *N*ω-nitro-l-arginine methyl ester (L-NAME), blunted the natriuretic and diuretic effects of leptin in healthy male rats (Villarreal et al. 2004), indicating that leptin-induced natriuresis involves an NO-dependent mechanism.

Despite the compelling evidence that leptin stimulates natriuresis (Serradeil-le Gal et al. 1997; Haynes et al. 1997b; Jackson and Li 1997; Villarreal et al. 1998), the physiological significance of the acute natriuretic effects of leptin remains incompletely understood, notably because these effects are exerted at hormone concentrations markedly exceeding the physiological range. Another major limitation of these studies is the absence of experiments in females which, on top of producing more leptin than males, express higher renal endothelial and inducible NO synthases than males and are less sensitive to NO synthase inhibition (Reckelhoff et al. 1998; Neugarten et al. 1997). Therefore, further studies are required to determine whether leptin similarly controls natriuresis in males and females and whether leptin-mediated natriuresis occurs in physiological conditions to balance the sympatho-mediated pressor effects of leptin.

Although several studies identified the kidneys as a potential organ counterbalancing the pressor action of leptin-mediated sympatho-activation, numerous animal

studies investigating the mechanisms whereby chronic leptin infusion elevates BP present the kidneys as one of the major effectors of the cardiovascular actions of leptin and associated leptin-mediated increases in BP with increased renal sympathetic nerve activity (Dunbar et al. 1997; Harlan et al. 2011a, b; Mark et al. 2009). However, the mechanism whereby the centrally mediated increase in renal SNA (Dunbar et al. 1997; Harlan et al. 2011a, b; Mark et al. 2009) elevates BP remains incompletely understood. Based on the evidence that leptin-mediated increases in renal sympathetic activity do not reduce renal vascular conductance (Dunbar et al. 1997) nor increase sodium retention in healthy rats (Kuo et al. 2001; Shek et al. 1998), it can be speculated that leptin-mediated increases in renal SNA stimulate renin secretion and elevate BP via renin-dependent mechanisms in male animals. This hypothesis finds its rational in the rate-dependent effects of sympathetic activity on renal function. Indeed, while high rates of renal nerve activity are required to increase fluid and sodium reabsorption and to impair renal hemodynamics, low rates of sympatho-excitation induce a prompt increase in renin secretion (Dibona 2000). Again, a major limitation of these studies is the absence of experiments in females, leading to a gap in our knowledge of the chronic effects of leptin on renal SNA activity and hemodynamics in females. The inhibitory effects of female sex steroids on renin secretion (Schunkert et al. 1997) might presage sex differences.

Collectively, these data suggest a dual role for leptin in the control of renal hemodynamics in male animals: a direct NO-mediated natriuresis counterbalancing centrally mediated pressor mechanisms likely stimulating renal sodium reabsorption via renin-dependent mechanisms.

Leptin Control of Vascular Function

Studies investigating the contribution of leptin to BP regulation have demonstrated that the effects of leptin are more evident when leptin is infused into a carotid artery (Shek et al. 1998) or directly into cerebroventricles (Dunbar et al. 1997). Chronic intravenous or subcutaneous leptin infusion induced modest to no increase in pressure (Haynes et al. 1997a) suggesting that, in these circumstances, the hypertensive influences of leptin might be affected by antagonistic actions of the hormone and that sympathetic activation is balanced by depressor mechanisms. In the previous paragraph, we discussed evidence supporting a role for leptin in the control of diuresis and natriuresis; however, other evidence suggests that leptin exerts additional depressor actions via modulating vascular tone. Early studies demonstrated that endothelial cells express the long (ObRb), signaling form of the leptin receptor (Sierra-Honigmann et al. 1998). Subsequent studies by *Lembo et al.* showed that leptin induces a dose-dependent vasorelaxation in aortic rings of Wistar-Kyoto rats (Lembo et al. 2000) and that leptin-mediated vascular relaxation is absent in endothelial-denuded vessels and abolished by exposure to the unspecific NO synthase inhibitor, L-NAME (Lembo et al. 2000; Kimura et al. 2000). This suggests that the vasodilatory response to leptin requires an intact endothelium and is

NO-mediated. In support of this concept, *Fruhbeck et al.* (Fruhbeck 1999) and *Beltowski et al.* (2002b) reported that leptin induces a dose-dependent increase in plasma NO that is absent in obese Zucker rats, which supports the dependence of this mechanism on the presence of functional leptin receptors (Fruhbeck 1999). Collectively, these data indicate that NO plays an important role in mediating the depressor action of leptin. The physiological significance of these observations remains, however, incompletely understood. Indeed, while leptin reduces BP in sympathectomized rats (Lembo et al. 2000), it has minimal effects on cardiovascular hemodynamics in the presence of L-NAME, despite continued sympatho-activation (Mitchell et al. 2001; Kuo et al. 2001). In accordance, it has been shown that leptin does not dilate hind limb vascular beds of conscious rats (Rahmouni et al. 2005). Contrarily, while experiments conducted in male human subjects confirm the vasodilatory properties of leptin, they challenged the NO dependence of the mechanism by reporting that high doses of acute leptin increase forearm blood flow (Nakagawa et al. 2002) and cause coronary vasodilatation (Matsuda et al. 2003) via mechanisms independent of NO. All together these studies may restrict the vascular, NO-mediated depressor effects of leptin to acute leptin exposure and minimize the physiological relevance of the direct stimulation of endothelial NO secretion induced by leptin.

As for many aspects of the physiology of leptin, the vascular effects of leptin have mainly been studied in male animals and subjects. Based on the unambiguous evidence that females have a better endothelial function than males (Green et al. 2016), and secrete higher leptin levels (Considine et al. 1996), it could easily be speculated that leptin-mediated vascular NO secretion would be exacerbated in females. Nevertheless, studies by our group suggest the contrary. We recently reported that increasing leptin sensitivity in mice, via genome-wide deletion of protein tyrosine phosphatase 1b (Ptp1b), a molecular brake on leptin signaling, markedly impaired endothelium-dependent relaxation in female mice only (Huby et al. 2016). Endothelial function was preserved in male animals despite hypersensitivity to leptin (Belin de Chantemele et al. 2009). Arguments to support a detrimental role for leptin in the control of endothelial function in female mice were provided by demonstrating that chronic leptin receptor blockade restored endothelial function in leptin-sensitized mice (Huby et al. 2016), while chronic leptin infusion dose-dependently impaired endothelial function in healthy wild-type female mice (Huby et al. 2015). Antagonism of aldosterone action via the mineralocorticoid receptor blocker spironolactone restored endothelial function in leptin-sensitized mice (Huby et al. 2016) and prevented leptin-mediated endothelial dysfunction in wild-type female mice (Huby et al. 2015), suggesting that leptin impairs endothelial function via indirect mechanisms involving mineralocorticoid receptor activation in female mice. Collectively, these studies indicate that leptin intervenes in the control of endothelial function via sex-specific mechanisms involving direct endothelial NO release in males and aldosterone-dependent activation of the mineralocorticoid receptor in females.

As suggested by the human studies conducted by the group of *Kazuaki Chayama* (Matsuda et al. 2003; Nakagawa et al. 2002), leptin-induced endothelium NO

production is likely not the only mechanism whereby leptin controls vascular function and exerts depressor activity in males. Several studies by our group demonstrated that increasing leptin sensitivity via Ptp1b deletion (Belin De Chantemele et al. 2009) or chronic elevation in leptin levels in healthy male mice (Belin de Chantemele et al. 2011) reduced vascular adrenergic contractility. Chronic leptin sensitization and infusion did not affect general vascular contractility but lowered vascular adrenergic contractility specifically likely via a reduction in vascular α-adrenergic receptor expression. Chronic sympatho-inhibition via α-adrenergic receptor blockade with prazosin restored vascular adrenergic contractility in leptin-sensitized mice and prevented leptin-induced vascular adrenergic desensitization (Belin de Chantemele et al. 2009). In addition, chronic α_1-adrenergic receptor stimulation with phenylephrine mimicked the effects of leptin on vascular adrenergic contractility and reduced vascular adrenergic contractility (Bruder et al. 2015). Collectively these data demonstrated that leptin specifically reduces vascular α-adrenergic signaling via sympatho-mediated mechanisms in male animals. This leptin-mediated reduction in vascular adrenergic contractility might represent another compensatory mechanism counterbalancing the pressor effects of leptin. The absence or low dose of leptin infused (0.3 mg/kg/day) supports the physiological relevance of this mechanism (Belin de Chantemele et al. 2009, 2011). Evidenced in several mouse models, this leptin-mediated reduction in vascular adrenergic contractility is absent in female mice (*AC. Huby & EJ. Belin de Chantemèle, unpublished data*). Leptin-mediated reduction in vascular adrenergic tone is sympatho-mediated in males. Therefore the lack of sympatho-activation reported in female mice in response to leptin sensitization and infusion (Huby et al. 2016) is likely the cause of the persevered vascular adrenergic reactivity. This sexual dimorphic response of the vasculature to leptin is therefore likely to be the result of the sexual dimorphic response of the sympathetic nervous system to leptin.

Although this chapter limits its focus on the role of leptin in the control of autonomic, renal, and vascular functions, the effects of leptin are not limited to these specific organs. Compelling evidence indicates that leptin receptors are also expressed in the heart and vascular smooth muscle cells and that leptin contributes to the regulation of these organs as well. However, whether leptin regulates the function of these organs via sex-specific mechanisms is currently unknown and remains to be investigated.

Leptin Contribution to Obesity-related Cardiovascular Disease

Excess body fat leading to overweight (25<BMI<30) and obesity (BMI>30) is the plague of our time. Currently more than 1.9 billion adults worldwide are overweight, of which 600 million are obese (World Health Organization). Besides the accumulation of metabolic alterations discussed in other chapters, one of the major

consequences of this worldwide increase in the prevalence of overweight and obesity is an enhanced cardiovascular morbidity and mortality, in part through development of hypertension (Go et al. 2013). The increased adipose mass associated with overweight and obesity is the source of marked increases in plasma leptin levels and the origin of the high circulating leptin levels of overweight and obese human subjects (Considine et al. 1996). As reviewed above, leptin is a hormone secreted in a sex-specific manner, females producing three to four times more leptin than males (Maffei et al. 1995). This sexual dimorphism in leptin levels is even further exacerbated with obesity. Notably, leptin levels rise 3.4-fold more rapidly as a function of BMI in women than in men (Kennedy et al. 1997). In the light of the cardiovascular actions of leptin described previously, leptin appears as an evident link between excess fat mass and cardiovascular disease, notably hypertension, in male and female patients.

Human Evidence

The first lines of evidence linking leptin to hypertension in the context of metabolic diseases were provided by *Agata and Hirose*, who reported a significant positive correlation between plasma leptin concentrations and BP in Japanese normal-weight insulin-resistant patients with essential hypertension as well as in Japanese obese male adolescents (Agata et al. 1997; Hirose et al. 1998). Using a larger population followed over an 8-year period, the group of *Pasquale Strazzullo* reported a graded positive relationship between leptin levels and BP which was independent of age, body mass, abdominal adiposity, fasting serum insulin, and creatinine levels (Barba et al. 2003). They further showed, in a sample of originally normotensive men, that circulating leptin levels were a significant predictor of the risk to develop hypertension, independent of BMI and insulin resistance (Galletti et al. 2008). The small size of the populations (Agata et al. 1997) and the absence of female participants (Hirose et al. 1998; Barba et al. 2003; Galletti et al. 2008) did, however, not allow the determination of the contribution of the biological sex to this positive association. Subsequent studies investigated whether the relationship between leptin and BP is sex-specific. A few studies conducted in Chinese and Italian populations reported elevations in leptin levels in male hypertensive patients only but no increase in circulating levels in hypertensive female patients although their leptin levels were higher than in males (Sheu et al. 1999; Mallamaci et al. 2000; Khokhar et al. 2010). These first studies suggested that leptin could contribute to the development of hypertension in males only. Large multicentric and multiethnic studies challenged this concept by identifying significant associations between plasma leptin levels and both BP and hypertension in pre- and postmenopausal women (Itoh et al. 2002; Ma et al. 2009; Shankar and Xiao 2010). The strength of this correlation was supported by a study by Itoh et al. demonstrating that changes in BP correlated with changes in leptin levels during a 3-month body weight reduction program in obese women (Itoh et al. 2002). The ethnicity and the reduced sample

size of the initial Chinese and Italian studies (Mallamaci et al. 2000; Sheu et al. 1999) might contribute to the discrepancy between studies.

Genetic factors also appear to intervene in the relationship between leptin and hypertension but with limited effects. Family-based association analyses in the National Heart, Lung, and Blood Institute Family Heart Study tested the hypothesis that leptin *(Ob)* gene variants and plasma leptin levels influence variability in BP and the risk of hypertension differently by sex (Ma et al. 2009). Significant associations between *Ob* single nucleotide polymorphisms with BP and hypertension were identified but in postmenopausal women only. Several other studies investigated the relationship between leptin receptor (*db*) gene polymorphisms (Lys109Arg, Gln223Arg, and Lys656Asn) and hypertension risk in different ethnic groups. The results are nevertheless controversial. Indeed, while *Liu* et al. demonstrated that the *LEPR* Gln223Arg polymorphism had an important role in a patient's susceptibility to hypertension in the Northern Han Chinese population (Liu et al. 2014), Rosmond et al. suggested that the variants of Lys109Arg and Gln223Arg protect from hypertension in the Swedish population (Rosmond et al. 2000). However, investigation of the contribution of both the biological sex and the degree of metabolic disease has been omitted in these two studies.

Mechanisms Linking Hyperleptinemia to Hypertension in Obese Males and Females

Mechanistic studies conducted in healthy volunteers and animals identified a role for leptin in the control of the autonomic nervous system and reported that, in several conditions, leptin-mediated increases in sympathetic activity are associated with BP elevations. Compelling evidence suggests that increased sympathetic nervous system activity plays a major role in obesity-associated hypertension in males. Indeed, when plasma and urinary catecholamine concentrations are increased in both obese human patients and animal models of obesity (Vaz et al. 1997; Esler et al. 2006), pharmacological blockade of the sympathetic nervous system and renal denervation markedly attenuate sodium retention and hypertension associated with a high-fat diet in experimental animals (Kassab et al. 1995; Rocchini et al. 1999; Asirvatham-Jeyaraj et al. 2016). In addition, while obesity increases MSNA and renal norepinephrine spillover in human subjects (Grassi et al. 1995; Vaz et al. 1997), body weight reduction decreases both MSNA and plasma noradrenaline levels in obese male individuals (Lambert et al. 2007). Arguments to support a role for leptin in this increased sympatho-activation associated with obesity were provided by demonstrating that leptin-deficient *ob/ob* mice have slightly lower BP than their wild-type controls despite profound obesity that would be expected to increase arterial pressure (Mark et al. 1999; Aizawa-Abe et al. 2000). Consistent with these findings, genetically based leptin deficiency due to a missense leptin gene mutation protects men and women from hypertension and sympatho-activation, despite

leading to severe obesity (Ozata et al. 1999). In addition, dysfunctional mutations of the hypothalamic melanocortin 4 receptor (MC4R), a major effector of the transduction of the hypothalamic leptin signal (Hall et al. 2015), revert the positive correlation usually found between leptin and MSNA in male and female individuals with intact MC4R and appear to protect MC4R-mutant individuals from sympathetic overdrive and consequent hypertension (Greenfield et al. 2009; Sayk et al. 2010). These findings suggest that obesity per se is not sufficient to raise sympathetic activity and BP and that leptin is required to mediate these effects.

Lines of evidence support a role for leptin in obesity-associated hypertension and sympatho-activation in males; however, whether obesity and leptin increase sympathetic activity in female is less evident. Healthy premenopausal women secrete three to four times more leptin than men (Considine et al. 1996; Kennedy et al. 1997) but exhibit lower sympathetic tone than men of the same age (Christou et al. 2005; Matsukawa et al. 1998). In response to obesity, women exhibit exaggerated increases in leptin levels compared with men (Kennedy et al. 1997) but with mild (Jones et al. 1996; Lambert et al. 2007) to no comparable increases in sympathetic tone (Bell et al. 2001; Messina et al. 2013; Tank et al. 2008). In addition, reduction in body weight decreases sympathetic activity in obese male individuals only (Lambert et al. 2007). Consistent with the observation that obesity is not associated with an increase in sympathetic activity in premenopausal women, our group recently reported that obesity does not elevate circulating catecholamine levels in yellow obese agouti female mice. We also showed that hypertension was associated with a reduced neurogenic control of BP, suggesting that obesity-mediated hypertension is independent of increases in sympathetic activity in females (Huby et al. 2016). Chronic leptin receptor blockade restored BP in yellow obese agouti female mice, supporting the leptin dependence of the hypertension and the dissociation between leptin and sympatho-activation reported in lean leptin-sensitized female mice (Huby et al. 2016). Consistent with these mouse data, *Matsumoto et al.* reported a similar dissociation between obesity and sympathetic activity in obese adult Japanese women (Matsumoto et al. 2003), reinforcing the concept that obesity-associated hypertension does not involve leptin-induced sympatho-activation in women.

Aldosterone in Obesity-related Hypertension

Besides sympatho-activation, excess production of the mineralocorticoid hormone aldosterone is another key characteristic of obesity (Calhoun and Sharma 2010). Interestingly, aldosterone, a major contributor to both cardiovascular and metabolic dysfunctions associated with obesity, has been reported to correlate with the degree of adiposity and hypertension in obese women but not in men (Kanashiro-Takeuchi et al. 2009; Vasan et al. 2004). Clinical evidence in support of a functional role for these high aldosterone levels was provided by reporting that antagonism of aldosterone action via mineralocorticoid receptor (MR) blockade is more effective at preventing cardiovascular disease in women compared to men (Kanashiro-Takeuchi

et al. 2009; Vasan et al. 2004). Until very recently the origin of the apparent hyperaldosteronism induced by obesity remained unknown. Neither angiotensin nor plasma potassium or adrenocorticotropic hormone, the three main regulators of aldosterone secretion (Hattangady et al. 2012), had been shown to contribute to the inappropriately high aldosterone levels observed in obese patients (Bochud et al. 2006; Rocchini et al. 1986). Because aldosterone levels are positively correlated with waist circumference, the contribution of adipocyte-derived factors had been investigated (Bochud et al. 2006; Rocchini et al. 1986) and the existence of as-yet-unidentified macromolecular stimuli of aldosterone synthesis released from cultured human adipocytes reported (Ehrhart-Bornstein et al. 2003). Recent work by our group identified leptin as the adipocyte-derived factor regulating adrenal aldosterone synthase expression and stimulating aldosterone production in females (Huby et al. 2015). Additionally this study provided convincing data supporting a key role for leptin-mediated aldosterone production in the development of obesity-related cardiovascular disease, notably hypertension. Consistent with the human studies, we reported that obesity induced a threefold elevation in plasma aldosterone levels and adrenal gland aldosterone synthase expression in yellow obese agouti female mice only. Chronic leptin receptor blockade restored plasma aldosterone levels and BP in female agouti mice, supporting a role for leptin in both obesity-associated hyperaldosteronism and obesity-induced hypertension. In parallel, chronic inhibition of aldosterone action via MR blockade restored BP, indicating that leptin raises BP via aldosterone-dependent mechanisms in obese female mice (Huby et al. 2016) and supporting the predominance of the leptin-aldosterone axis over the leptin-mediated sympatho-activation pathway in obese female mice. In the process of investigating the mechanisms whereby aldosterone elevates BP, we reported, in agreement with the human literature showing that obese female patients are more prone to vascular dysfunction than obese men (Safar et al. 2013), that yellow obese agouti female mice exhibited endothelial dysfunction when males did not, despite presenting similar BP levels as obese female mice. Leptin receptor and MR blockade restored endothelial function in female animals supporting a role for leptin-mediated aldosterone secretion in endothelial dysfunction and, likely, the contribution of endothelial dysfunction to the development of hypertension. Collectively, these studies provide a body of evidence to support the new concept that leptin drives hypertension via sex-specific mechanisms in obesity: sympatho-activation in males and activation of the aldosterone-MR axis in females (concept summarized in Fig. 1).

Sex Differences in Selective Leptin Resistance in Obesity

A common feature of obesity is the development of a resistance to the anorexigenic effects of leptin, leading to a failure of leptin to promote weight loss in obesity despite high leptin levels (Considine et al. 1996). In light of the observations that leptin-mediated stimulation of brown adipose tissue innervation is lost with obesity,

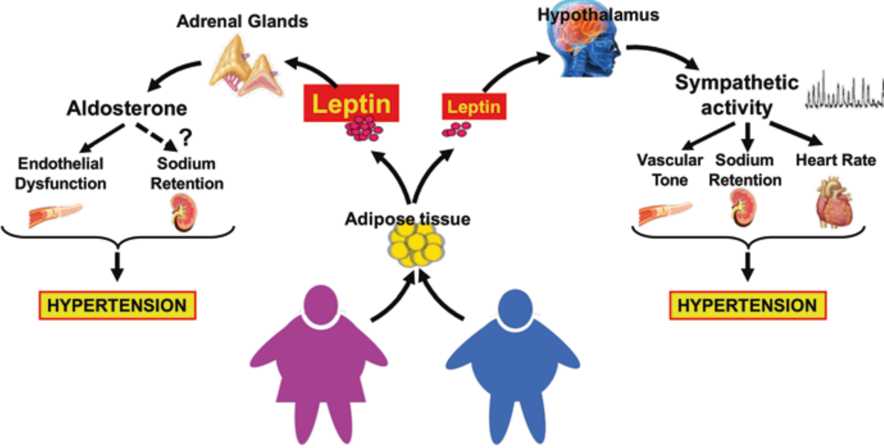

Fig. 1 Leptin drives hypertension via sex-specific mechanisms in obesity. Summary of the potential mechanisms whereby leptin elevates blood pressure in male and female with obesity

but leptin-mediated activation of renal sympathetic nerve activity is preserved, the group of *Allyn Mark* developed the concept of "selective leptin resistance" in male animals (Correia et al. 2002; Mark 2013). The results of these studies, which were restricted to renal sympathetic activity, were expanded by the work from our group that reported preserved sensitivity to leptin-mediated increases in neurogenic control of the vasculature and leptin-induced aldosterone production in obese male animals (Belin de Chantemele et al. 2011). In a recent study, we demonstrated that leptin-mediated adrenal aldosterone production is also preserved in obese female mice that are resistant to the anorexigenic effects of leptin (Huby et al. 2015). This observation led to the conclusion that the concept of selective leptin resistance is not sex-specific and can be extended to females as well.

Conclusion and Clinical Perspective

The discovery of leptin in 1994 was followed by the almost immediate demonstration that leptin is secreted in a sex-specific manner and contributes, when secreted in excess with overweight and obesity, to cardiovascular disease in both men and women. While an impressive body of literature immediately tackled the task of identifying the mechanisms whereby hyperleptinemia contributes to obesity-related hypertension in males, thus implicating leptin-induced sympatho-activation as a major contributor, more than two decades have been necessary to see the emergence of studies investigating the consequences of the sexual dimorphism in leptin secretion on the cardiovascular function. The recent studies involving female animals led to the discovery of a dissociation between leptin levels and sympathetic activity in

obese females, followed by the demonstration that leptin is a direct regulator of aldosterone synthase expression, and to the finding that excess leptin associated with obesity drives hypertension via activation of the aldosterone-mineralocorticoid receptor axis. These findings led to the new concept that leptin induces hypertension via sex-specific mechanisms in obesity: sympatho-activation in males and activation of the aldosterone-mineralocorticoid receptor axis in females. These novel findings, which likely contribute to explain the higher responsiveness of obese women to mineralocorticoid receptor blockade, might represent one more step toward personalized and sex-specific medicine.

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Sex Effects at the Ramparts: Nutrient- and Microbe-Mediated Regulation of the Immune-Metabolic Interface

Nyrie Israelian and Jayne S. Danska

Abstract The relationships between dietary compounds, derivative metabolites, and host metabolism and immunity are controlled by diverse molecular mechanisms. Essential contributions to these dynamics come from the community of microbes (the microbiome) inhabiting the human digestive tract. The composition and function of the microbiome are shaped by available nutrients, and reciprocally, these organisms produce an as yet poorly defined repertoire of molecules that communicate with the epithelial barrier and the mucosal immune system. We present evidence that diet-derived vitamins and lipids regulate immunity and metabolic function and highlight the diverse mechanisms through which these effects are impacted by sex. We discuss exciting new data emerging from studies using high-throughput sequencing technology, specialized mouse models, and bio-specimens, and clinical data from human subjects that have begun to reveal the complexity of these interactions. Also profiled in this chapter are the striking sex differences in pathways by which dietary nutrients and gut microbes modify metabolism, immunity, and immune- and inflammation-mediated diseases. Although the incidence, severity, and therapeutic responses of many autoimmune diseases differ by sex, the molecular mechanisms of these effects remain poorly understood.

Sexual dimorphism is displayed at multiple levels of immune system regulation. In general, adult females mount stronger immune responses than males, are more resistant to infection than males, and exhibit more robust responses to vaccines than

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males (Klein and Flanagan 2016). The Janus-faced nature of the stronger female immune response is demonstrated in their increased susceptibility to inflammatory and autoimmune disease compared to males. The diverse effects of immunological sex differences manifest not only in anti-infectious and antineoplastic functions but also in the converging effects of microbes, nutrients, and metabolites.

Multiple mechanisms contribute to sex-based immunological differences. Sex steroid hormones affect multiple aspects of immunity. Estrogens and estrogen receptor (ER) activity display dose- and context-dependent effects on multiple lineages of immune cells, where they can both augment and dampen immune signaling pathways (Klein and Flanagan 2016). Androgens exert an overall immunosuppressive effect on the immune system, mediated through the androgen receptors (AR) (Trigunaite et al. 2015). Genes encoded on the X and Y chromosomes also mediate sex differences in immune responses. For example, a significant number of X chromosome genes regulate immune function, including cell proliferation and activation, inflammatory signaling, and immune tolerance (Libert et al. 2010). Polymorphism in Y chromosome genes may also contribute to infectious and autoimmune disease susceptibility (Case et al. 2013).

There are sex differences in the incidence, progression, and severity of many immune-mediated diseases. A strong female bias is observed in the prevalence of many autoimmune diseases, with female-to-male ratios highest in Hashimoto thyroiditis, Graves' disease, systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), and multiple sclerosis (MS) (Ngo et al. 2014). Autoimmune disease severity may also differ between sexes. Although MS is more prevalent in females, the course of MS in males is often more severe, with more rapid progression of disability and poorer recovery from relapse (Golden and Voskuhl 2017). Excluding the reproductive organs, many cancers demonstrate significant sex bias in incidence, prognosis, and response to treatment (Dorak and Karpuzoglu 2012). Males are disproportionately affected by cancer across many geographical regions. Furthermore, for the majority of cancers, males have almost twofold greater risk of dying from certain cancers than females. While gender differences in behavior undoubtedly contribute to these differential risks, biological mechanisms also contribute to this male bias, including sex differences in hormonal regulation and tissue-specific gene expression.

The Microbiome

A critical part of the network linking sex and immunity are the communities of microbes known as "the microbiome." The microbiome is comprised of trillions of bacteria, viruses, and fungi that colonize human mucosal surfaces. The intestinal microbiome is critical for the development and maturation of the immune system (Round and Mazmanian 2009). Reciprocally, the gut epithelial and associated immune tissue response to the microbiome impacts microbial composition and function in the intestine. The dynamic cross talk between the microbiota and host

tissues shapes both mucosal and systemic immune homeostasis. Diversity and richness of microbial community composition and function influence the regulation of immune and metabolic health of the host. Gut microbiome composition is shaped both by host genetics and the environment, particularly diet and xenobiotic exposure. Perturbation resulting in an "altered" microbial composition, termed dysbiosis, has been identified as a likely causal factor of metabolic and autoimmune diseases (Belkaid and Hand 2014).

Interactions between immune and metabolic function are pivotal in the health of both systems, and the microbiome is a key actor in this interplay. Gut microbes are essential for the production of diet-derived metabolites that control host metabolic and immune functions (Rowland et al. 2017; Sonnenburg and Backhed 2016). Diet shapes gut microbial composition and function, and these effects are strongly implicated in the burgeoning incidence of chronic diseases including obesity, diabetes, and autoimmunity. The Western diet characterized by high consumption of processed foods, salt, and saturated fats, and a deficiency of fruits and vegetables, is associated with increased risk of type 1 diabetes (T1D) and type 2 diabetes (T2D) (Zimmet et al. 2014; Paun et al. 2017). In comparison to hunter-gatherer or rural farming populations, the gut microbial composition of Western diet-consuming populations displays reduced diversity and loss of specific taxonomic groups of bacteria (Sonnenburg and Backhed 2016). Dietary deficiencies also impact the microbiome, with attendant effects on health. Impaired development of the gut microbial community is causally related to undernutrition in childhood (Subramanian et al. 2014). Transplantation of gut microbiota from undernourished children to completely sterile, germfree (GF) mice transmitted impaired growth, altered bone morphology, and metabolic abnormalities in the muscle, liver, and brain, compared to GF mice that received microbiota from healthy age-matched donors (Blanton et al. 2016). These and other studies support the role of the gut microbiome as a producer and interpreter of dietary compounds and metabolites.

Microbiome composition and function are also subject to sex effects that influence immunity and metabolic health. Recent human studies provide evidence that microbial composition differs between males and females (Haro et al. 2016; Dominianni et al. 2015). Twin studies revealed that microbial composition of opposite-sex twins becomes more divergent after puberty, in comparison to same-sex twins, likely in response to circulating sex hormones (Yatsunenko et al. 2012). The authors and others have also demonstrated the influence of sex hormones on the gut microbiome and immunity in mouse models. The nonobese diabetic (NOD) mouse model of spontaneous autoimmune diabetes, when housed in specific pathogen-free (SPF) settings, displays a 2:1 female to male sex bias in disease incidence. Under GF conditions, diabetes incidence is equal between the sexes, indicating that the microbiome is a requirement for the sex bias (Markle et al. 2013). Microbiome composition of NOD males and females is similar until puberty and subsequently diverge (Markle et al. 2013; Yurkovetskiy et al. 2013). Transfer of adult male intestinal microbiota into weaning-age female mice resulted in strong protection from diabetes, as well as increased serum testosterone levels, altered serum metabolites, and an altered microbial composition (Markle et al. 2013). The

effects of male microbiome transfer were abrogated in androgen receptor antagonist-treated female recipients, indicating that testosterone was critical in the transfer of protection. An independent study demonstrated that colonization of GF NOD females with specific bacterial taxa enriched in SPF males also induced elevated serum testosterone levels (Yurkovetskiy et al. 2013). Together these results demonstrate that sex and androgen action contribute to microbiome composition and function, which in turn affects immune responses and progression to autoimmunity.

Dietary Compounds and Derivative Metabolites Influence the Gut Microbiome and Host Immune System

As we have described for the immune system, there are many examples of sex differences in metabolic homeostasis, T2D, and obesity (Mauvais-Jarvis 2015). Investigations into the biological mechanisms that drive such sex differences need to consider how sex affects these critical immune-metabolic interactions. Of particular interest is the role of sex variation in the interplay between diet, immunity, and metabolism. Immune cells express a variety of cell surface and nuclear receptors which sense external cues, such as those from diet, and mediate signals which influences the development, function, and regulation of these immune cells. Sex hormones can influence the expression and downstream action of these nutrient receptors, with consequences for immune and metabolic processes. In the following sections, we profile dietary components and metabolites that mediate the cross talk between diet, the gut microbiome, immunity, and metabolism and exemplify the diversity of mechanistic impacts that sex can have on these systems.

Diet is a key regulator of the complex network required for human immune and metabolic homeostasis (Maslowski and Mackay 2011). Dietary choices are an identified risk factor for T2D, inflammatory bowel disease, some autoimmune disease, and certain cancers. Some of these effects are mediated by mucosal microbial communities in the digestive tract and the lung (Thorburn et al. 2014). Food-derived compounds and their metabolites have broad immunomodulatory functions. They can promote pro- or anti-inflammatory immune responses both through direct interaction with immune cells and effects on gut microbial composition and epithelial barrier function (Veldhoen and Brucklacher-Waldert 2012). Essential to understanding the interplay between diet, mucosal microbes, and immune and metabolic responses is consideration of the influence of biologic sex differences. Here we will profile the evidence that diet-derived vitamins and lipids regulate immunity and metabolic function and highlight the diverse mechanisms through which these effects are impacted by sex.

Vitamin D

Vitamin D is an important regulator of immunity, gut microbial composition and function, and complex metabolic processes (Dimitrov and White 2017; Clark and Mach 2016; Wimalawansa 2016). The major source of vitamin D production results from skin exposure to UBV radiation resulting in conversion of the precursor 7-hydrocholesterol into pre-vitamin D3, which is nonenzymatically converted to vitamin D3 (cholecalciferol) (Tavera-Mendoza and White 2007). Fatty fish, liver, and cheese are rich dietary sources of vitamin D3, and modern foods such as milk products and breakfast cereals are often fortified with vitamin D2 (ergocalciferol) (Clark and Mach 2016). Ingested vitamins D3 and D2 absorbed in the intestinal tract, and sunlight-derived vitamin D3 in the skin, are converted by the liver into the active hormone 1,25$(OH)_2$D3. This active metabolite binds and signals through the cell surface vitamin D receptor (VDR). VDR expression is in turn regulated by gut bacteria, fatty acids, and hormones including estrogen (Pilon et al. 2015).

While vitamin D is a well-known regulator of bone health, it also is important for intestinal mucosal homeostasis. Studies in both mouse models and humans demonstrate that vitamin D shapes microbial composition, fortifies the intestinal epithelial barrier, and promotes protective immunity (Barbachano et al. 2017; Meeker et al. 2016). For example, C57BL/6 mice fed a vitamin D-insufficient diet showed increases in the relative abundances of fecal *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Gammaproteobacteria*, in comparison to mice fed a vitamin D-sufficient diet (Assa et al. 2014). Further evidence of vitamin D's ability to regulate the gut microbiome was demonstrated in mice genetically deficient in either VDR or the hepatic enzyme Cyp27B1 which converts vitamin D precursors into active 1,25$(OH)_2$D3. VDR- and Cyp27B1-deficient mice displayed higher relative abundance of *Bacteroidetes* and *Proteobacteria* and lower abundance of the phyla *Firmicutes* and *Deferribacteres* compared to wild-type littermates (Ooi et al. 2013). Independent studies in C57BL/6 mice revealed that vitamin D-deficient diets decreased total colonic bacterial load and reduced microbial diversity (Lagishetty et al. 2010). Vitamin D also promotes gut barrier integrity. Wild-type mice fed a vitamin D-insufficient diet or mice genetically deficient in VDR or Cyp27B1 displayed heightened disease severity in a dextran sodium sulfate (DSS)-induced model of colitis compared to vitamin D-sufficient or wild-type mice, respectively (Assa et al. 2014; Ooi et al. 2013; Lagishetty et al. 2010). Human studies demonstrate similar effect of vitamin D on microbial composition. In healthy human volunteers, oral vitamin D3 supplementation reduced the relative abundance of *Gammaproteobacteria* and increased bacterial diversity in the upper gastrointestinal tract (Bashir et al. 2016). In patients with multiple sclerosis (MS), vitamin D3 supplementation increased the abundance of immune tolerance-promoting *Akkermansia*, as well as *Faecalibacterium* and *Coprococcus*, which produce the anti-inflammatory short-chain fatty acid (SCFA) butyrate (Cantarel et al. 2015). Moreover, vitamin D3 treatment increased *Enterobacteria* abundance in both MS patients and healthy controls, and in relapsing-remitting MS patients, treatment

altered the relative abundances of *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (Mielcarz and Kasper 2015). Together, these data suggest that dietary vitamin D impacts both gut microbial composition and mucosal integrity.

Vitamin D also exerts potent anti-inflammatory immunomodulatory effects. Many immune cell types express VDR including monocytes and macrophages, dendritic cells (DC), and T cells (Dimitrov and White 2017). In monocytes, vitamin D downregulates production of the inflammatory cytokines including tumor necrosis factor (TNF) and interleukins (IL-) 1β, 6, and 8 (Giulietti et al. 2007; Neve et al. 2014). Vitamin D promotes the maturation and survival of DC, which respond to local environmental signals and capture and present antigens to T cells (Adorini 2002). The nature of antigens presented by DC to naïve T cells and the surrounding microenvironment determines T cell differentiation into various "helper" subtypes with distinct effector activities controlled by master regulatory transcription factors (Brucklacher-Waldert et al. 2014). Vitamin D was found to impair the effector T-helper 17 (Th17) cell activity. Th17 cells are generally pro-inflammatory and play significant roles in the pathology of several immune-mediated diseases (Adorini and Penna 2008; Di Rosa et al. 2011; Penna et al. 2005; van der Aar et al. 2011). Vitamin D also impacts T regulatory cell (Treg) frequency and function; this T cell subset plays a nonredundant role in maintenance of immune tolerance to harmless dietary antigens and to the commensal microbiome (Chambers and Hawrylowicz 2011; Bollrath and Powrie 2013). Vitamin D increased the frequency of activation-induced Treg cells cocultured with DC likely via effects on the DC (Jeffery et al. 2009; Penna et al. 2005). VDR agonists also enhance the immune suppressive activity of Treg and promote their recruitment to sites of inflammation (Gorman et al. 2007; Adorini and Penna 2008). As mentioned above, intestinal barrier integrity is affected by vitamin D. Other mechanisms of these effects include induction of antimicrobial peptide expression, a "first-line" defense against invading microbes, as well as expression and release of antimicrobial defensins by secretory intestinal Paneth cells and expression of tight junction proteins by gut epithelial cells (Muehleisen and Gallo 2013; Lucas et al. 2014; Su et al. 2016).

Numerous studies have begun to examine the effects of vitamin D on metabolic and autoimmune disease including insulin resistance, metabolic syndrome, T1D, and T2D (Nasri et al. 2014; Boucher 2011). Vitamin D has beneficial effects on pancreatic islet cell function, on insulin release, and on decreasing insulin resistance by target tissues. Indeed vitamin D deficiency may contribute to both islet beta cell dysfunction and insulin resistance, both of which modify diabetes risk (Wimalawansa 2016). An inverse association was observed between low serum levels of 25(OH)D3 and rates of obesity, metabolic syndrome, and T2D (Wimalawansa 2016; Mohr et al. 2008). For T1D, a global geographic association exists between low UVB radiation exposure and higher incidence rates of T1D (Wimalawansa 2016; Mohr et al. 2008). There is also evidence that links vitamin D levels with both the incidence and severity of autoimmune diseases (Dankers et al. 2016). Vitamin D insufficiency has been identified as a risk factor for SLE, ankylosing spondylitis, and systemic sclerosis, among others (Borba et al. 2009; Kamen et al. 2006; Durmus et al. 2012; Caramaschi et al. 2010; Vacca et al. 2009). Further supporting the role

of vitamin D in autoimmune disease is an association between VDR gene polymorphisms and risk for autoimmune disease (Dankers et al. 2016). Multiple clinical trials using vitamin D supplementation have been conducted in cohorts of autoimmune disease patients. Although the results of most of these trials do not reach significance, some efficacy was reported in reducing SLE severity suggesting that more highly powered trials are warranted (Abou-Raya et al. 2013). Of particular interest is vitamin D as a risk factor for MS susceptibility (Pierrot-Deseilligny and Souberbielle 2017). Several studies have reported that higher serum levels of 25(OH)D3 was correlated with lower disease risk, fewer relapses, and attenuated disease severity (Alharbi 2015). Genetically induced low 25(OH)D3 serum levels, as well as polymorphisms in genes that participate in vitamin D metabolism, are also linked to MS risk (International Multiple Sclerosis Genetics et al. 2011; Ramasamy et al. 2014; Mokry et al. 2015; Rhead et al. 2016). Evidence from both animal models and humans indicate that vitamin D has immunomodulatory affects that are beneficial against MS, likely through the induction of Tregs and anti-inflammatory cytokines, the inhibition of pro-inflammatory Th17 cells and their related cytokines, and the reduced B cell immunoreactivity. Whether and if so how the microbiome may influence cross talk between vitamin D and immunomodulation in MS patients is an important area for future investigation.

Interestingly, vitamin D levels may exert sex-dependent effects in immune-mediated disease. One potential confounder in identifying these effects is a conflicting literature concerning sex and gender differences in basal vitamin D levels (Hagenau et al. 2009; McCullough et al. 2010; Jungert and Neuhauser-Berthold 2015). Since vitamin D is fat-soluble and can be sequestered in adipose tissue, sex differences may be affected by differences in body fat composition and distribution (Wortsman et al. 2000; Lagunova et al. 2009; Johnson et al. 2012). As discussed above, many autoimmune diseases exhibit sex bias in incidence and clinical manifestations. Vitamin D may influence sexual dimorphism in disease risk and/or severity (Rubtsova et al. 2015; Vasile et al. 2017). Vitamin D deficiency may affect the primary pathogenesis of Sjögren's syndrome, especially in females where one study found that patients have lower vitamin D levels than healthy controls (Erten et al. 2015). The higher incidence of some autoimmune diseases in women may also reflect links between vitamin D deficiency and increased synthesis of estrogens (Cutolo 2013; Kovats 2015). Estrogens may act in concert with vitamin D to reduce incidence and disease severity in MS. Evidence in mouse models of experimental autoimmune encephalomyelitis (EAE), which resembles some features of human MS, links vitamin D, estrogen, and protection against disease. Vitamin D supplementation inhibited EAE in sham-operated compared to ovariectomized females and did not affect disease in males (Spach and Hayes 2005). This protective effect likely results from 17b-estradiol (E2)-mediated enhancement of VDR gene transcription in the central nervous system (Nashold et al. 2009). In humans, higher serum 25(OH)D3 levels were correlated with reduced disease risk and severity in women, but not in men (Kragt et al. 2009). It was suggested that these effects could be due to vitamin D-mediated modulation of Treg cell maintenance of immune tolerance. In T1D, a randomized crossover clinical trial suggested that vitamin D

supplementation enhances Treg frequency in male but not in female patients (Bogdanou et al. 2017). The knowledge gap identified by these animal model and human studies reporting vitamin D effects on autoimmune disease is what are the mechanisms responsible for these disease-specific, sex-dependent differences?

Vitamin A

Vitamin A and its metabolites are well known for their importance in development and biological processes, including immune function. Vitamin A deficiency is a primary cause of compromised immune function worldwide, increasing risk for early childhood diarrhea, infections, and mortality (Schaible and Kaufmann 2007; Christian et al. 2001; Sommer 2008; West and Mehra 2010). Vitamin A is a fat-soluble vitamin obtained exclusively from the diet and processed into its active forms in the gastrointestinal tract (Harrison 2005). Animal sources such as dairy products, eggs, and meat contain vitamin A metabolites retinol and retinol esters (Czarnewski et al. 2017). Plants contain the nonbiologically active provitamin A known as carotenoid or beta-carotene, which must be converted into retinal and then reduced to retinol, to enable cellular uptake. In the cell cytoplasm, retinal dehydrogenases (RALDH) generate the predominant metabolite all-trans-retinoic acid (atRA) and 9-cis-retinoic acid. The expression patterns of retinal dehydrogenases by specific cell types limit action of RA (Kumar et al. 2012). RA binds to members of the steroid/thyroid/retinoid receptor family, consisting of the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Rochette-Egly and Germain 2009). RAR heterodimerize with RXR generating ligand-dependent transcription factors. They bind to retinoic acid-responsive elements (RAREs) located in promoters, introns, or more distant intergenic regions, to regulate target gene expression (Bono et al. 2016). Intestinal DC express the retinal dehydrogenase 2 (RALDH2) and are important producers of atRA for the immune system (Coombes et al. 2007; Jaensson-Gyllenback et al. 2011). Intestinal epithelial cells can also generate RA, through constitutive expression of the retinal dehydrogenase 1 (RALDH1) isoform (Lampen et al. 2000).

RA has broad immunomodulatory activities that are influenced by cell type, retinoic acid receptor isoforms, and environmental milieu (Czarnewski et al. 2017). In the intestine, RA signaling functions as a rheostat to fine-tune immune responses, balancing pro- and anti-inflammatory responses (Larange and Cheroutre 2016). RA modulates intestinal DC antigen presentation and reduces their production of inflammatory cytokines. In contrast, under conditions of infection, RA-mediated signals promote pro-inflammatory responses by both DC and CD4+ T cells (Hall et al. 2011; Coombes et al. 2007; Jaensson-Gyllenback et al. 2011). RA has multiple effects on T cell function. Through intestinal DC, RA can imprint gut-homing specificity on T cells by inducing expression of C-C chemokine receptor type 9 (CCR9) and the a4β7 integrin on T cells (Iwata et al. 2004). RA is particularly important for modulating T cell responses during infection. Acting through the receptor RARα,

RA drives pro-inflammatory T-helper 1 (Th1) cell-mediated immunity and phagocyte-dependent responses to intracellular bacteria and viruses (Hall et al. 2011; Pino-Lagos et al. 2011). RA also plays critical roles in optimal effector CD8+ "cytotoxic" and effector memory CD8+ T cell differentiation at mucosal sites. These T cell subsets support immune defense against intracellular pathogens and tumor cells (Allie et al. 2013). Conversely, RA promotes immune tolerance by contributing to differentiation of induced Treg cells in response to transforming growth factor beta (TGFβ) and reciprocal inhibition of the Th17 fate (Coombes et al. 2007; Mucida et al. 2007; Hall et al. 2011; Bergstrom et al. 2010; Kang et al. 2007). Of particular importance, RA signaling influences immunoglobulin (Ig) class switching by B cells, promoting IgA antibody production and controlling polymeric Ig receptor expression on intestinal epithelial cells (Mora et al. 2006). Secretory IgA is essential to protect the intestinal epithelium by binding directly to microbes and can regulate microbial community composition, diversity, and gene expression (Mantis et al. 2011).

To date there are only limited studies of the effect of vitamin A deficiency on human gut microbial composition and function. Some of the evidence suggests that there is cross talk between RA and the microbiome, most likely through effects on mucosal DC. Acute vitamin A deficiency dramatically affects human bacterial community structure and gene expression compared to diets lacking other micronutrients including folate, iron, and zinc (Hibberd et al. 2017). Direct tests of causality in rodent studies have demonstrated microbial community alteration in response to vitamin A. RA administration significantly altered the composition of the mouse gut microbiome likely due to the action of RA on intestinal immune populations (Lee and Ko 2016). B cells deficient in RA signaling display abrogated antigen-specific IgA responses after oral immunization, as well as altered microbiome composition compared to wild-type controls (Pantazi et al. 2015). Reciprocally, the microbiome affects the immunomodulatory actions of RA. Microbial stimulation through toll-like receptors (TLR) expressed on DC is necessary for normal RALDH expression which is required for RA biosynthesis (Iwata et al. 2004; Mora et al. 2006; Wang et al. 2011; Bakdash et al. 2015). Specific bacterial strains may promote retinoic acid synthesis and function. For example, *Bifidobacterium infantis* in mice results in elevated numbers of mucosal DC that can metabolize vitamin A into RA (Konieczna et al. 2013).

Vitamin A also plays a role in both T1D and T2D. It affects pancreas development, improves pancreatic beta cell functions, and influences adipocyte homeostasis (Rhee and Plutzky 2012; Amisten et al. 2017). The literature is conflicting as to whether vitamin A is pro- or antidiabetic (Trasino and Gudas 2015). High serum vitamin A levels were reported to be associated with elevated risk of T2D (Sun et al. 2014). However, others studies saw no relationship between serum vitamin A levels and T2D, while others reported that high serum vitamin levels were associated with protection against insulin resistance (Dakshinamurti 2015). Retinoids may have therapeutic potential in T2D (Meerza et al. 2016). In a retrospective Danish study, vitamin A dosing during fetal development was associated with lower T2D risk to offspring later in life (Keller et al. 2017). In mouse models, atRA improved T2D

through production of VEGF-A by pancreatic islets (Chien et al. 2016). RA also has beneficial effects on T1D, where atRA protected against disease in vivo and protected beta cells from IL-1-mediated damage in cell culture models (Zunino et al. 2007; Kang et al. 2004).

Retinoic acid generating enzymes are reported to have effects on multiple aspects of metabolism, including the regulation of female-specific fat distribution, sex-specific diet-induced thermogenic regulation, sex dimorphism of white adipose tissue, and risk for chronic disease (Yasmeen et al. 2013; Wang et al. 2001; Trasino et al. 2007; Li et al. 2004; Reichert et al. 2011; Petrosino et al. 2014). Many vitamin A metabolizing enzymes are differentially expressed in males and females in a tissue-specific manner, although the molecular mechanisms of these differences remain unclear (Kos et al. 2011). RALDH isoform expression and function are controlled in both a tissue- and sex-specific manner (Kumar et al. 2012; Petrosino et al. 2014; Duester 2008). Obese men and women are dimorphic in RALDH expression, and sex hormones appear to underpin this regulation (Yasmeen et al. 2013). Estrogen response elements (ERE) reside in the RALDH2 promoter, while RALDH3 is positively regulated by androgens (Wang et al. 2001; Trasino et al. 2007). Further studies demonstrate that E2 suppresses both RALDH2 and RALDH3 in adipose tissue (Yasmeen et al. 2013). As a result, RA production in males depends on both RALDH1 and RALDH2, whereas, in females, RALDH1 is the predominant enzyme catalyzing RA production. This distinction has reported consequences for sex differences in visceral adiposity (Reichert et al. 2011; Yasmeen et al. 2013). RALDH1 deficiency impaired visceral adipose tissue (VAT) formation in female mice more than in males, and RALDH1 contributed to sex differences in high-fat diet-dependent cytokine production (Gushchina et al. 2013). Given vitamin A's extensive role in modulating immunity and metabolism, more in-depth investigation into sex effects on RA synthesis and function is clearly warranted.

Dietary Lipids

Dietary fats can have both pro- or anti-inflammatory effects on the host and are associated with modulation of intestinal and metabolic health. Metabolism of dietary fats produces fatty acids (saturated, mono- and polyunsaturated) and their derivatives mono-, di-, and triglycerides, phospholipids, and sterols including cholesterol (Veldhoen and Brucklacher-Waldert 2012). Meat and dairy products, prominent in Western diets, are the primary sources of saturated fatty acids (SFAs). Monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), including omega-3 and omega-6 PUFAs, are present in oils, nuts, fruits, seeds, and animal products (Veldhoen and Brucklacher-Waldert 2012; Schwingshackl and Hoffmann 2012). Diets rich in saturated fats are associated with increased white adipose tissue (WAT) inflammation and metabolic disease, while diets rich in unsaturated fats can counter inflammation and promote a lean and metabolically healthy phenotype (Kennedy et al. 2009; Buckley and Howe 2009; Calder 2006). Fats are

digested and absorbed by the intestine. Following emulsification by bile salts in the duodenum, they are hydrolyzed by pancreatic lipases in the intestinal lumen (Hussain 2014). The digestive products, fatty acids (FAs) and 2-monoacylglycerol (MAG), can then be absorbed from the small intestine (D'Aquila et al. 2016). FAs are taken up by intestinal epithelial cells, are released into the lamina propria, and transported through the mesenteric lymph to enter systemic circulation.

Receptors for Dietary Lipids

Dietary lipids have complex mechanisms of actions mediated through specific receptors. Additional proteins participate in the uptake and transport of FAs, including transport proteins (FATPs) that mediate cellular uptake and intracellular fatty acid-binding proteins (FABPS) that modify FA signaling (Gimeno 2007; Hotamisligil and Bernlohr 2015). G-protein-coupled receptors (GPCRs) serve as lipid metabolite sensors and recognize FA with different affinities and expression patterns (Thorburn et al. 2014). Ligand-activated transcription factors are another group of lipid receptors that play important roles in lipid homeostasis and immunity. The peroxisome proliferator-activated receptors (PPARs) regulate cellular differentiation, development, and metabolism by acting as transcriptional modifiers (Veldhoen and Brucklacher-Waldert 2012). The three members of the PPAR family, PPARα, PPARβ/δ, and PPARγ, exhibit distinct expression patterns and bind to a variety of fatty acids and their metabolites. PPARα is highly expressed in brown adipose tissue and the liver and to a lesser extent in the heart, kidney, and intestine. PPARβ/δ is highly active in skeletal muscle and is also expressed in the skin, gut, placenta, adipose tissue, and the brain. PPARγ regulates both white and brown adipocyte differentiation and is expressed broadly across tissues, including the gut, brain, and immune cells, with highest expression in fat (Ahmadian et al. 2013; Wahli and Michalik 2012; Gross et al. 2017). Cholesterol and its metabolites, oxysterols and bile acids, activate the nuclear receptors liver X receptor (LXR) and farnesoid X receptor (FXR), respectively (Janowski et al. 1999; Makishima et al. 1999). These receptors are expressed in metabolically active tissues such as liver, intestine, and adipose tissues and are essential to integrate lipid metabolism with liver and intestinal homeostasis.

Mechanisms of Lipid Receptor Effects on Immune Pathways

Dietary lipids can regulate immune and inflammatory responses directly through lipid sensors expressed on the surface and in the cytoplasm of immune cell subsets.

There has been much interest in the potent influence omega-3 PUFAs exert on the immune system, specifically the modulation of macrophage function.

Macrophages phagocytose apoptotic cells and microbial pathogens, produce pro- or anti-inflammatory effector molecules, and promote tissue repair. They exhibit high plasticity and can change functional phenotype depending on the environmental milieu. One functional extreme is the classically activated pro-inflammatory "M1" macrophage, which functions in defense against pathogens and also in chronic inflammation, tissue damage, and autoimmunity (Italiani and Boraschi 2014; Martinez et al. 2009). Omega-3 PUFAs exerts its anti-inflammatory effects by binding to G protein-coupled receptor 120 (GPR120) expressed on pro-inflammatory macrophages. GPR120 activation triggers several signaling cascades which block pro-inflammatory signaling through inhibition of both TLR and cytokine-mediated signaling pathways (Hirasawa et al. 2005; Oh et al. 2010), as well as inhibiting NOD-, LRR- and pyrin domain-containing (NLRP) inflammasomes (Yan et al. 2013). GPR120-mediated signaling was also found to decrease macrophage chemotaxis into adipose tissue in high-fat diet conditions and shift the overall gene expression profile of adipose tissue macrophages toward an anti-inflammatory phenotype (Oh et al. 2010; Lackey and Olefsky 2016).

Role of PPARs

PPARs are widely expressed by various immune cells, including macrophages, dendritic cells, and lymphocytes, and their diverse array of lipid ligands have demonstrated potent anti-inflammatory and immune tolerance-promoting effects (Park and Choi 2017). PPARs predominately form obligate heterodimers with RXR and, in the absence of ligand, are bound to DNA response elements where they repress target gene expression. Ligand binding induces a conformational change which alleviates repression and allows for the initiation of target gene expression (Kidani and Bensinger 2012). Notably, in macrophages, PPARs inhibit inflammation through ligand-dependent transrepression of genes. Rather than direct binding to DNA sequences, PPARs bind to and antagonize the action of other classes of transcription factors (Straus and Glass 2007). Other family members PPARα and PPARγ mediate inhibition of master regulators of immune activation NF-κb, activator protein 1(AP-1), nuclear factor of activated T cells (NFAT), and signal transducers and activators of transcription (STAT) (Wahli and Michalik 2012). PPARβ/δ exerts effects by association with the transcriptional repressor B cell lymphoma 6 (BCL-6) (Lee et al. 2003). PPARβ/δ in its unbound state sequesters the repressor and, upon ligand binding, releases BCL-6, which then acts to inhibit expression of the pro-inflammatory mediators iNOS, COX2, and TNFa and chemokines MCP-1 and MCP-3 (Varga et al. 2011; Chinetti-Gbaguidi and Staels 2017).

PPARs also regulate T cell function and differentiation. Both PPARα and PPARγ can act as negative T cell regulators. PPARα ligands inhibit IL-2, TNFα, and IFNγ production by activated CD4 T cells (Marx et al. 2002). Further, PPAR/T cells are hyperresponsive to T cell receptor stimulation (Dunn et al. 2007). PPARγ can negatively regulate T cell activation following TCR stimulation through inhibition of

NFAT and IL-2 production (Clark et al. 2000; Yang et al. 2000). In support of PPARs' negative regulatory role, colitis mouse models have demonstrated that PPARα or PPARγ ligand treatment inhibits inflammatory cytokine production and reduces disease severity (Lee et al. 2007; Azuma et al. 2010; Hontecillas and Bassaganya-Riera 2007). PPARs have also been demonstrated to have an important role in T cell differentiation. PPARα promotes effector T-helper 2 (Th2) responses through the regulation of IL-4 and IL-5 gene expressions (Gocke et al. 2009). PPARγ also promotes Th2 immune responses while inhibiting Th1 effector function and Th17 cell differentiation (Tontonoz and Spiegelman 2008; da Rocha Junior et al. 2013; Straus and Glass 2007; Klotz et al. 2007). PPARγ appears to be critical for Th2 immunity, because PPARγ-deficient mice do not develop allergic pathology and are poorly protected against parasite infection (Chen et al. 2017). Finally, in vitro evidence suggests that in both mice and humans, PPARα is critical for maintaining the frequency and function of T regulatory cells (Dubrac et al. 2011; Lei et al. 2010; Hichami et al. 2016) and PPARγ expression by visceral adipose tissue Tregs is crucial for their accumulation, phenotype, and function (Cipolletta et al. 2012; Chen et al. 2017).

Roles of LXR and FXR

The classical functions of LXR and FXR are regulation of cholesterol and bile acid metabolism, but they also regulate inflammatory immune responses and gut barrier integrity. Similarly to PPARs, LXR and FXR influence gene expression by forming obligate heterodimers with RXRs to repress gene expression and, through transrepression of other transcription factors, can inhibit the production of pro-inflammatory cytokines (Veldhoen and Brucklacher-Waldert 2012; Kidani and Bensinger 2012). LXRs are expressed in numerous cell types, including macrophages, where they control cholesterol homeostasis as well as regulate macrophage responses to bacterial infection, apoptotic cell clearance, and other pro-inflammatory functions (Joseph et al. 2003; Matalonga et al. 2017). LXR inhibits the transcription of pro-inflammatory genes including IL-6, IL-1b, and iNOS through transrepression of NF-κb or AP1 (Pascual-Garcia and Valledor 2012). LXR has also been shown to be a negative regulator of IL-18, a key cytokine in the pathogenesis of autoimmune and inflammatory disease (Pourcet et al. 2016). FXR is an important factor in intestinal innate immunity and mucosal homeostasis. FXR is expressed predominantly in the liver and in intestinal epithelial cells, and in addition to its role in regulating bile acid homeostasis, fat and glucose metabolism is required for gut barrier integrity and limiting bacterial growth (Assa et al. 2014). Along with LXR, FXR can also negatively regulate monocyte and macrophages. FXR activation was reported to repress macrophage TLR4 gene expression and pro-inflammatory cytokine production (Vavassori et al. 2009). FXR can also negatively regulate the NLRP-3 inflammasome by preventing assembly of the NLRP-3 and caspase-1 complex via physical interaction (Hao et al. 2017; Garcia-Irigoyen and Moschetta 2017).

While LXR signaling has been best studied in macrophages, it also mediates lymphocyte proliferation and differentiation. Ligand-activated LXR signaling was found to negatively regulate T cell antigen receptor-driven clonal expansion and homeostatic proliferation (Bensinger et al. 2008). LXR may also mediate Th17 cell differentiation. LXR signaling inhibits *IL-17* expression as well as suppresses the expression of other Th17 cytokines IL-21 and IL-22. Furthermore, ligand activation of LXR inhibited the expression of RAR-related orphan receptor gamma (RORγt) and aryl hydrocarbon receptor (Ahr), which are the master transcription factors of the Th17 program (Xu et al. 2009; Cui et al. 2011; Heller et al. 2011). Thus, lipid-mediated signaling through a series of receptors mediates cross talk between host metabolism and immune function. This is a fertile area for ongoing studies to advance our mechanistic understanding of how these nuclear receptors control essential, interrelated physiological functions.

Dietary Lipids and the Gut Microbiome in Metabolic Disease

Multiple epidemiological studies conclude that increased fat intake is associated with higher prevalence of T2D (Fujimoto et al. 1983; Kawate et al. 1979; Stern et al. 1992; Tsunehara et al. 1990; Marshall et al. 1991; van de Laar et al. 2004). This relationship is greatly but not exclusively determined by increased adiposity and obesity (Feskens et al. 1995; Lindstrom et al. 2006). Conversely, dietary omega-3 PUFAs may have protective and even therapeutic effects on metabolic disease. In animal studies, omega-3 PUFAs treatment improved obesity-associated insulin resistance (Lamping et al. 2013; Flachs et al. 2014; Lalia and Lanza 2016). There is contradictory evidence in human studies on the relationship between omega-3 PUFAs and risk of T2D, likely due to genetic, lifestyle, and different dietary patterns which may influence the effect on omega-3s in preventing and/or ameliorating the disease (Jafari et al. 2013). In most studies, high-fat diet (HFD) decreased gut microbial diversity. Mice on HFD displayed extensive changes in microbial composition in comparison to mice fed with normal chow (Hildebrandt et al. 2009; Turnbaugh et al. 2008). This diet-induced compositional shift occurred within 24 h (Turnbaugh et al. 2009). The effect of diet on microbial composition shifts may contribute to the low-grade chronic inflammation observed in mice with HFD-induced obesity. Strikingly, completely germfree mice were protected against HFD-induced obesity and exhibited reduced WAT inflammation and insulin resistance compared to colonized mice (Backhed et al. 2007; Caesar et al. 2012; Ding et al. 2010; Rabot et al. 2010). The gut microbial community of obese compared to lean mice has an increased capacity for energy harvest and storage. Transplantation of microbiota from HFD obese donors caused increased adiposity in germfree recipient mice compared to transfer of microbes from lean mice (Turnbaugh et al. 2008). HFD resulted in compromised intestinal tight junction protein expression, increased intestinal permeability, increased markers of WAT inflammation, and increased body fat compared to mice fed a normal chow diet. These differences were

abolished by antibiotic treatment, indicating that the microbiota is necessary to confer these phenotypes (Cani et al. 2008). HFD-induced breakdown in gut barrier integrity may elevate systemic circulating levels of bacterial lipopolysaccharide (LPS) and TLR4-mediated immune activation provoking chronic low-grade inflammation and "metabolic endotoxemia" (Shen et al. 2014). Finally, the type of dietary lipids can differentially affect gut microbiota composition, and this contributes to phenotypic differences between mice fed with SFAs derived from lard and mice fed with omega-3 PUFA-rich fish oil (Caesar et al. 2015). Mice fed with lard had increased TLR activation, increased WAT inflammation, and reduced insulin sensitivity compared to fish oil-fed mice, in a manner dependent on diet-induced changes in microbial composition. Thus, interactions between dietary lipids and the gut microbiota have profound effects on host health.

PPAR in Immunity: Sex Effects on Therapeutic Targets

PPARs are defined targets for metabolic diseases and are emerging as therapeutic targets for treatment of T1D, SLE, and MS. In this context, it will be important to understand PPAR-mediated sex differences in immune responses (Park and Choi 2017). For example, PPARα's effect on EAE is sexually dimorphic, likely due to sex differences between its expression and function. Male PPARα-deficient mice had greater disease susceptibility and severity compared to wild-type males, whereas there was no genotype-dependent differences in females (Dunn et al. 2007). PPARα protein was more abundant in male compared to female T cells. This finding may indicate that PPARα is a more potent negative regulator of T cell function in males compared to females (Dunn et al. 2007). Insight into how sex-dependent regulation emerged from evidence that the androgen receptor interacts with the PPARα gene promoter. In the EAE mouse model, androgens were demonstrably essential for maintaining PPARα expression and to enforce inhibition of Th1 responses (Zhang et al. 2012; Bebo et al. 1998). Sex differences in PPARα function were also demonstrated in experiments that used small interference RNA-mediated "knockdown" of PPARα gene expression in T cells. In male T cells, PPARα knockdown resulted in increased IFNγ production, whereas similarly treated female T cells exhibited augmented IL-17 expression compared to controls (Zhang et al. 2012). These data suggest that PPARα-mediated control of T cell effector functions differs by sex and that at least in one model of autoimmune disease, this mechanism is central to the sex differences in disease pathogenesis.

PPARγ agonists are first-line drugs for the treatment of dyslipidemia and insulin resistance and are under investigation for management of autoimmune disorders. Recent observations demonstrate sex-dependent differences in PPARγ effects on T cell differentiation and function in mouse models. In contrast to PPARα, PPARγ is more highly expressed in female compared to male T cells, likely due to the effects of estrogen (Zhang et al. 2012; Dunn et al. 2007; Park et al. 2016). Male mouse T cell exposure to estradiol increased PPARγ expression. In female mice, PPARγ

expression levels correlated with phases of the estrus cycle (Park et al. 2016). T follicular helper cells (TFH) are critical to assist B cell responses to foreign antigen during the germinal center reaction in lymph nodes. Treatment of female mice with a PPARγ agonist reduced TFH responses to an injected foreign antigen. However, in males, co-administration of E2 and PPARγ agonist was required to reduce TFH responses to immunization (Park et al. 2016). Sex differences in PPARγ function have also shown impact in mouse models of autoimmune syndromes. Female mice rendered PPARγ deficient in the CD4+ T cell compartment displayed spontaneous autoantibody production, glomerular inflammation in the kidney, increased TFH cell frequency, and germinal center reactions. In contrast, these phenotypes were reduced or absent in male mice. These effects of PPARγ on regulation of TFH were dependent on estrogen, which acts to increase expression of PPARγ (Park et al. 2014, 2016).

PPARs are an attractive clinical target for immune and metabolic disease; however, these findings of robust sex differences in the immune effects of PPARα and PPARγ indicate that substantive study of biological mechanism is essential for clinical development of PPAR agonists for management of immune-mediated disease.

Summary

Diverse molecular mechanisms are involved in mediated the relationships between dietary compounds, metabolites, and effects on host metabolism and immunity. The intestinal microbiota is a dynamic community shaped by the availability of nutrients and generators of an as yet incompletely understood repertoire of molecules that bolster the intestinal barrier and signal to neighboring immune cells just beyond this one cell thick epithelium. Here we have profiled exciting recent data, fueled by high-throughput sequencing technologies, use of specialized mouse models, and studies in human subjects that are beginning to reveal the complexity of these interactions. Notably, many of the signaling pathways that mediate dietary and gut microbe effects on metabolic and immune homeostasis differ significantly as a function of biological sex. Thus, studies motivated by mechanistic discovery as well as translation to clinical practice must be designed and powered to observe sex differences. Within these sex differences lie opportunities to discover novel pathways of physiological regulation; to advance the precision of disease prevention, diagnosis, and treatment; and limit adverse outcomes.

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Sexual Dimorphism and Estrogen Action in Mouse Liver

Sara Della Torre, Federica Lolli, Paolo Ciana, and Adriana Maggi

Abstract Recent studies have demonstrated that in mice, the estrogen receptor alpha (ERα) is expressed in the liver and has a direct effect on the regulation of the hepatic genes relevant for energy metabolism and drug metabolism. The sex-related differential expression of the hepatic ERα raises the questions as to whether this receptor is responsible for the sexual differences observed in the physiopathology of the liver.

List of Abbreviations

AA	Amino acids
CARB	Carbohydrates
ERα	Estrogen receptor alpha
L	Lipids
SC	Subcutaneous
VLDL	Very-low-density lipoproteins

The Role of Chance in Scientific Discovery

In research the experimental results may lead to significantly diverge from the initial aims toward unanticipated fields of investigation; this happened to our group when we generated a novel transgenic mouse engineered to express the luciferase under the control of an estrogen-driven promoter (named ERE-Luc mouse) (Ciana et al. 2001).

The initial studies with the new mouse model demonstrated that the strategy pursued had led to the creation of a mouse where the luciferase was faithfully

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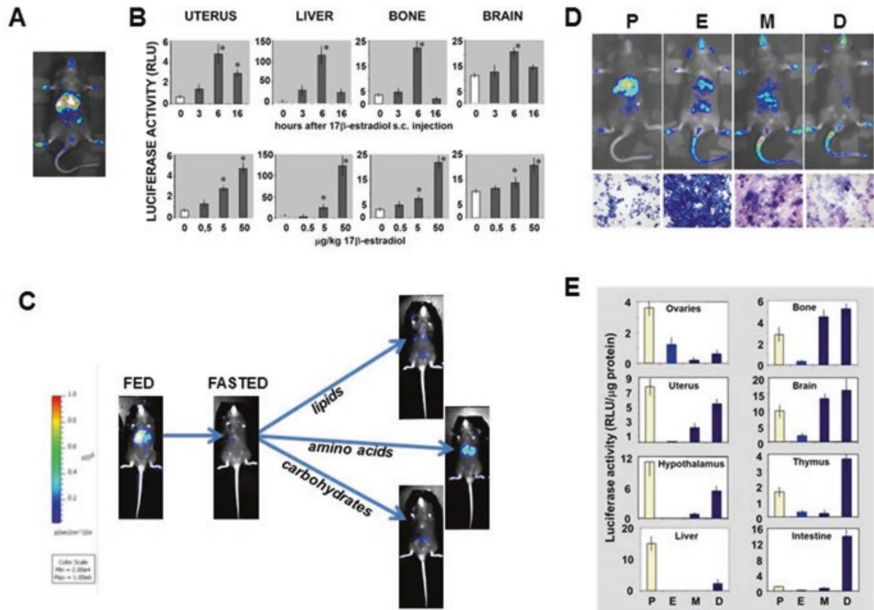

Fig. 1 In the ERE-Luc reporter mouse, the expression of luciferase parallels estrogen receptor transcriptional activity. In vivo and ex vivo study. (**a**) Pseudocolor image of photon emission from a male ERE-Luc mouse. (**b**) Luciferase enzymatic activity measured in the various tissues from ERE-Luc mice treated with E2; upper panel time-course study in female mice treated with 5 μg/kg 17β-estradiol for 6 h, dose-response: the hormone was injected SC (Adapted from Ciana et al. 2001). (**c**) Pseudocolor image of photon emission from one representative female ERE-Luc mouse in feeding and fasting conditions and after oral administration of isocaloric amounts of lipids, amino acids, or carbohydrates. (**d**) Whole-body imaging of a representative ERE-Luc mice in different phases of the estrous cycle; the H&E staining of the vaginal smears corresponding to each phase of the estrous cycle. (**e**) Luciferase enzymatic activity measured in the various tissues from ERE-Luc female mice in different phases of the estrous cycle (Adapted from Ciana et al. 2003)

reproducing estrogen receptor (ER) transcriptional activity in all body tissues; on the other hand, this mouse gave origin to surprising developments because it clearly indicated that the liver was a major target for estrogens. This result was unequivocal as obtained by biochemical localization of luciferase expression confirmed by whole-body in vivo imaging (Ciana et al. 2001, 2003) (Fig. 1a).

At the time, common belief was that the autonomic nervous system, together with pituitary hormones, were responsible for the physiological regulation of liver activities including glucose and energy homeostasis. Vagal afferents regulated glucose homeostasis (Pocai et al. 2005) and sympathetic transmission participated in the negative regulation of lipid synthesis by directly acting on the mitochondrial oxidation and on the production of VLDL particles (Jensen et al. 2013; Yamauchi et al. 1998). According to this line of thoughts, the well-known effects of circulating estrogens on hepatic lipid metabolism and synthesis of transport proteins (Abbott et al. 1983; Barnes et al. 2002) were considered as a mere consequence of the

hormone signaling in the brain. Thus, the results gained with the ERE-Luc mouse were in opposition with this dogma because pointed to the liver as a direct target for estrogens; in addition, this organ appeared to be a major estrogen target as the amount of luciferase measured in the liver was comparable or higher than in the reproductive tissues. This led us to further investigate on estrogen action on the hepatic transcriptional capabilities.

Regulation of the Transcriptional Activity of the Estrogen Receptor in the Liver

Prior studies had shown that in the liver, the α isoform of ER (ERα) is the predominant form. To establish the extent to which the luciferase reporter reproduced the state of ERα transcriptional activity, we measured luciferase activity at different times after the SC injection of 17β-estradiol or after the administration of increasing concentrations of the hormone. The hepatic response, in terms of time and dose dependency, was superimposable with the other tissues known to be a target of estrogen action as the uterus, bone, and brain (Fig. 1b). However, in the course of these studies, we were puzzled by the fact that the extent of liver luciferase content in the liver of unstimulated mice was very variable and this was not observed in the other organs; furthermore, in general, the hepatic ER transcriptional activity was higher in experiments done in the morning than in those done late in the evening (Ciana et al. 2005). We explored the possibility of a circadian effect or an association with food intake, and immediately, we could establish that liver ERα-dependent transcription was activated by food intake and was significantly decreased in case of starvation or calorie restriction (Fig. 1c). A detailed analysis of the macronutrient responsible for this effect was done by feeding overnight starved ERE-Luc mice with an equicaloric bolus containing either lipids (L), carbohydrates (CARB), or amino acids (AA). AA, but not CARB or L, were able to induce the liver to synthesize luciferase. The fact that this phenomenon could be inhibited by the administration of a specific antagonist of the receptor provided the final demonstration that ER was activated by AA intake.

Next, to demonstrate that the luciferase reporter was sufficiently sensitive to provide information on ER transcriptional activity under physiological conditions, we applied whole-body in vivo imaging to female ERE-Luc mice in the course of estrous cycle progression. Figure 1d shows that in the transgenic mouse, the luciferase activity measured in the reproductive tissues and in the liver paralleled circulating estrogen levels, indicating, as predicted, that the peak of ER transcriptional activity occurred at proestrus when circulating estrogens are highest. In contrast, in tissues such as the bone and brain, the peak of ER activity was observed at diestrus. Further quantitative analysis of luciferase activity in different tissues (Fig. 1e) supported the in vivo study by indicating that the hepatic ER activity was

always in synchrony with ER activity measured in the reproductive tissues (Ciana et al. 2003).

This observation suggested to us, for the first time, the potential for a functional link between the liver and the reproductive tissues and led us to verify whether experimental data supported the existence of such an association in animals other than the mouse. A detailed literature search in species other than mammals such as invertebrates (e.g., the Cephalochordata Amphioxus) or vertebrates (e.g., reptiles, amphibian, fish, and birds) demonstrated that commonly the organs with the highest expression of the ER were the gonads and the liver, further stressing the existence of an operational association between these two tissues (Griffin et al. 1999; Nagler et al. 2000; Todo et al. 1996; Della Torre and Maggi 2017).

From Genes to Function, in Search of the Role Played by the Hepatic ER in the Control of Liver Activities

With the aim to gain a better insight on the role of estrogens in liver functions, we applied the power of whole genome 'omics to verify the extent of the impact of the estrous cycle on liver transcriptome. RNA-Seq analysis showed that in the course of the estrous cycle, 1178 genes (9.1% of the entire hepatic transcriptome) change their expression between P and M (Fig. 2a); Chip-on-chip studies showed that out of the 919 DNA fragments that coprecipitated with ERα, 366 were specifically related to P, 479 to M, and only 74 (corresponding to 8%) were in common between the two phases. These studies indicated that ERα had a direct influence on liver gene expression and that the activity of the hepatic ER differed significantly in the two phases of the cycle. Bioinformatics studies allowed to conclude that at M, several of the sequences identified by the Chip-on-chip were in the proximity to genes involved in lipid and carbohydrate metabolism, whereas at P, most of the sequences recognized could be associated with genes encoding proteins necessary for transcriptional regulation such as zinc finger proteins, histone demethylases, homeobox proteins, and genes for the regulation of reproductive functions (Villa et al. 2012) (Fig. 2b). We further explored the extent to which the hepatic ERα could be involved in lipid metabolism in the LERKO mice where the hepatic ERα had been ablated specifically in the liver by breeding mice expressing the Cre recombinase under the albumin promoter with ERα-floxed mice (Della Torre et al. 2011). In the LERKO mice, we demonstrated that ERα exert a major control on liver lipid synthesis by downregulating the biosynthesis of fatty acids and cholesterol (Villa et al. 2012); in addition, estrogens regulate the turnover of transport proteins such as ApoA1 and ApoE and their receptors (Della Torre et al. 2016). Part of these effects might be mediated by the hepatic LXRα which abundance in the liver changes significantly in the different phases of the estrous cycle and that was found to cross-couple with ERα in the course of the ovulatory cycle.

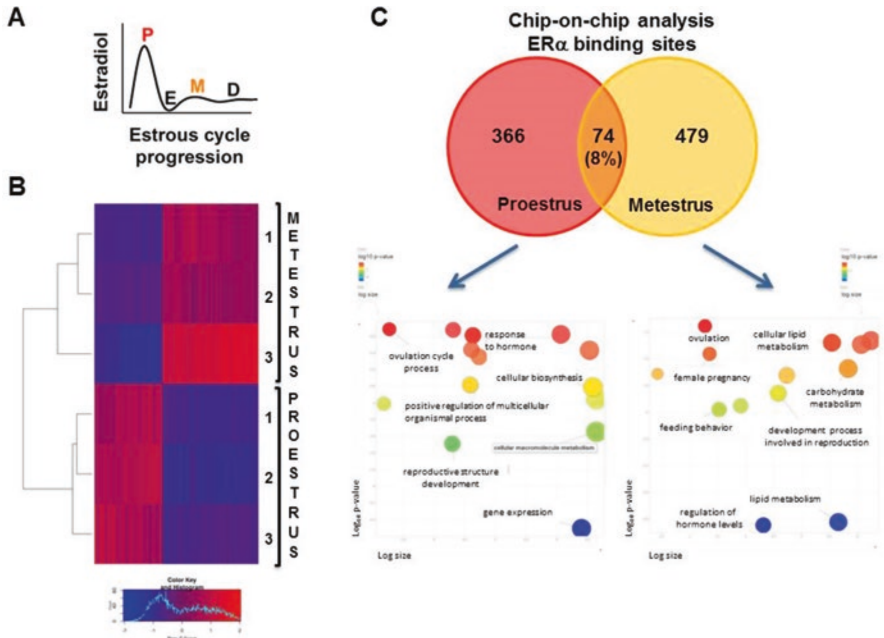

Fig. 2 RNA-Seq and chromatin immunoprecipitation reveal that, relative to the phase of the ovulatory cycle, the hepatic ERα has a different transcriptional impact on mouse liver. (**a**) Plasma levels of E2 change during the mouse estrous cycle progression. (**b**) Heatmap of the clustering of the 1178 DEGs (FDR < 0.05) from RNA-Seq analysis done in the livers of females at metestrus, M, and proestrus, P (3 mice/group). The heatmap was generated using the web interface shinyheatmap (http://shinyheatmap.com). (**c**) Upper panel, Venn diagram summarizing the overlap between ERα binding sites in the liver of females at proestrus (P, red circle on the left) and at metestrus (M, yellow circle on the right). Lower panel, Gene Ontology classification of the DNA fragments coprecipitated with ERα and located within 20 kb of known genes. The scatterplots have been generated using the web interface REVIGO (http://revigo.irb.hr/)

The combination of these results most heavily argued for a significant role of the hepatic ERα in the control of liver metabolism, also in relation to the ovulatory cycle, and raised the question as to whether this receptor constituted an important sensor of gonadal functions necessary to connect metabolism and reproduction.

Liver ERα Integrates Metabolic and Reproductive Functions in Mammals

Molecular mechanisms to limit fertility in case of famine are essential for animal survival, and throughout evolution, the strategies to associate fertility to food availability that were devised by living organisms are very well conserved. Detailed biochemical studies identified the pathways coupling nutrition and reproduction in

oviparous; in these animals, vitellogenins are the key proteins for the regulation of fertility because responsible for fat storage and mobilization; in addition, vitellogenins are important in the composition of the egg yolk because these proteins provide the nutrients necessary for the development of the embryo (e.g. AA, CARB, phosphates, and sulfates) (Jasmani et al. 2004). Vitellogenin synthesis occurs in the female liver (or organs acting like the liver in less evolved animals, like the intestine in nematodes) and is transcriptionally upregulated by endocrine and nutritional cues (Romano et al. 2002). The gonads synthesize the hormones regulating vitellogenin synthesis, and dietary AA participate in this control through the activation of an evolutionary conserved nutritional signaling cascade (target of rapamycin pathway) (Della Torre et al. 2014). Estrogen transcriptional control of the vitellogenin gene has been largely explored in the liver of female *Xenopus* becoming a paradigm for studies on hormonally regulated transcription (Tata 1988), and the relevance of AA for fertility has been elucidated in insects (Attardo et al. 2005; Hansen et al. 2005). Thus, in oviparous females, either a deficiency in ovarian functions or a prolonged starvation may block the maturation of the egg preventing reproduction.

The mechanisms reducing fertility in mammals are poorly investigated. Yet, it is well known that the liver plays a role, and mutants with impaired synthesis of VLDL or Apo-B (apolipoprotein B-100 is a member of the vitellogenin family) show reduced fertility and incapacity to generate a viable placenta. The fact that the transcriptional activity of the hepatic ER could be induced by AA led us to investigate on the consequences of AA-dependent activation of liver ER on fertility and to experimentally establish that AA-dependent activation of the hepatic ERα in the mouse is necessary for the synthesis of the IGF-1 necessary for the progression of the estrous cycle toward ovulation.

The study demonstrated that the ancestral mechanisms linking fertility to liver metabolic functions are well conserved in mammals (Della Torre et al. 2011).

The Delicate Balance of Energy Metabolism and Reproduction in Female Mammals

Differently from oviparous, in mammals the mother continues with her reproductive responsibilities well after ovulation by bearing gestation and then lactation. These duties, not present in oviparous, must have required a significant adaptation of the subtle mechanisms linking fertility to nutrition to enable the mother to respond to the variable energy demands of each stage of the reproductive cycle (periodic ovulation, gestation, and lactation). Indeed, experimental evidence in animals and humans showed that female reproductive functions may induce significant changes of liver metabolism, during the ovulatory cycle (as we described before) like during pregnancy or during lactation.

In case of pregnancy, a female liver, in the initial period, maximizes its lipogenic potential and uses all substrates available, including AA, to increase the production

of lipids and the transport proteins necessary to store energy in safe deposits; this function is necessary to provide the mother with the energy supply necessary for the final growth of the embryo (Baardman et al. 2013; Woollett 2011). In the late gestation, the liver limits lipid synthesis and preserves the AA and glucose for the fetus, while it facilitates the mobilization of lipids from their deposits (Ghio et al. 2011). Thus, the liver is responsible for the switch from anabolic to catabolic metabolism reported at mid-end gestation in most species. Then, in the course of lactation, the liver is again engaged for the massive production of glucose to be taken up by the mammary gland for the production of milk sugars (lactose) and the glycerol backbone of milk fat (triglycerides) (Burton and Fowden 2015; Butte 2000).

In order to perfect the control mechanisms necessary for the integration of the hepatic metabolic pathways into the intricacies of mammalian reproductive functions, the livers of female mammals must have evolved significantly in 120 million years since their first appearance on the surface of the earth. This likely explains why in all mammals, including humans, the liver has such a high degree of sexual dimorphism documented by a long series of biochemical, pharmacological, and clinical studies.

Liver, the Sexually Dimorphic Organ "Par Excellence"

A large number of clinical and preclinical studies have demonstrated that the male and female liver is highly dimorphic. It is well known that several P450 enzymes are expressed sex specifically; for instance, in women CYP3A4 content is much higher than in males, and this explains the major sex differences in the catabolism of endogenous hormones such as cortisol and testosterone, that undergo 6β hydroxylation by the CYP3A3/4, as well as several drugs (e.g., tricyclic antidepressants, benzodiazepines, and others) (Waxman and Holloway 2009; Williams et al. 2004). More recently, it became clear that compared to males, fertile females have higher rates of hepatic FA uptake, esterification, VLDL-TG synthesis and secretion, and a more efficient use of cholesterol, furthermore, very significant differences are found in the expression of genes involved in these pathways (Fig. 3a, b). Indeed, transcriptome and proteome studies have indicated that up to 72% of the genes in the liver may have characteristic of sex specificity; thus, the liver appears to be, together with some brain areas, the organ most sexually differentiated in mammals (Yang et al. 2006). Considering the large extent of liver sexual dimorphism in the regulation of metabolic functions, the medical implications of this phenomenon demand for a better understanding of the strategies and underlying mechanisms adopted by the male and female livers.

So far it was believed that the main hormonal factors for liver sexual differentiation are sex steroids and the pituitary growth hormone (GH). In the adult males, GH is secreted in pulses of 3–4 h intervals, while females have pulses of lower height resulting in lower GH content in plasma. Sex hormones, together with endogenous signaling associated with the nutritional status (fasting, exercise, glucose levels),

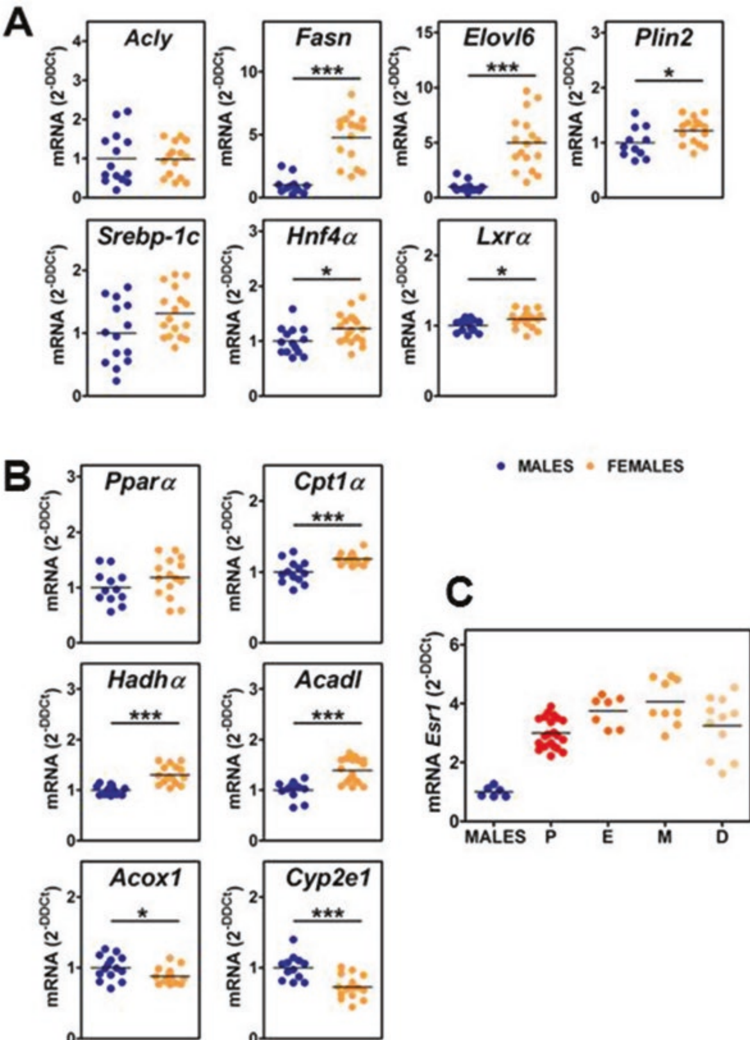

Fig. 3 Liver metabolism is sexually dimorphic. (**a**, **b**) Male and female comparative analysis of liver content of the mRNAs encoding proteins involved in hepatic metabolism. Each dot represents a single animal. $^{**}p < 0.05$ and $^{***}p < 0.001$ by t-test. (**c**) ERα mRNA content in the liver of adult males and female mice. Female livers were dissected in each phase of the cycle: proestrus (P), estrus E, metestrus (M), diestrus (D)

regulate GH secretion that affects primarily the liver where GH promotes lipolysis, decreased glucose uptake, gluconeogenesis, and increased protein synthesis. This suggests that this hormone may play a major role in determining the sex-specific expression of hepatic genes (Gustafsson et al. 1983; Chowen et al. 2004; Fernández-Pérez et al. 2013).

The finding that the hepatic ERα is directly involved in the regulation of the genes controlling lipid metabolism imposes a revision of the role of GH in controlling hepatic functions. Preliminary studies using the LERKO mice have in fact clearly shown the essential role played by liver ERα in coupling hepatic and reproductive functions and pointed to the fact that, together with GH indirect effects on the liver, a direct action of ER contributes to the maintenance of liver sex differences.

Thus, a better understanding of the hepatic ERα regulation and functions becomes very relevant for the full understanding of liver functions in the two sexes.

To this aim, we recently extended our studies to male liver. Interestingly, according to prior data in the literature, our transcriptomic analysis showed that the mouse male liver does not express aromatase; this renders the male liver an unlikely target for sex hormones. In addition, quantitative analysis of liver mRNA (Fig. 3c) and protein (Della Torre et al. 2016) shows that in male liver, the content of ERα is significantly lower than in females. In males, like in females, the liver expresses ERβ mRNA at the concentration that is several times lower than ERα. In spite of these findings, studies in the ERE-Luc model show that in males the hepatic ERα is transcriptionally activated after food ingestion (Ciana et al. 2005) and after administration of exogenous estrogens (Ciana et al. 2001). In keeping with this, studies in LERKO have shown that also in males the ablation of the hepatic ERα has significant metabolic consequences suggesting that also in males this receptor plays a role that requires further studies.

Health Consequences for the Dimorphic Hepatic Functions in Humans

The finding that ERα is a major target for estrogens in mammalian liver allows to better understand the physiological regulation of female metabolism and the strict interactions among nutrition-reproduction-metabolism. The studies carried out so far indicate that, at least in the female liver, the ER is a sensor of reproductive functions necessary to control the hepatic metabolism. Because of this, the cessation of ovarian functions due to pathology has a severe repercussion on liver metabolism with major consequences for the entire organism as underlined by the increased prevalence of metabolic, cardiovascular, skeletal, immune, and brain disorders in climacteric women as reviewed by us and others (Della Torre et al. 2014; Della Torre and Maggi 2017 and references therein). The understanding of the role exerted by ER in liver functions may explain how the impairment in its signaling triggers a series of dysfunctions characterizing women aging, thus providing the conceptual bases for future therapeutic interventions.

In addition, these studies provided novel theories of the origin of the significant, well-known hepatic sexual dimorphism. The discovery that, at least in mice, the hepatic ER is transcriptionally regulated by food intake (with particular reference to

AA) in both sexes may be stimulus for a better comprehension of the section of the hepatic transcriptome that is susceptible to AA-induced modulation via the ERα and of the consequences of a protein-enriched, unbalanced, diets in both females and males. This may also help in the identification of the mechanisms that provide a fertile woman with a selective health benefits toward men and provide novel therapeutic targets or relevant information for a dietary intervention.

Finally, the study presented further stresses the necessity to consider sex as a biological variable of paramount relevance for the design of personalized dietary and pharmacological therapies.

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Sex Differences in Muscle Wasting

Lindsey J. Anderson, Haiming Liu, and Jose M. Garcia

Abstract With aging and other muscle wasting diseases, men and women undergo similar pathological changes in skeletal muscle: increased inflammation, enhanced oxidative stress, mitochondrial dysfunction, satellite cell senescence, elevated apoptosis and proteasome activity, and suppressed protein synthesis and myocyte regeneration. Decreased food intake and physical activity also indirectly contribute to muscle wasting. Sex hormones also play important roles in maintaining skeletal muscle homeostasis. Testosterone is a potent anabolic factor promoting muscle protein synthesis and muscular regeneration. Estrogens have a protective effect on skeletal muscle by attenuating inflammation; however, the mechanisms of estrogen action in skeletal muscle are less well characterized than those of testosterone. Age- and/or disease-induced alterations in sex hormones are major contributors to muscle wasting. Hence, men and women may respond differently to catabolic conditions because of their hormonal profiles. Here we review the similarities and differences between men and women with common wasting conditions including sarcopenia and cachexia due to cancer, end-stage renal disease/chronic kidney disease, liver disease, chronic heart failure, and chronic obstructive pulmonary disease based on the literature in clinical studies. In addition, the responses in men and women to the commonly used therapeutic agents and their efficacy to improve muscle mass and function are also reviewed.

Introduction

Reductions in muscle mass are often associated with aging and/or an underlying illness and result in poor physical function and quality of life. With aging and other muscle wasting diseases, skeletal muscle undergoes pathological changes such as

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increased inflammation, enhanced oxidative stress, mitochondrial dysfunction, satellite cell senescence, elevated apoptosis and proteasome activities, and suppressed protein synthesis and myocyte regeneration. In addition, reduced food intake, physical activity, and circulating hormones such as sex steroids and growth factors can also contribute to muscle wasting.

Sarcopenia is defined as the age-related loss in muscle mass and strength or function (Cruz-Jentoft et al. 2010). When it is accompanied with excessive accumulation of fat mass, it is termed "sarcopenic obesity" (Narici and Maffulli 2010). The loss of muscle mass begins at the age of 50 and is continued until the end of life (Dennison et al. 2017). By age 80, the average muscle loss is about 40% of the peak muscle mass usually attained in the 20s (Janssen et al. 2000). Sarcopenia leads to multiple adverse outcomes such as higher risk of falls and fractures, increased drug toxicity, impaired quality of life, and decreased survival (Evans 2010; Fried et al. 2001; Morley 2016; Morley and Malmstrom 2013). In the United States (US), the medical-related cost of sarcopenia in 2000 was approximately 1.5% of the total healthcare expenditure (Janssen et al. 2004b). In contrast, cachexia is defined as edema-free unintentional weight loss of at least 10% in 12 months or less, or 5% in 6 months or less, or body mass index (BMI) <20 kg/m^2 with 2% weight loss over any period of time, in the presence of underlying illness (Fearon et al. 2011). Muscle wasting in cachexia commonly occurs with fat loss and a combination of inflammation, reduced muscle strength and function, and/or anorexia (Reid et al. 2016). It was recently reported that the median duration of hospital stay was 6 days for patients with cachexia but was 3 days for non-cachexia patients in 2009 (Arthur et al. 2014). Similarly the median cost was more than 10,000 dollars per cachexia admission compared to 6,000 dollars for non-cachexia admissions (Arthur et al. 2014).

Muscle wasting occurs when protein synthesis is reduced and/or protein degradation is increased to the extent that the balance results in net protein loss. The transcriptional and translational mechanisms regulating protein synthesis and degradation are largely coordinated by the phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway and the ubiquitin proteasome system (UPS; see Fig. 1) (Glass 2005). The mTOR pathway regulates protein synthesis and is activated by PI3K (Backer 2008; Gulati et al. 2008) and Akt, which are activated by growth factor signaling such as insulin-like growth factor (IGF)-I (Miyazaki and Esser 2009; Sandri 2008). Akt also regulates the influential Forkhead box-O (FOXO) atrogenes (atrophy-inducing genes). FOXO genes are transcription factors that induce the UPS by upregulating gene expression of two muscle-specific ligases, atrogin-1 and muscle RING finger 1 (MuRF-1) (Gross et al. 2008; Huang and Tindall 2007; Jang et al. 2007; Stitt et al. 2004). Akt phosphorylates FOXO genes, causing FOXO translocation into the cytoplasm where transcriptional activity is inhibited (Calnan and Brunet 2008; Huang and Tindall 2007; Nakae et al. 2008; Salih and Brunet 2008). Akt activity decreases during catabolic conditions, allowing FOXO genes to relocate to the nucleus and resume transcriptional activity (Sacheck et al. 2007). Atrogin-1 tags protein for degradation by the UPS targets the eukaryotic initiation factor 3 subunit 5 and the myogene MyoD for proteolysis,

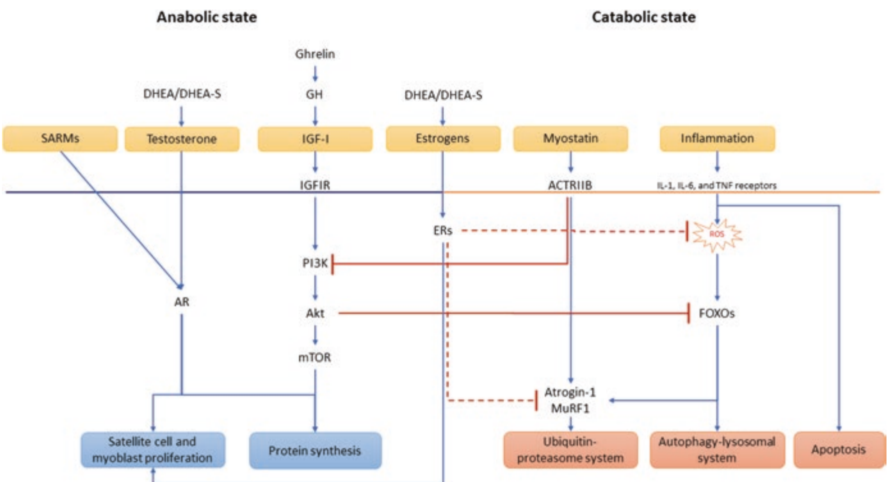

Fig. 1 Inhibition (red lines) and activation (blue arrows) signaling pathways involved in muscle anabolism (blue boxes) and catabolism (red boxes). Muscle wasting is induced by an imbalance between the anabolic and catabolic states. For the anabolic state: Dehydroepiandrosterone (DHEA) and its sulfur ester (DHEA-S) are the precursors for testosterone and estrogen. Testosterone and selective androgen receptor modulators (SARMs) bind to the androgen receptor (AR) that induce satellite cell and myoblast proliferation and activate protein synthesis. Ghrelin stimulates the release of growth hormone (GH) and further promotes the secretion of insulin-like growth factor I (IGF-I). IGF-I binds to the IGFI receptor (IGFIR) which leads to induction of protein synthesis via activation of the phosphoinositide 3-kinase–serine/threonine-protein kinase–mammalian target of rapamycin (PI3K–Akt–mTOR) signaling. Also, Akt inhibits the activation of the transcription factors forkhead boxOs (FOXOs). Estrogens act on estrogen receptors (ERs) alpha and beta to activate satellite cell and myoblast proliferation. For the catabolic state: the major three catabolic systems are ubiquitin-proteasome system (UPS), autophagy-lysosomal system, and apoptosis. Most of the myofibrillar proteins (e.g., myosin and actin) in skeletal muscle are degraded through the UPS. Under the catabolic conditions, myostatin binds to the activin receptor type IIB (ACTRIIB) which activates the UPS via E3 ligases (Atrogin-1 and MuRF1) and suppresses protein synthesis through inhibition of Akt signaling. Inflammatory cytokines, including tumor necrosis factor (TNF), interleukin (IL)-1β, and IL-6, promote generation of reactive oxygen species (ROS) and activate FOXO proteins which further contribute to activation of the UPS. In addition, FOXOs are responsible for the activation of autophagy-lysosomal system, which is a catabolic system for removing protein aggregates, dysfunctional mitochondria, and endoplasmic reticulum membranes. The inflammatory cytokines also contribute to the activation of apoptosis through caspases, which mediate the loss of muscle fibers. In contrast, estrogens have an anti-catabolic by inhibiting ROS and E3 ligases; however, the ER-mediated mechanisms are largely uncharacterized at this time (dashed lines)

resulting in muscle atrophy (Lagirand-Cantaloube et al. 2008, 2009). MuRF-1 targets the myosin heavy chain, an integral structural protein, for degradation (Clarke et al. 2007; Cohen et al. 2009). Another atrogene, myostatin, is a powerful negative regulator of muscle mass that binds to the activin receptor IIB (ActRIIB) (Lee and McPherron 2001). Myostatin reduces activation of Akt and mTOR signaling, inhibiting protein synthesis in concert with the actions of atrogin-1 and MuRF-1 which promote protein degradation (Amirouche et al. 2009).

Sex hormones play important roles in maintaining skeletal muscle homeostasis. Under normal conditions, the different roles of androgens and estrogens contribute to the gender disparity in skeletal muscle morphology and function. In young healthy adults, men have larger muscles, faster velocity, and more fast-twitch fibers than women. As a result, force generation and relaxation time are also faster in men during fatigue, while women have increased endurance and faster recovery than men (Haizlip et al. 2015). Testosterone is a potent anabolic factor promoting protein synthesis and muscular regeneration through androgen receptor signaling (Fig. 1). In skeletal muscle, the androgen receptor is expressed in mesenchymal stem cells, satellite cells, myocytes, and fibroblasts (Dubois et al. 2012; Sinha-Hikim et al. 2004). Testosterone has a potent anabolic effect on skeletal muscle. One of the mechanisms is through increasing the muscular expression of IGF-I which, in turn, activates the Akt signaling pathway to induce muscle hypertrophy. In humans, testosterone deficiency is associated with a decrease in circulating and intramuscular IGF-I, adversely affecting muscle size and strength (Carson and Manolagas 2015; Grinspoon et al. 1996; Mauras et al. 1998).

The influence of estrogens on the balance of skeletal muscle catabolic and anabolic processes is a relatively new field of study in muscle physiology. Estrogen receptors α and β are expressed in skeletal muscle satellite cells, myofibers, and endothelial cells. Estradiol has been shown to reduce long-term muscle atrophy in postmenopausal women (Pollanen et al. 2010; Ronkainen et al. 2009; Sipila et al. 2001; Sorensen et al. 2001; Teixeira et al. 2003) and reduce exercise-induced muscle fiber damage in young women (Carter et al. 2001; Roth et al. 2000). The influence of estrogens on skeletal muscle inflammatory and catabolic pathways has been previously examined in small preliminary studies (Dieli-Conwright et al. 2009; Dieli-Conwright et al. 2012; Williamson et al. 2010). In the basal state, elderly women (~85 years of age) not receiving hormone replacement therapy (HRT) with presumably very low estrogen levels displayed lower cytosolic phosphorylated (P)-FOXO3a and higher total nuclear FOXO3a than young women (~24 years of age) (Williamson et al. 2010). In addition, basal mRNA levels of the atrogenes MuRF-1 and myostatin decreased after 12 weeks of resistance training (RT) in the young women, while myostatin mRNA levels increased in the elderly women (Williamson et al. 2010). In an acute exercise setting, HRT users experienced smaller exercise-induced muscular mRNA increases in the inflammatory genes interleukin (IL)-6, IL-8, IL-15, and tumor necrosis factor (TNF)-α (Dieli-Conwright et al. 2009) and experienced greater exercise-induced muscular mRNA decreases in myostatin and ActRIIB (Dieli-Conwright et al. 2012). These observations may suggest an anti-inflammatory and anti-catabolic influence of estrogens on skeletal muscle in women, especially after exercise. However, evidence supporting a significant effect of estrogens on muscle mass is lacking, and the link between estrogen receptor signaling and downstream anti-inflammatory and anti-catabolic effects is uncharacterized at this time (Fig. 1).

Age- and/or disease-induced alterations in sex hormone levels are major contributors to muscle wasting. Hence, men and women may respond differently to

catabolic conditions because of their different hormonal profiles. Here we review the similarities and differences between men and women with common wasting conditions including sarcopenia and cachexia due to cancer, end-stage renal disease (ESRD)/chronic kidney disease (CKD), liver disease, chronic heart failure (CHF), and chronic obstructive pulmonary disease (COPD) based on the literature in clinical studies. In addition, the responses in men and women to the commonly used therapeutic agents and their efficacy to improve muscle mass and function are also reviewed.

Age-Related Muscle Wasting: Sarcopenia

The prevalence of sarcopenia is approximately 5–13% in 60- to 70-year-old individuals and increases to 11–50% for people aged 80 or older (von Haehling et al. 2010). Sarcopenia is evidenced by reduced fiber size (atrophy) and fiber number (hypoplasia), preferentially affecting fast-twitch type IIB fibers (Mitchell et al. 2012; Narici and Maffulli 2010). The decrease in fiber number is caused by a loss in motor units, which starts at age 60 and continues at a rate of 3% per year (Campbell et al. 1973). The decreased fiber size is additionally due to decreased protein turnover, with a decreased ratio of protein synthesis to degradation. With aging, the levels of anabolic hormones such as IGF-I and testosterone dramatically decline in men and women resulting in net protein loss. In addition, age-related decline in insulin sensitivity is associated with impaired IGF-I action (Perrini et al. 2010). On the other hand, the systems for protein degradation, including the UPS, autophagy, and apoptosis, are activated by elevated catabolic factors such as the inflammatory cytokines TNF-α and IL-6 and excessive production of reactive oxygen species (ROS) with aging (Thompson 2009; Baumann et al. 2016). Other age-related factors, such as malnutrition and decreased physical activity, also contribute to the development of sarcopenia via protein depletion and decreased neuromuscular stimulation. Although most factors contributing to sarcopenia are similarly affected in men and women, some processes can be different due to the dramatic changes in sex steroids during aging, such as menopause in women. It is important to understand the sex-related differences in sarcopenia in order to identify therapeutic targets which may be sex dependent.

Sex Differences in Sarcopenia

Muscle Mass and Body Composition

While the decrease in muscle mass, muscle strength, and physical function and increase in fat mass are more prominent in men compared to women, the prevalence of sarcopenia is higher in women given that their absolute muscle mass is

smaller to begin with. Women have 40% less upper body and 30% less lower body muscle mass than men (Janssen et al. 2000), and with aging, the absolute and relative decrease in muscle mass is less in women (Churchward-Venne et al. 2014). For example, the losses in total appendicular skeletal muscle mass in men and women were 4 kg (~15%) and 2 kg (~11%), respectively, over five decades between the ages of 20 and 70 years (Gallagher et al. 1997). In a different study in elderly participants, the absolute loss in appendicular muscle mass was approximately 1 kg in men and 0.6 kg in women over a 7-year period (Gallagher et al. 2000). In addition, a study on the rate of muscle atrophy in elderly subjects (68–78 years) showed lower rates of muscle mass loss (leg and appendicular muscle) in women compared to men over a 2-year period (Zamboni et al. 2003). The large magnitude of muscle loss in men can only be partially explained by the larger initial muscle mass in men.

An increase in fat mass with aging is also seen in both men and women. In general, older women have higher body fat and lower lean body mass (LBM) than the same-aged men. In the Health, Aging and Body Composition (Health ABC) study (Tseng et al. 2014), a cohort of 3,075 well-functioning 70- to 79-year-old men and women were analyzed for body composition by using dual-energy X-ray absorptiometry (DEXA). Older women showed higher body fat mass than men in the same cohort (women vs. men, 28.9 kg vs. 24.1 kg), whereas appendicular lean mass was lower in women (women vs. men, 16.5 kg vs. 23.9 kg). Similarly, in a study involving 79 community-dwelling age- (75–76 years) and BMI-matched men and women, women had lower total mineral-free lean mass (MFLM) and lower-body MFLM but higher percent body fat than men (Straight et al. 2015). However, age-associated changes in fat were more prominent in older men than in women. In the Health ABC study, the absolute and relative increases in total fat were both higher in men compared to women (Goodpaster et al. 2006).

Muscle Strength and Quality

Deterioration in muscle strength occurs in both men and women with aging. Although older men have greater absolute muscle strength than same-aged women, the loss of muscle strength with aging is greater and faster in men (Tseng et al. 2014). In the Health ABC study, older men lost twice as much absolute knee extensor strength as women over a 3-year period (Goodpaster et al. 2006). Similarly, it has been reported that eccentric peak torque is better preserved in older women than in older men. Specifically, the age-related decline in eccentric peak torque of knee extensor was 31% in men and 22% in women (Lindle et al. 1997). Nevertheless, in the same study, the age-induced decreases in concentric peak torque of knee extensor were equal in men and women (33% vs. 35%, respectively) (Lindle et al. 1997).

Physical Function

Physical activity and functional fitness decline with aging in men and women. A study compared 60- to 69-year-old and 70- to 80-year-old individuals for functional fitness, including back scratch, chair sit and reach, 8-ft up and go, chair stand-up for 30 s, arm curl, and 2-min step test (Milanovic et al. 2013). Significant age-associated declines were found in all tests in men. Similar changes were found for women, except no significant differences were detected for the chair sit and reach and 2-min step test. The results indicated equal age-associated declines in physical function for men and women though women were more flexible than men (Milanovic et al. 2013). Other studies indicate that, functionally, sarcopenia affected women more than men. In people aged 65–74 years, the likelihood of limitations in functional activities was higher in women than men (Newman and Brach 2001). Clinical studies showed that functional impairment, development of frailty, and loss of independence were more likely related to sarcopenia in elderly women than men (Janssen et al. 2004a).

Age-Related Changes in Testosterone

Approximately 60% of circulating testosterone is bound to sex hormone-binding globulin (SHBG), and this fraction is not available to bind the androgen receptor. The free (~2%) and albumin-bound (~38%) testosterone fractions are biologically active (Garcia et al. 2006). With aging, there is a decrease in total testosterone (total T) and free testosterone (free T) and an increase in SHBG in both sexes (Baumgartner et al. 1999). The circulating level of total T declines in men about 1% per year after the age of 40 (Sipila et al. 2013). Total T and free T levels in the serum of independently living elderly men (73–94 years) were about 255 ng/dL (8.83 nmol/L) and 1 ng/dL (0.03 nmol/L), respectively (van den Beld et al. 2000), whereas the Endocrine Society Guidelines suggest the lower limits of the normal total T and free T range in healthy young men are 300 ng/dl and 5 ng/dl, respectively (Bhasin et al. 2006b). In older men, there is a higher prevalence of low T levels which is related to the decline in total T and free T levels. In the Baltimore Longitudinal Study of Aging (Harman et al. 2001), low T levels were defined by using the date-adjusted total T level, which was less than 325 ng/dl (the 2.5th percentile of values for the men at 21–45 years). By this definition, the prevalence of low T was about 12%, 19%, 28%, and 49% in men greater than 50, 60, 70, or 80 years of age, respectively (Harman et al. 2001).

In women, total T levels decline from 20 to 40 years of age, whereas the age-related changes in free T are minimal. By the age of 40, testosterone levels in women are about 50% of levels at the age of 20 (Zumoff et al. 1995). At the time of menopause, typically early in the fifth decade of life, there is no further decrease in tes-

tosterone, although the level is still lower than in premenopausal women (Morley and Perry 2003). After the age of 65, free T levels slightly increase due to the estradiol-associated decrease in SHBG. Specifically, total T and free T levels in women older than 65 were about 20 ng/dL and 0.28 ng/dL (Cappola et al. 2007), respectively, whereas these values were 35 ng/dL and 0.37 ng/dL in 20- to 45-year-old healthy premenopausal women, respectively (Sinha-Hikim et al. 1998).

As testosterone and IGF-I decrease with age (Carson and Manolagas 2015; Grinspoon et al. 1996; Mauras et al. 1998), the faster muscle loss seen in aged men compared to women is likely associated with the greater decrease in testosterone levels in men (Mitchell et al. 2012). In addition, preclinical studies demonstrated a role of androgens in satellite cell and myoblast proliferation. Genes involved in terminal myogenic differentiation (Cdkn1c and Igf2) in the muscles of male androgen receptor knockout mice were upregulated, whereas a negative regulator of muscle differentiation, Itgb1bp3, was decreased compared to wild-type muscles. These results indicated a role of androgens in maintaining myoblasts in the proliferative state and delayed differentiation (MacLean et al. 2008). Androgens also play a role in the inflammatory stage of muscle regeneration. Castrated mice presented lower concentration of myosin heavy chain in soleus muscle after injection of snake venom (Ferry et al. 1999) and impaired phagocytosis with the implantation of mouse skeletal muscle grafts (Grounds 1987). In humans, testosterone targets satellite cells to prevent the age-related myocyte apoptosis. Administration of testosterone increases mitochondrial area and satellite cell number, leading to increased muscle size (La Colla et al. 2015).

Low free T levels are associated with development of mobility limitation. In a longitudinal study, the progression of mobility limitation was more likely to be present in men with low free T levels than in men with normal free T levels (Basualto-Alarcon et al. 2014; Krasnoff et al. 2010). Many studies have shown benefits from testosterone therapy on increasing muscle size and strength (Sipila et al. 2013). These studies are discussed later in this chapter.

Age-Related Changes in Estrogens

Estrogens decline in women after menopause around the age of 50 (Horstman et al. 2012; Morley and Perry 2003). The concentration of estrogens, including estradiol and estrone, dramatically decreases within a 6-month period around menopause (Sipila et al. 2013) and continues for 3 years postmenopause. Estrogen deficiency is associated with decreased muscle strength and increased inflammation which has been primarily reported in preclinical studies. The ovariectomized (OVX) mouse, an animal model of low estrogen levels, showed a decline in force generation in skeletal muscles that was reversible with estradiol replacement (Haizlip et al. 2015; Moran et al. 2007). Also, estradiol successfully attenuated the inflammatory response in exercise-induced muscle damage (La Colla et al. 2015). Moreover, estradiol has antiapoptotic effects through the PI3K/Akt signaling (Boland et al. 2008). In vitro

studies showed that estradiol-pretreated C2C12 cells can prevent H_2O_2-induced apoptotic changes, such as nuclear fragmentation, mitochondrial reorganization, and increased cytochrome c (La Colla et al. 2015; Vasconsuelo et al. 2011).

The decrease in estrogens during menopause is associated with a higher injury risk and a decrease in LBM (Haizlip et al. 2015; Bea et al. 2011). Although post-menopausal estradiol-containing HRT does not have as strong an anabolic effect as testosterone, HRT in women improved muscle function (Sipila et al. 2013). After 1-year trial of HRT, 50- to 57-year-old postmenopausal women showed ~15% greater muscle power, 32% greater plantar flexor twitch torque, and 2–7% greater running/walking speed than the non-HRT counterparts. In the same study, HRT prevented fat infiltration and loss of muscle mass in the thigh muscles (Sipila et al. 2013). However, HRT is not commonly used in postmenopausal women because of its multiple side effects: increased risk of cardiovascular disease, higher risk of breast cancer, and deep vein thrombosis. It is possible that selective estrogen receptor modulators (SERMs) can be developed in the future to exploit the benefits from estrogens with a more favorable safety profile.

Sex as a Factor Modulating Response to Therapies

To date, there is no approved pharmacologic treatment for sarcopenia. Resistance exercise is commonly used and it is known to promote muscle hypertrophy. There is also evidence showing aerobic exercise improves muscle function in the lower extremities in older men and women with sarcopenia (Pahor et al. 2014). Non-pharmaceutical supplements to manage sarcopenia include vitamin D (Beaudart et al. 2014), antioxidants (Khor et al. 2014), and nutritional supplements (Welch 2014) although their effectiveness has not been confirmed in large randomized trials. The therapeutic effects of hormonal interventions on men and women with sarcopenia are reviewed here.

Testosterone and Anabolic Steroids

The decrease in testosterone levels with age is associated with muscle atrophy and weakness (Baumgartner et al. 1999). Many studies show that administration of testosterone preserves muscle mass and improves muscle strength in men (Snyder et al. 1999; Herbst and Bhasin 2004; Bhasin et al. 2006a; Chen et al. 2005). At lower doses, testosterone increases protein synthesis and results in muscle hypertrophy (Wolfe et al. 2000; Ferrando et al. 2003). At higher doses, testosterone also improves satellite cell recruitment and decreases adipose stem cells (Kovacheva et al. 2010). To investigate the effects of testosterone therapy on physical performance, sexual function, and energy on older men with low testosterone, the Testosterone Trials (T Trials) were conducted at 12 sites in 790 men over 65 years old in the United States. Participants in the study received either testosterone or matching placebo gel which

was applied to the skin daily. Sexual function was improved in the testosterone-treated group compared to the placebo group. No significant benefits from testosterone were detected in walking distance (Snyder et al. 2016). The number of adverse events was similar between groups. However, testosterone was associated with a significantly greater increase in coronary artery noncalcified plaque volume, as measured by coronary computed tomographic angiography (Budoff et al. 2017). Larger studies are needed to understand the clinical implications of this finding.

While small studies have suggested that testosterone may be efficacious for the treatment of sarcopenia (Page et al. 2005), potential side effects include increased adverse cardiovascular events, fluid retention, and higher risk of prostate cancer. Basaria et al. studied the effect of daily testosterone gel on >65-year-old community-dwelling men with mobility limitation (TOM trial) (Basaria et al. 2010). This study was terminated at 6 months due to increased cardiovascular-related events in the testosterone-treated group (testosterone vs. placebo, 22% vs. 5%), including syncope, myocardial infarction, peripheral edema, and elevated blood pressure.

In women, few studies have investigated the anabolic effect of testosterone on sarcopenia. Sixteen-week treatment with Estratest® (methyltestosterone and esterified estrogens) improved LBM and lower body strength in postmenopausal women compared to women receiving esterified estrogen alone with no noteworthy side effects (Dobs et al. 2002). However, testosterone therapy in women can cause masculinization (excessive facial hair, balding, deepening of the voice, and clitoromegaly) (Mooradian et al. 1987; Morley and Perry 2003). In addition, other side effects of testosterone such as increased adverse cardiovascular events and fluid retention have also been reported in women (Morley and Perry 2003). Although some epidemiology studies showed a higher risk of breast cancer associated with elevated androgen levels, other studies indicated that testosterone had a protective function on the breast tissue (reviewed in Glaser and Dimitrakakis 2013).

Selective Androgen Receptor Modulators (SARMs)

Although testosterone promotes muscle anabolism in both sexes, the potential for side effects such as adverse cardiac events, prostate cancer in men, and virilization in women limits its use. Selective androgen receptor modulators (SARMs) are a class of tissue-selective androgen receptor ligands that activate androgenic signaling via binding to androgen receptors (Bhasin and Jasuja 2009). SARMs preserve the anabolic effects on skeletal muscles but may potentially have a more favorable safety profile than testosterone. In addition, SARMs are orally available, whereas oral administration of testosterone is limited by its hepatic side effects. The anabolic effects of SARMs have been well characterized in preclinical studies. JNJ-28330835, a nonsteroidal SARM, successfully prevented muscle atrophy in rats after orchiectomy (Jasuja and LeBrasseur 2014; Allan et al. 2007). MK0773, a 4-aza-steroidal SARM, was safe and well tolerated and showed anabolic effects on men and women. In a phase IIA randomized, placebo-controlled clinical trial, MK0773 increased

muscle mass, bilateral leg press, and stair climbing power in women >65 years old with sarcopenia and moderate physical dysfunction (Papanicolaou et al. 2013). However, serum transaminase levels were higher in the MK-0773-treated group versus placebo. Also, increased hemoglobin, hematocrit, and systolic blood pressure were observed in the MK-0773-treated group. GTx-024 (enobosarm) is a nonsteroidal SARM with a strong efficacy and safety profile. In a 12-week randomized, double-blind, placebo-controlled study, GTx-24 showed a dose-dependent increase in LBM and improvement in physical function (stair climb) in elderly men and postmenopausal women (aged >60 years) compared to the placebo group (Dalton et al. 2011). GTx-024 was well tolerated with lower risks of developing cardiovascular disease and insulin resistance in this study.

Dehydroepiandrosterone (DHEA) and Its Sulfate Ester (DHEAS)

Dehydroepiandrosterone (DHEA) is a precursor of various sex steroids, such as testosterone and estradiol. The sulfate ester of DHEA (DHEAS) is also a circulating androgen in humans (Percheron et al. 2003). In 20- to 35-year-old adults, the levels of DHEA and DHEAS are 10–20% higher in men compared to women (Flynn et al. 1999). With aging, there is a decline in DHEA and DHEAS in both sexes starting at the age of 30. At the age of 70, the level of DHEA is about 20% of the peak level at the age of 20–30 (Orentreich et al. 1992). It has been reported that lower levels of DHEA and DHEAS are correlated with decreased muscle mass and strength, mobility, and a higher risk for falls (Reviewed in Samaras et al. 2013), and some groups have proposed that DHEA could promote anabolism by elevating testosterone and estradiol levels modestly and with minimal androgenic effects (Nair et al. 2006).

DHEA administration in older adults improves the serum levels of IGF-I (Morales et al. 1998; Igwebuike et al. 2008) and sex hormones (Morales et al. 1998; Igwebuike et al. 2008; Kenny et al. 2010) and insulin sensitivity (Kenny et al. 2010; Villareal and Holloszy 2004; Jedrzejuk et al. 2003). In turn, LBM (Jedrzejuk et al. 2003; Villareal and Holloszy 2004), muscle strength, and function are improved by administration of DHEA in some studies. However, these studies are small-sized, single-centered studies, and the results are inconsistent between studies. For instance, in a randomized controlled trial, administration of DHEA for 6 months improved insulin sensitivity and decreased visceral and subcutaneous fat in 65- to 78-year-old men and women (Villareal and Holloszy 2004). Similarly, in another study in elderly frail women (~76-year-olds) with low DHEA levels, administration of DHEA combined with calcium and cholecalciferol and gentle exercise increased DHEAS, estradiol, estrone, and testosterone levels. Also, muscle strength and function were significantly improved in these women in the lower extremities after DHEA treatment in a placebo-controlled study (Kenny et al. 2010). However, other clinical trials have not confirmed these results on muscle mass and function in response to the treatment of DHEA. For example, in a 2-year, placebo-controlled, randomized, double-blind study, no significant improvement was found in body

composition, physical performance, insulin sensitivity, or quality of life with DHEA administration in elderly men and women with lower levels of DHEAS compared to placebo (Nair et al. 2006). Also, a double-blind trial of DHEA administration for 1 year versus placebo in healthy 60- to 80-year-old men and women showed significantly increased DHEAS levels but no improvements in muscle strength and myocyte cross-sectional area (CSA) when compared to placebo (Percheron et al. 2003). In addition, Igwebuike et al. reported the effects of exercise with or without DHEA in postmenopausal women (Igwebuike et al. 2008). Although the results showed exercise + DHEA improved the levels of IGF-I and sex hormones compared to the exercise + placebo group, there was no additional benefit from DHEA on body composition or functional measures. Regarding the role of gender, Morales et al. reported a sex dimorphism in 50- to 65-year-old subjects in response to a higher dose of DHEA supplement. After 6 months of administration of DHEA, body composition was improved in both sexes with increases in LBM and reduction in fat mass. However, muscle strength only moderately increased in men, and testosterone levels only increased in women (Morales et al. 1998). DHEA administration has been well tolerated in general. Minimal adverse effects have been detected in women, including acne, facial hair growth, and edema (Legrain et al. 2000; Traish et al. 2011). One concern in men is that the prostate may be affected by the androgen transformed from DHEA. However, no prostatic adverse events were reported after 2-year administration of DHEA in older men (Nair et al. 2006). Taken together, although some biomarkers of anabolism appear to improve with DHEA treatment, the functional impact of these changes is equivocal. More data is needed before this intervention can be recommended.

Growth Hormone (GH) and Insulin-Like Growth Factor-I (IGF-I)

In preclinical studies, overexpression of IGF-I in rodents significantly improved muscle mass and strength (McMahon et al. 2014). The mechanism was mainly through the activation of IGF-I/Akt signaling. In turn, mTORC1 activation induces protein synthesis, and the ubiquitin-proteasome system is suppressed, resulting in less protein degradation. In humans, growth hormone (GH) administration increases LBM in both sexes (Rudman et al. 1990). However, no strength improvement has been found with GH alone, although the combination of GH and testosterone did improve strength (Blackman et al. 2002). In a 26-week randomized, double-blind, placebo-controlled parallel-group trial in 65- to 88-year-old community-dwelling US women and men (Blackman et al. 2002), the effects of four different combinations of GH and sex steroids were studied: GH + sex steroids, GH + placebo sex steroids, sex steroids + placebo GH, or placebo GH + placebo sex steroids. The sex steroids used for women were transdermal estradiol + oral medroxyprogesterone acetate, during the last 10 days of each 28-day cycle; for men, the sex steroid was

testosterone enanthate, given biweekly through intramuscular injections. The results showed significant increase in LBM and decreased fat mass in GH-treated groups with or without sex steroid in men and women. The improvement in one repetition maximum strength was only presented in men with a combination of GH and testosterone by week 17 (Blackman et al. 2002). No significant strength improvement was found in women with GH and/or hormone replacement. Nevertheless, these studies are small, and the results have not been replicated in a larger, controlled study yet. Hence, because the efficacy of GH is still debatable as a treatment for sarcopenia, further studies are needed before this can be recommended as an option. In addition, adverse effects have been noted in GH treatment including carpal tunnel syndrome, edema, arthralgias, and glucose intolerance (Abs et al. 1999; Cohn et al. 1993).

Ghrelin and Ghrelin Mimetics

Ghrelin is a hormone that stimulates food intake and GH release by binding to its receptor in the hypothalamus and somatotroph cells in the pituitary. Although women showed higher fasting ghrelin levels than men in both young and older groups, the levels of ghrelin declined with age in both sexes (Serra-Prat et al. 2015). In the elderly, individuals with sarcopenia have lower ghrelin levels compared to those without sarcopenia (Serra-Prat et al. 2015). Agonists of the ghrelin receptor (also known as GH secretagogues or ghrelin mimetics) stimulate appetite and increase GH secretion similarly to ghrelin. Ghrelin mimetics present the advantage of having a longer half-life and good oral availability.

Several studies evaluated the anabolic effects of ghrelin mimetics on healthy older men and women. MK-0677 (ibutamoren mesylate), a ghrelin mimetic, successfully increased IGF-I levels in older adults (>60-year-olds) to young-adult levels with daily administration. Although there was an increase in LBM, no functional improvements were seen (Nass et al. 2008). In >65-year-old hip fracture patients, serum levels of IGF-I increased 84%, and the performance of repeated chair rise was significantly improved with daily administration of MK-0677 (Bach et al. 2004). In another hip fracture study, 24 weeks of MK-0677 treatment improved gait speed and stair climbing power but not other physical functions in ambulatory patients ≥60 years old with a recent unilateral hip fracture (Adunsky et al. 2011). This study was terminated early due to increased incidence of heart failure. Another ghrelin mimetic, capromorelin, induced a dose-related increase in IGF-I in 65- to 84-year-old “pre-frail” men and women in a placebo-controlled study (White et al. 2009). Six months of capromorelin treatment increased lean mass and tandem walk speed, and an improvement in stair climb was noted after 12 months of treatment (White et al. 2009). No sex differences have been reported in response to ghrelin or ghrelin mimetic treatment so far, although the studies were not powered to detect a difference. Adverse events from ghrelin mimetics were mild to moderate and included increases in fasting glucose, glycosylated hemoglobin, and indices of insulin resistance (Adunsky et al. 2011; Ali and Garcia 2014). In summary, ghrelin and

its mimetics appear to increase LBM, appetite, and IGF-I in older men and women, but their safety and efficacy in the setting of sarcopenia have not been fully established.

Disease-Related Muscle Wasting: Cachexia

Cancer

Sex Differences in Incidence and Mechanisms of Muscle Wasting

Muscle wasting in cancer is a symptom of cachexia, a multifactorial metabolic syndrome of ongoing muscle loss, with or without fat loss, not fully reversed by nutritional supplements and resulting in physical function decrements (Fearon et al. 2011). Tumor burden and host response increase production of cytokines and catabolic factors (Ali et al. 2013; Batista et al. 2012; Plata-Salaman 2001), decrease anabolic tone (Del Fabbro et al. 2010; Garcia et al. 2006), and centrally suppress appetite (Mondello et al. 2015; Plata-Salaman 2001). This promotes lipid mobilization and protein degradation, decreased protein synthesis, and increased resting energy expenditure (REE), ultimately reducing body weight, muscle mass, fat mass, and quality of life (QOL) (Fearon et al. 2011; Quinten et al. 2009). These symptoms are typically exacerbated with chemotherapy/radiotherapy treatment. The initial clinical presentation of cancer cachexia is usually involuntary weight loss (>10% in 12 months or less, >5% in 6 months or less, or BMI <20 kg/m^2) often accompanied by fatigue and loss of appetite. Prevalence of cachexia is greatest in gastric, pancreatic, and esophageal cancer (80%), followed by head and neck cancer (HNC; 70%) and lung, colorectal, and prostate cancer (60%) (Laviano and Meguid 1996). The incidence of cachexia is roughly 40–60% in men and 30–50% in women across various cancer types (Baracos et al. 2010; Nakamura et al. 2015; Prado et al. 2009; Stephens et al. 2012; Wolf et al. 2006). For example, incidences in some cohorts are reported as 43% (men) and 47% (women) in gastrointestinal cancer (Stephens et al. 2012), 60% (men) and 49% (women) in large B-cell lymphoma (Nakamura et al. 2015), and 61% (men) and 31% (women) in non-small cell lung cancer (Wolf et al. 2006), suggesting that gender may play a role in this setting.

In tumor-bearing mice, males lose greater percent body weight than female mice, not due to difference in tumor burden (Hetzler et al. 2017; White et al. 2011), but due to rapid loss of muscle and fat mass. In female mice, fat mass declined early in the cachexia process, while muscle loss was more gradual. Cessation of estrous cycles occurred in roughly 40% of tumor-bearing female mice and was associated with muscle inflammatory gene expression and severe cachexia (Hetzler et al. 2017). In addition, the prevalence of hypogonadism has been reported between 40% and 90% of male patients with cancer (Garcia et al. 2006; Strasser et al. 2006). However, the causal relationship between hypogonadism and cancer cachexia has

not been observed consistently (Del Fabbro et al. 2010; Simons et al. 1999; Taira et al. 2009; Utech et al. 2012).

Sex Differences in Therapeutic Effects

Testosterone and SARMs have been used as hypertrophic treatments in the cancer setting. Men with advanced cancer of various types and hypogonadism received testosterone or placebo every 2 weeks for 10 weeks in a phase II trial with the goal of achieving bioavailable testosterone levels between 70 and 270 ng/dL (Del Fabbro et al. 2013). There were no differences between groups in body composition, muscle strength, or physical function, although libido and performance status improved with testosterone. The SARM enobosarm (3 mg/day) was well tolerated in a phase II trial and increased muscle mass and stair climb power (SCP) compared to placebo after 16 weeks in men and women with various tumors and ≥2% weight loss in the prior 6 months (Dobs et al. 2013). These results were not replicated in two large 5-month long phase III trials in men and women with non-small cell lung cancer (NSCLC). The change in LBM was significantly greater than the change in the placebo group; however, there was no difference on SCP (Crawford et al. 2014). It is unknown whether there is a difference between the response of men and women in the Enobosarm trials in cancer patients at this time.

Similar to these reported anabolic hormone interventions, exercise and nutritional supplementation individually have largely been ineffective at reducing muscle loss or increasing muscle mass in the cancer setting (Anderson et al. 2017). Other interventions such as ghrelin/mimetics, β2-agonists, or anti-inflammatory agents may have more potential as individual therapies or in combination. Ghrelin improved muscle mass after 8 weeks in men and women with gastrointestinal cancer and cachexia in a phase II trial, although there was no placebo group for comparison (Lundholm et al. 2010). Anamorelin, a ghrelin mimetic, increased muscle mass and appetite rating compared to placebo after 12 weeks in men and women with advanced cancer and cachexia in phase II and III trials (Garcia et al. 2015; Temel et al. 2016). However, the increases in handgrip strength (HGS) seen in the phase II studies were not seen in phase III trials. Espindolol, an agent with partial β2-agonist activity, improved muscle mass compared to placebo in men and women with advanced colorectal or NSCLC in a multicenter phase II trial (Coats et al. 2016). Espindolol is suggested to alleviate cachexia via anti-catabolic (reduced catabolism through β-receptor blockade and reduced fatigue and thermogenesis through central antagonism of a serotonin subtype (5-HT1a) receptor) and anabolic mechanisms (β2-receptor agonism) (Coats et al. 2016). Anti-inflammatory therapies show some promise at improving cachexia. ALD518 (anti-IL-6) attenuated loss of muscle mass after 12 weeks in patients with advanced NSCLC in a phase II trial; participant sex was not reported (Rigas et al. 2010). MABp1 (anti-IL-1a) showed potential for increasing muscle mass after 8 weeks in men and women with metastatic cancer in a dose-escalation phase I study (Hong et al. 2015). Omega-3 fatty acids like eicosapentaenoic acid (EPA) counteract the inflammatory effects of

cyclooxygenase. EPA improved muscle mass compared to an isocaloric diet after two chemotherapy cycles in NSCLC with cachexia in a phase II/III trial (Sanchez-Lara et al. 2014) but have not been successful otherwise (Fearon et al. 2006). It is currently unknown whether there is a difference between the response of men and women in these ghrelin/mimetic, beta agonism, or anti-inflammatory therapeutic trials in cancer patients.

There is currently no approved treatment for cancer cachexia, and individual therapeutic modalities have shown limited promise at alleviating cachexia symptoms. Current studies have not been powered to address sex differences in therapeutic response, which may be important in cancer cachexia due to the potentially reduced incidence of cancer cachexia in women. In addition, future research should explore the potential of multimodal therapy to reduce cachexia symptoms in both men and women.

Chronic Obstructive Pulmonary Disorder (COPD)

Sex Differences in Incidence and Mechanisms of Muscle Wasting

Skeletal muscle wasting is experienced by approximately 15–50% of chronic obstructive pulmonary disorder (COPD) patients with an incidence in men and women reported between 15–38% and 16–25%, respectively (Schols et al. 1995; Vestbo et al. 2006; Sergi et al. 2006). Patients with more than one acute COPD exacerbation per year experience greater decline in muscle mass compared to patients with less than one acute COPD exacerbation per year (Hopkinson et al. 2007). Patients on maintenance oral steroids also experience greater decline in muscle mass than those not on maintenance steroids, but those who cease smoking may experience an increase muscle mass (Hopkinson et al. 2007). COPD patients also often display increased abdominal adiposity and reduced appendicular muscle mass (van den Borst et al. 2012). Suggested mechanisms for cachexia in COPD include increased whole-body myofibrillar protein breakdown (Rutten et al. 2006), satellite cell senescence (Theriault et al. 2012), and increased MAFbx and myostatin expression (Plant et al. 2010). Elevated resting energy expenditure (Sergi et al. 2006) and increased circulating myostatin, IL-6, TNF-α, and C-reactive protein (CRP) (Ju and Chen 2012; Vogiatzis et al. 2007; Yende et al. 2006) have also been observed in COPD patients with or without muscle wasting compared to healthy controls. Altered oxidative stress, muscle autophagy, and regenerative capacity in COPD are not consistently reported (Gosker et al. 2003; Plant et al. 2010; Pomies et al. 2015; Puig-Vilanova et al. 2015; Theriault et al. 2012). Sex differences in these cachexia-associated observations are largely still unknown. Increased whole-body myofibrillar protein breakdown and satellite cell senescence have only been investigated in men. Increased MAFbx and myostatin expression and increased circulating myostatin, CRP, and cytokines have been observed in cohorts including both men and women without comparison between genders. However, circulating myostatin was

associated with low muscle mass in men only (Ju and Chen 2012). At this point, there is insufficient evidence to determine if a sex difference exists in the mechanisms or presentation of muscle wasting in men and women with COPD.

Sex Differences in Therapeutic Effects

Common therapies for improving muscle mass or reducing muscle dysfunction in COPD patients are not available. However, potential treatments include ghrelin/mimetics, testosterone/derivatives, and exercise. Ghrelin levels in underweight men and women with COPD are higher than in normal-weight men and women with COPD (Itoh et al. 2004), while ghrelin levels in men with COPD have been reported as lower (Luo et al. 2005) or higher (Uzum et al. 2014) than in healthy men when body weight was not taken into account. Ghrelin treatment in men and women with COPD and cachexia has also provided inconsistent results. Three weeks of intravenous ghrelin treatment (2 μg/kg twice a day) in men and women with COPD and cachexia increased body weight, muscle mass, HGS, and 6 MWT (6-min walk test) distance in a small, uncontrolled, single intervention study (Nagaya et al. 2005). In a different study, 3 weeks of the same regimen in men and women with COPD and cachexia did not increase body weight or muscle mass, but did increase 6 MWT distance compared to placebo (Miki et al. 2012). After 12 weeks of the ghrelin mimetic, SUN11031 (20 μg/kg or 40 μg/kg twice a day), in men and women with COPD and cachexia, body weight and muscle mass were increased in both doses compared to placebo, whereas there was no difference between any groups in 6 MWT distance or HGS (Levinson 2012). In this study, patients with severe cachexia showed a trend for improving 6 MWT distance and HGS with 40 μg/kg twice a day dose (Levinson 2012). No gender comparison was provided for any of these ghrelin/mimetic trials.

Testosterone and/or its derivatives have shown potential for increasing muscle mass in patients with COPD. While not currently FDA-approved for this indication, a review of anabolic androgen interventions in COPD patients suggested that intramuscular nandrolone decanoate of 50–200 mg per week for 12 weeks may increase muscle mass in patients with severe COPD (Velema et al. 2012). The efficacy of this regimen requires adequately powered clinical trials before it can be recommended. In COPD patients with cachexia, the testosterone derivative oxandrolone increased body weight in men and women with COPD after 4 months (no placebo) (Yeh et al. 2002), and another testosterone derivative, stanozolol, increased body weight and muscle mass after 4 months compared to placebo in a study of COPD in men only (Ferreira et al. 1998). In reports of COPD patients where muscle wasting was not specified, 8 weeks of nandrolone increased muscle mass with no influence on muscle function compared to placebo in men in one phase II trial (Creutzberg et al. 2003), but 16 weeks of nandrolone did not influence body weight, fat mass, muscle mass, or 6 MWT distance in men and women compared to placebo in a different phase II trial (Sharma et al. 2008). There was no gender analysis provided, but the discrepancy may be explained by the inclusion of women in the latter trial. However,

a 6-month testosterone supplementation trial in men with COPD without cachexia increased muscle mass compared to placebo (Svartberg et al. 2004).

Testosterone in combination with exercise also may increase muscle mass, but it is unclear whether the magnitude of increase is greater than that from testosterone alone. After 10 weeks of testosterone ± resistance training (RT) or placebo ± RT in men with low testosterone and COPD without cachexia, muscle mass increased in the testosterone and testosterone + RT groups compared to the placebo groups; only testosterone + RT improved strength compared to the non-RT groups (Casaburi et al. 2004). Similarly, muscle mass increased in men and women with COPD without cachexia after 8 weeks of aerobic exercise + nutrition + nandrolone compared to exercise + nutrition + placebo or exercise + placebo (Schols et al. 1995). In this same trial, COPD patients with cachexia in the exercise + nutrition + nandrolone group saw maintenance of muscle mass compared to the placebo groups which lost muscle mass. There were no differences between genders in any outcomes (Schols et al. 1995). There is not clear evidence to indicate whether or not sex differences exist in the response to testosterone alone or in combination with exercise in patients with COPD.

It is unclear whether exercise alone may have the potential to increase muscle mass in men and women with COPD. Thigh muscle mass increased after 8 weeks of RT with or without nutritional supplementation in men and women with COPD with or without cachexia in a phase II trial. These changes were not different from the healthy control groups undergoing the same interventions; no gender comparison was provided (Constantin et al. 2013). Combination resistance and aerobic programs in men and women with COPD have shown similar increases in muscle strength and aerobic capacity as RT or aerobic training alone, respectively (Ortega et al. 2002; Vonbank et al. 2012; Zambom-Ferraresi et al. 2015). These trials did not report changes in muscle mass, nor did they specify if patients were cachectic or not, and no gender comparisons were provided. It is still unknown whether sex differences exist in the response to traditional exercise in COPD patients.

A nontraditional exercise modality, neuromuscular electrical stimulation (NMES), may have the potential to improve muscle dysfunction in COPD patients who have difficulty exercising, for example, during hospitalization due to acute exacerbation. One month of NMES plus usual care in men and women with COPD and severe cachexia increased strength more than usual care alone (Vivodtzev et al. 2006; Zanotti et al. 2003). Muscle mass and 6 MWT distance increased after NMES plus usual rehab but not after rehab alone (Vivodtzev et al. 2006). Similarly, 6 weeks of NMES increased midthigh and calf muscle CSA, strength, and 6 MWT distance as compared to sham training in men and women with severe COPD (Vivodtzev et al. 2012). In addition, decreased muscular expression of atrogin-1 protein and increased phosphorylation of P70S6K were observed, possibly indicating an increase in muscle anabolism (Vivodtzev et al. 2012). No gender differences were reported for these NMES trials. The majority of exercise intervention trials in COPD patients have not reported muscle mass nor provided gender comparisons. NMES has shown some potential for improving muscle wasting in patients with severe

COPD, but there is no evidence to determine whether sex differences exist in the response to this therapeutic modality.

There is not currently approved treatment for cachexia due to COPD, and current studies have not provided sufficient evidence to determine whether sex differences exist in treatment modalities. However, current studies have not been powered for providing gender analyses, which may be an important consideration in future research due to the potentially reduced incidence of cachexia in women compared to men with COPD.

Chronic Heart Failure

Sex Differences in Incidence and Mechanisms of Muscle Wasting

Cachexia in the context of heart failure (HF) is defined as ≥5% non-edematous involuntary body weight loss, while sarcopenia in this context is defined as loss of muscle mass and strength which may occur with or without concomitant fat loss. Sarcopenia is thought to develop in the initial stages of cachexia (Anker et al. 1999; von Haehling 2015); however, it is not known whether the degree of muscle loss differs with sex. The incidence of cachexia in chronic HF (CHF) is reportedly 8–36% overall, with incidences in men and women reported from 17.5% to 35% and 5% to 44%, respectively (Anker 1997; Anker et al. 2003; Bekfani et al. 2016; Christensen et al. 2013; Fulster et al. 2013; Rossignol et al. 2015; Valentova et al. 2016). Patients with cachexia in CHF may exhibit increased adiponectin (Araujo et al. 2009; Lindberg et al. 2014; McEntegart et al. 2007; Szabo et al. 2014), angiotensin II (Cabello-Verrugio et al. 2012), ghrelin (Lund et al. 2009), GH (Cicoira et al. 2003; Morley et al. 2006), IL-6 (Fulster et al. 2013), and tumor necrosis factor (TNF) (Levine et al. 1990; Rauchhaus et al. 2000) and decreased circulating IGF-I (Anker et al. 2001; Ebner et al. 2014), myostatin (Biesemann et al. 2014; Christensen et al. 2013), and leptin (McEntegart et al. 2007; Murdoch et al. 1999) compared to CHF patients without cachexia. Adiponectin, which is normally higher in healthy women than healthy men, appears to be increased more so in men with CHF as no differences were detected between men and women with CHF (Szabo et al. 2014); it is unknown whether there are sex differences in CHF patients with cachexia. No sex difference was observed in circulating myostatin in CHF patients with cachexia (Christensen et al. 2013). In addition, skeletal muscle of CHF patients with cachexia displays increased skeletal myocyte apoptosis, thought to be induced by increased TNF or other cytokines, compared to CHF patients without cachexia (Adams et al. 1999; Vescovo et al. 2000). It is unknown whether there are sex differences in myocyte apoptosis in patient with CHF and cachexia.

Sex Differences in Therapeutic Effects

Therapeutic interventions with potential to improve cachexia in CHF include nutritional supplementation, exercise, and hormone administration. Supplementation with omega-3 polyunsaturated fatty acids for 18 weeks decreased TNF-α with no change in body weight or muscle mass in men and women with severe HF in a phase II trial (Mehra et al. 2006). Protein supplementation increased muscle mass after 6 weeks but was not maintained at 18 weeks in men and women with CHF and cachexia in a pilot trial (Rozentryt et al. 2010). However, there was no control group, and it is not known if sex differences were observed in muscle mass changes. Protein supplementation for 2 months improved body weight but not muscle mass compared to a no-supplement control in men and women with CHF and cachexia; there were no sex differences in body weight change (Aquilani et al. 2008). Nutritional interventions in CHF patients to date have not been designed to assess sex differences.

Resistance training has not proven effective at improving muscle mass in CHF; however, interventions are sparse and have not specifically included patients with cachexia. Three months of RT and protein supplementation was not more effective than RT alone at improving body weight in men and women with CHF without cachexia in a phase II trial (Pineda-Juarez et al. 2016). Change in muscle mass was not reported and gender analysis was not provided. Ten weeks of RT in women with CHF increased muscle strength and endurance, with no difference in total muscle mass or muscle fiber area changes, compared to a stretching control group (Pu et al. 2001).

In contrast, aerobic exercise may have more potential to improve muscle wasting in CHF, although trials have also not specifically included patients with cachexia. Twelve weeks of aerobic exercise reduced proteasome activity in men and women with CHF in a phase II study compared to a no-exercise control group; a gender comparison was not provided (Cunha et al. 2012). Twelve weeks of aerobic training also increased thigh muscle area and reduced muscular myostatin mRNA and protein content in men with CHF compared to healthy men without CHF in another phase II trial (Lenk et al. 2012). After 4 months of aerobic training, testosterone, or both, in men with CHF, muscle mass increased in both exercise groups and decreased in the testosterone group in a phase IV trial. In addition, type I and type II muscle fiber areas increased in the aerobic training plus testosterone group compared to the testosterone-only group (dos Santos et al. 2016). Six months of aerobic training in men with CHF increased aerobic capacity and reduced muscular expression of IL-6, TNF-α, and IL-1β mRNA compared to a sedentary control group (Gielen et al. 2003). There was no report of body weight or muscle mass change in this pilot trial. In a phase II trial, there were no differences between groups for change in body weight or performance measures after 12 weeks of aerobic training plus testosterone or aerobic training plus placebo in men with CHF (Stout et al. 2012). Exercise interventions in CHF have been predominantly conducted in men, particularly aerobic exercise interventions; therefore, there is insufficient evidence to determine whether sex differences exist in the response of skeletal muscle or physical function.

Trials with testosterone supplementation alone have shown a tendency to improve exercise capacity in patients with CHF despite not specifically including patients with muscle wasting (Toma et al. 2012). Twelve weeks of testosterone supplementation increased distance walked in the shuttle walk test with no effect on muscle mass or strength compared to the placebo group in one pilot study in men with CHF (Pugh et al. 2004). Distance walked in the 6 MWT also increased with no effect on muscle mass compared to placebo in men with CHF in a different pilot trial (Mirdamadi et al. 2014). Similarly, 12 weeks of testosterone increased body weight and improved aerobic capacity and muscle strength in men with CHF in a phase II trial; however, muscle mass was not reported (Caminiti et al. 2009). Six months of testosterone increased distance walked in the shuttle walk test with no effect on muscle mass, HGS, or circulating TNF compared to placebo in men with CHF in a phase II trial (Malkin et al. 2006). In women with CHF, 6 months of testosterone increased 6 MWT distance, muscle strength, and aerobic capacity compared to placebo with no change in body weight in either group; muscle mass was not reported in this pilot study (Iellamo et al. 2010).

These testosterone-associated improvements in exercise capacity may apply to both men and women with CHF; however, the effect of testosterone on improving muscle mass in either gender is still unknown. Similarly, the investigations of GH therapy in patients with CHF have included both men and women, without provision of gender comparisons, and have not specifically included patients with cachexia. Three months of physiologic GH either improved exercise capacity (Fazio et al. 1996) or showed no effect on exercise capacity (Isgaard et al. 1998; Osterziel et al. 1998) and did not influence body weight in these preliminary studies. These GH interventions were conducted in men and women without reporting muscle mass or providing any gender analysis. It is unlikely that GH will influence muscle wasting in men or women with CHF as its direct anabolic effect is limited. There is also limited evidence to support an effect of testosterone on muscle wasting in men or women in CHF as muscle mass is unchanged or not reported in most interventions in CHF patients. In addition, the vast majority of testosterone interventions in CHF patients do not include women.

Other potential anabolic interventions in CHF patients include ghrelin and β2-adrenoreceptor agonists. Three weeks of ghrelin administration to men and women with CHF and cachexia improved aerobic capacity, HGS, and LBM which were all unchanged in the placebo group in this pilot study (Nagaya et al. 2004). It is unknown whether there is a sex difference in the response to ghrelin treatment in CHF patients. Preclinical data suggests that ghrelin mimetics have the potential to improve LBM as much as ghrelin itself (Palus et al. 2013), potentially due to decreased muscular myostatin expression (Lenk et al. 2013), in male rats, but this has yet to be tested in CHF patients with or without cachexia. Body weight, exercise capacity, muscle strength, and thigh muscle area were not influenced after 3 weeks of the β2-agonist, salbutamol, or placebo in men with CHF in one pilot study (Harrington et al. 2000). In another pilot study, there was no difference between groups in strength increases after 12 weeks of another β2-agonist, clenbuterol, or placebo in men and women with CHF; no gender analysis was provided

(Kamalakkannan et al. 2008). Clenbuterol decreased exercise endurance and increased lean mass, while endurance increased with no change in LBM in placebo (Kamalakkannan et al. 2008). It is still unclear whether there are sex differences in the lean mass response to β2-agonist administration in patients with CHF and cachexia; moreover, this modality may be of limited clinical use as beta-blockade is part of the standard of care in CHF. In summary, there is no currently approved treatment for cachexia in CHF, and data suggests that there is a similar incidence of cachexia in both men and women. However, current reports are not powered to analyze gender differences in response to therapeutic modalities.

End-Stage Renal Disease/Chronic Kidney Disease

Sex Differences in Incidence and Mechanisms of Muscle Wasting

Protein-energy wasting (PEW) is the proposed terminology for the malnutrition/sarcopenia clinical presentation in end-stage renal disease (ESRD)/chronic kidney disease (referred to collectively herein as ESRD) (Fouque et al. 2008). The incidence of PEW is roughly 40–60% for men and 35–70% for women with ESRD, with muscle wasting being a key component of the syndrome (Cianciaruso et al. 1995; Heimburger et al. 2000; Marcen et al. 1997; Qureshi et al. 1998). Mechanisms of muscle wasting in ESRD are acidosis, chronic low-grade inflammation, comorbidities (i.e., hypogonadism, diabetes, etc.), hemodialysis treatment, inactivity, protein malnutrition, appetite loss, low insulin-like growth factor-I (IGF-I), and corticosteroid use (Carrero et al. 2013; Ikizler et al. 2002; Kaizu et al. 2003; Mitch 2007; Qureshi et al. 1998; Raj et al. 2004). Hypogonadism has been reported between 33% and 66% in men with ESRD (Gungor et al. 2010). It is largely unknown whether the mechanisms of muscle wasting differ between men and women with PEW in ESRD. However, women may experience a larger degree of muscle wasting than men (Carrero et al. 2008). In addition, HGS was a significant predictor of survival after kidney transplant in men but not in women. In a gender comparison study, decreased survival rate was observed in men with LBM below the study-reported median than men with LBM above the median (Stenvinkel et al. 2002). There was no difference in survival rate between women with LBM above or below the median (Stenvinkel et al. 2002). These observations suggest that there may be an inherent difference in the effect on muscle quality in men and women with muscle wasting in ESRD.

Sex Differences in Therapeutic Effects

Various therapeutic modalities have been proposed to combat muscle wasting in ESRD patients including anabolic hormones, exercise, and caloric/nutrient supplementation. Four weeks of recombinant human GH did not increase mid-arm muscle circumference compared to a control group in men and women with ESRD; no functional outcomes were measured (Iglesias et al. 1998). However, 24 weeks of

treatment with the testosterone derivative oxymetholone increased muscle mass and HGS compared to placebo in men and women with ESRD (Supasyndh et al. 2013). Liver enzymes were elevated; however, incidence of values >3× upper limit of normal was not different from placebo. It is unknown whether there are sex differences in the lean mass response between men and women in these anabolic hormone interventions.

Generally, 12–24 weeks of RT alone or in combination with nutritional supplements or testosterone does not confer benefit on muscle mass when compared to a control group in men and women with ESRD (Dong et al. 2011; Cheema et al. 2007a, b; Johansen et al. 2006; Kopple et al. 2007). However, increases in rectus femoris CSA and volume were reported after 8 weeks of progressive RT in men and women with ESRD in a different study (Watson et al. 2015). It is unknown whether there were sex differences in the muscle changes between men and women in these reports. Nutrition interventions alone have also not been effective at alleviating muscle wasting in ESRD (Stratton et al. 2005). However, bicarbonate supplementation for acidosis reduction may increase arm muscle circumference after 2 years in men and women (de Brito-Ashurst et al. 2009). In addition, 1 year of vitamin D treatment increased muscle mass in men, but not in women with ESRD (Mori et al. 2013). As there are no trials aimed at exploring the effect of sex on muscle wasting therapies, it is unknown whether there are sex differences in the lean mass response in exercise, hormonal, or nutritional interventions in patients with ESRD.

One area showing promise for therapeutic benefit is mitochondrial energetics. Stimulation of mitochondrial biogenesis has the potential to influence muscle mass, as mitochondrial dysfunction has been linked to muscle loss during periods of disuse (Powers et al. 2012), and activators of sirtuins, such as resveratrol, can attenuate muscle atrophy in mice with ESRD (Sun et al. 2017). The influence of gender/sex on mitochondrial dysfunction and the response to sirtuins in ESRD have not been explored. It is also important to consider that, oftentimes, therapeutics appear promising in preclinical studies but fail to translate to significant clinical findings.

Taken together, the data suggests that the prevalence of cachexia in ESRD is roughly the same in men compared to women, with limited evidence suggesting women experience a larger magnitude of cachexia. Sex differences in mechanisms of cachexia are unknown; however, some evidence suggests that muscle quality may be more adversely effected in men than women. Sex differences in response to treatments are also unknown as current reports were not powered for gender analyses.

Liver Disease

Sex Differences in Incidence and Mechanisms of Muscle Wasting

The major contributors to muscle wasting in liver disease are altered metabolism wherein fatty acid oxidation and gluconeogenesis are elevated, hyperammonemia leading to increased skeletal muscle catabolism, and endotoxemia due to altered

digestive function (Dasarathy and Merli 2016). Other relevant factors are decreased food intake, decreased physical mobility, and increased fatigue (Dasarathy and Merli 2016) and, in men, hypogonadism due to increased aromatase activity and reduced IGF-I (Zietz et al. 2003). Due to the impact of anabolic hormones on muscle mass, low muscle mass is reported in 50–80% of men and 20–50% of women with advanced liver disease (Hanai et al. 2015; Montano-Loza 2014; Peng et al. 2007; Sinclair et al. 2016b; Tandon et al. 2012). Hypogonadism is reported in roughly 60–90% of men with advanced liver disease (Grossmann et al. 2012; Sinclair et al. 2016a), and average total testosterone is reportedly lower than healthy control values (Zietz et al. 2003). Low testosterone is also correlated with increased mortality and may be a better predictor of mortality than low muscle mass (Grossmann et al. 2012).

In women, low estrogens may contribute to the development of liver disease, specifically nonalcoholic fatty liver disease in postmenopausal women and women with polycystic ovarian syndrome (Grobe et al. 2010). Some evidence in animal studies suggests that ovarian senescence and hepatic inflammation arise due to hypoestrogenemia (Kamada et al. 2011; Turola et al. 2015), suggesting that estrogens may have a protective effect on the liver, in addition to skeletal muscle as discussed in section "Age-Related Changes in Estrogens," in premenopausal women. This may be a mechanism by which incidence of muscle wasting is reduced in women with liver disease.

Sex Differences in Therapeutic Effects

Anabolic androgen supplementation for treatment of hypogonadism appears to be well tolerated in men with liver disease (Bonkovsky et al. 1991; Fenster 1966; Gluud 1986; Mendenhall et al. 1984, 1993; Puliyel et al. 1977; Sinclair et al. 2016b; Wells 1960). Primary benefits appear to be increased albumin and reduced incidence of gynecomastia with no difference in survival or adverse events between the treatment group and control/placebo. However, the effect of androgen interventions on muscle wasting in liver disease is still unclear. In the only report to assess muscle mass, computed tomography determined that appendicular and total muscle mass increased after 1 year of testosterone replacement compared to placebo (Sinclair et al. 2016b), thus providing evidence for testosterone as a treatment for muscle wasting in liver disease in men. It is still unknown whether testosterone treatment in women would have similar potential as a therapeutic strategy. Estradiol treatment in healthy postmenopausal women improved liver enzymes (Codes et al. 2007) and reduced fibrosis in women with hepatitis C (McKenzie et al. 2006); however, estrogens are not a likely candidate for treatment of muscle wasting due its potential for cardiovascular and cancer risks (Rossouw et al. 2002). Whether SERMs may be

beneficial in this setting is unknown. Caloric supplementation is a commonly used strategy in attempts to improve body weight and muscle mass. While caloric supplementation alone has not proven effective at reversing or ameliorating muscle wasting in liver disease, ingestion of late evening or early morning supplements has potential to improve muscle mass in men and women with cirrhosis (Plank et al. 2008). It is currently unknown whether there is a sex difference in the skeletal muscle response to nocturnal supplementation.

Taken together, the data suggests that the prevalence of cachexia in the liver is greater in men compared to women, likely due to the high incidence of hypogonadism in men. Sex differences in response to treatments are still unknown as current reports primarily targeted hypogonadism treatment in men and/or were not powered for gender analyses.

Summary and Conclusion

In general, deficiency of sex hormones is one of the main contributors to the process of muscle wasting under the conditions of aging, cancer, and other catabolic diseases. As a result, men and women respond differently to these conditions due to different hormonal profiles of androgens and estrogens. The sex differences in the mechanisms of muscle wasting and effects of therapeutic modalities are summarized in Table 1.

Sex hormones play important roles in the growth and maintenance of skeletal muscles. Testosterone is a potent anabolic factor for maintaining muscle mass through promoting protein synthesis and muscular regeneration, which is abundant in healthy young men. With aging and/or other diseases, testosterone deficiency leads to a more robust catabolic response in men than women (e.g., sarcopenia and cachexia). On the other hand, estrogens may have a protective effect on preserving muscle mass and function through anti-inflammation and anti-catabolism. Estrogen deficiency is more likely related to sarcopenia in postmenopausal women than in older men though most evidence exists in preclinical studies. Up to now, testosterone and other sex hormones or receptor agonists (SARMs, DHEA) have been studied because of their potential anabolic effects. However, the benefits of testosterone and other sex hormone mediators have been mostly observed in men, whereas the effects on women are minimal or unknown. Also, adverse effects of testosterone and other sex hormone mediators appear differently in men and women because of their influences on reproductive tissues (e.g., prostate, breast) and the fact that virilization may be a desirable effect in men but a limiting side effect in women. Although other anabolic hormones and their mediators (GH, ghrelin, and ghrelin mimetics) have been studied in men and women, the sex differences in efficacy and safety are not clear yet.

Table 1 Summary of sex differences in mechanisms of muscle wasting and effects of therapeutic modalities

	Men	Women
Sarcopenia		
Mechanisms	Testosterone is a major factor regulating skeletal muscle anabolism in men. The greater extent of age-related decrease in testosterone levels in older men leads to more pronounced catabolic responses in muscles: greater losses in muscle mass and strength, higher increase in percent body fat, and more deterioration in physical activities compared to older women	Women live longer but have a higher likelihood of functional deterioration with aging. It is likely due to lower muscle mass and strength, higher percent body fat, and increased osteoporosis compared to same-aged men
		Lower estrogen levels induced by menopause are also associated with decreased muscle strength and function. Estrogen may modulate inflammation in this setting, although most of the evidence is preclinical. Benefits from HRT are questionable due to multiple adverse effects
Treatment effects	*Testosterone*: more studies investigated the effects of testosterone in men. Sex-different adverse effects: influence on prostate tissues in men, virilization in women, CV risk potential in both	
	SARMs: more data is needed. Similar effects as testosterone but less adverse effects in both sexes	
	Growth hormone: lack of evidence in improving muscle strength by acting alone, especially in women. Some safety concerns with long-term use on both sexes	
	Ghrelin and mimetics: more data is needed. No sex differences in ghrelin/mimetic treatment effects reported	
	DHEA/DHEAS: improvement in anabolic hormone levels and body composition in both sexes. Effects on muscle strength are unclear, especially in women. More data is needed	
Cachexia		
Mechanisms	Incidences of cachexia are greater in men in some conditions, especially in cancer and liver disease, likely due to high prevalence of hypogonadism in these cases and to the increased prevalence of lung and GI cancer in men	It is speculated that estrogen may provide anti-inflammatory effects on the liver and muscle, which may reduce cachexia incidence in liver disease
		Lower incidence of cachexia in breast cancer may contribute to slightly lower overall incidence of cancer cachexia in women

(continued)

Table 1 (continued)

	Men	Women
Treatment effects	Insufficient evidence to determine if sex differences exist in the response to treatment in the most settings	
	Testosterone may have potential to increase muscle mass in cachectic patients, shown primarily in men; it is unknown if testosterone has the same potential in cachectic women	
	Ghrelin mimetics or exercise may also have potential to increase muscle mass in cachectic patients, but there is insufficient evidence to determine if sex differences exist	
	More data is needed for any treatment before they can be recommended or any conclusion can be drawn regarding their gender-specific safety and therapeutic effects	

HRT hormone replacement therapy, *CV* cardiovascular, *SARM* selective androgen receptor modulator, *DHEA/DHEAS* dehydroepiandrosterone and its sulfate ester, *GI* gastrointestinal

In conclusion, most preclinical and clinical studies are not designed or powered to explore sex-based differences in spite of the different physiologic mechanisms regulating muscle mass in men and women. In addition, therapeutic options for women with muscle wasting are limited because most therapeutic targets have been developed based on translational studies with males only. The recent requirements by National Institutes of Health and Food and Drug Administration in studying both sexes are an important first step in this direction. Thus, understanding the underlying mechanisms of sex disparities in muscle wasting induced by aging and/or diseases will provide potential therapeutic options for both sexes.

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Origins and Functions of the Ventrolateral VMH: A Complex Neuronal Cluster Orchestrating Sex Differences in Metabolism and Behavior

William C. Krause and Holly A. Ingraham

Abstract The neuroendocrine brain or hypothalamus has emerged as one of the most highly sexually dimorphic brain regions in mammals, and specifically in rodents. It is not surprising that hypothalamic nuclei play a pivotal role in controlling sex-dependent physiology. This brain region functions as a chief executive officer or master regulator of homeostatic physiological systems to integrate both external and internal signals. In this review, we describe sex differences in energy homeostasis that arise in one area of the hypothalamus, the ventrolateral subregion of the ventromedial hypothalamus (VMHvl) with a focus on how male and female neurons function in metabolic and behavioral aspects. Because other chapters within this book provide details on signaling pathways in the VMH that contribute to sex differences in metabolism, our discussion will be limited to how the sexually dimorphic VMHvl develops and what key regulators are thought to control the many functional and physiological endpoints attributed to this region. In the last decade, several exciting new studies using state-of-the-art genetic and molecular tools are beginning to provide some understanding as to how specific neurons contribute to the coordinated physiological responses needed by male and females. New technology that combines intersectional spatial and genetic approaches is now allowing further refinement in how we describe, probe, and manipulate critical male and female neurocircuits involved in metabolism.

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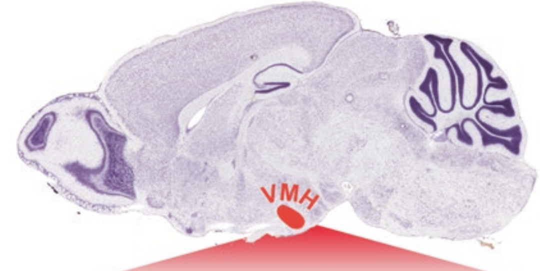
Introduction

If one asks the general question as to why sex differences are needed in metabolic circuits, the obvious answer is to preserve and enhance reproductive capacity. Embedded in this conclusion is the overall objective in terms of evolutionary pressure on males and females to reproduce. This topic was recently reviewed by Torre and Maggi (Della Torre and Maggi 2017), which describes how in females, but not males, evolutionary pressure in a changing environment is needed to optimize energy intake and expenditure with reproduction. This pressure is evident in invertebrates as reviewed by (Mccall 2004), with demands increasing in placental and lactating vertebrates. Over the last two decades, works from several labs have created an overall narrative as to how neurons in the VMHvl help to control the sexually dimorphic male and female behavioral and metabolic responses (Fig. 1). The VMHvl is one of the many brain regions that expresses a major detector of the sex steroid estrogen, estrogen receptor alpha (ERα, encoded by *Esr1*), where expression increases in females postnatally (Fig. 2a). Other ERα-expressing regions within the medial basal hypothalamus (MBH) include the nearby arcuate nucleus (ARC), as well as the medial preoptic area (MPOA), and the bed nucleus of the stria terminalis (BnST) (Brock et al. 2015). In the VMHvl, this nuclear receptor is predominantly nuclear, and thus, one can reasonably assume that nearly all estrogen signaling in the VMHvl occurs through genomic rather than non-genomic actions. One of the striking features of ERα is the sexually distinct pattern in rodents (Koch 1990), with both transcripts and protein levels much higher in the female VMHvl; refer to



Sex-Dependent Functions:	**VMH Subtype Marker:**
Energy expenditure	ERα, NKX2-1, TAC1
Aggression	ERα, PR
Reproductive behavior	ERα, PR
Sex-Independent Functions:	
Energy expenditure	LEPR, IR, SF-1
Fear	SF-1
Anxiety	SF-1
Glucose homeostasis	SF-1

Fig. 1 Sex-dependent VMH functions are mediated by ERα-expressing VMHvl neurons. Within the hypothalamus, the VMH (red-shaded region) controls multiple aspects of metabolism and behavior. Whereas leptin receptor (LEPR) and insulin receptor (IR) expression overlap with SF-1 and regulate metabolism in both males and females, sex-dependent functions of the VMH are mediated by ERα-expressing VMH neurons (Nissl-stained image adapted from the Allen Brain Atlas)

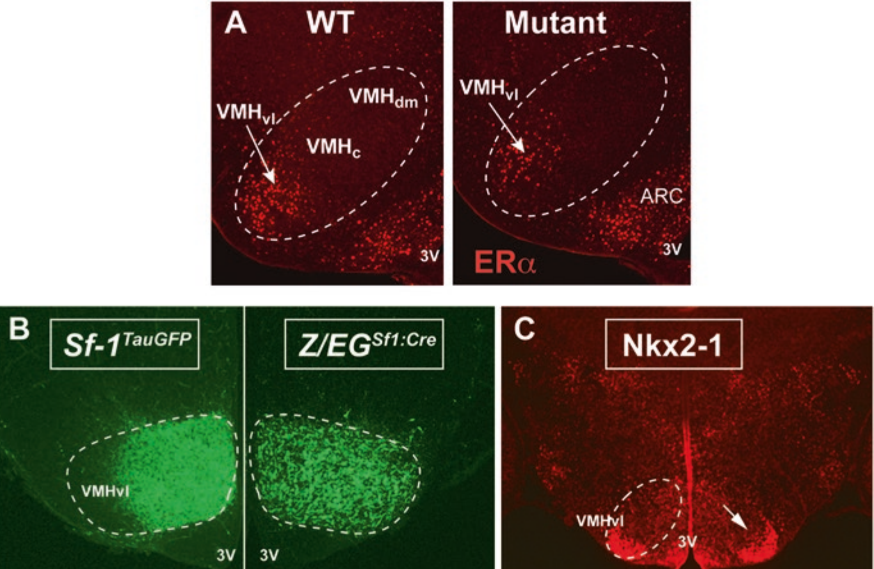


Fig. 2 Development of the VMHvl module that modulates energy homeostasis in females. **a**) ERα immunostaining demonstrates that expression is restricted to the VMHvl in female mice as well as the arcute nucleus (ARC). Fewer $VMH^{ER\alpha}$ neurons are born in the *Nkx2-1*$^{Sf1\text{-}Cre}$ mutant females (Mutant) compared to *Nkx2-1*$^{fl/fl}$ control females (WT), as described in text. Third ventricle (3V). **b**) VMHvl neurons do not express SF-1, as illustrated by the lack of GFP-positive neurons using a knock-in reporter (*Sf-1*)^{TauGFP}. However, Cre-mediated lineage tracing (*Z/EG*)$^{Sf1:Cre}$ reveals that most VMHvl neurons derive from SF1-expressing precursors. **c**) Postnatal NKX2-1 expression is largely restricted to ERα-positive and SF-1-negative VMHvl neurons

(Correa et al. 2015) and references within. Contribution by the two other estrogen receptors is assumed to be minimal as evidenced by the exceeding low transcript levels for both estrogen receptor beta (ERβ) and the 7-transmembrane G-protein-coupled estrogen receptor 1 (GPER-1), formerly referred to as GPR30 (Chimento et al. 2014; Prossnitz et al. 2008). Indeed, expression of ERβ (Zuloaga et al. 2014) and GPER (Brailoiu et al. 2007) is sparse or undetectable in the adult VMHvl. The VMHvl also expresses *Cyp19A1* that encodes aromatase, an essential enzyme needed for local conversion of androgens to estrogens (Stanic et al. 2014; Wu et al. 2009). In males, expression of aromatase in the VMHvl region is thought to be critical for the early masculinization of the male brain via estrogen, as reviewed in (Yang and Shah 2014). Indeed, the loss of aromatase in males impairs aggression as measured by the frequency and duration of attacks in a standard resident/intruder assays.

Another nuclear receptor, the progesterone receptor (PR), is also expressed in the VMHvl. The PR gene is a well-established transcriptional target of ERα. Interestingly, while PR has emerged as one of the sexually dimorphic transcripts

detected in the adult VMHvl (Yang et al. 2013), at earlier stages prenatally, PR expression is not confined to the VMHvl but instead is unrestricted and found throughout the entire VMH (Correa et al. 2015). Thus, its pattern of expression in the VMH is not necessarily identical to that of ERα at all developmental stages. Indeed, while one observes a clear sex-dependent pattern of ERα expression beginning postnatally, the same cannot be said for PR. In this instance, PR transcripts are not differentially expressed until adulthood (Hagihara et al. 1992; Simerly et al. 1990).

The VMH Mediates Sex Differences in Energy Expenditure

This chapter will focus primarily on estrogen effects on the VMHvl, with emphasis on female metabolism. Others have reviewed some of the more recent work on the VMHvl in male sexual behavior (Yang and Shah 2014). When appropriate, we will highlight general statements inferred from studies in both rats and mice. Much of the differences noted for females emerged over a century ago with the seminal observations by Strominger. Using methods of the day and challenged by their limited access to reagents post-WWII, they noticed a tight correlation between the estrus cycle and peaks of activity concomitant with decreased food intake (Brobeck et al. 1947). If we fast forward to the next century, genetic manipulations achieved by either SiRNA- or Cre-mediated disruption of estrogen signaling clearly highlight the VMHvl as a center for sex differences in female metabolism. Indeed, ShRNA knockdown of ERα in the rat VMH increases food intake and decreases diet-induced thermogenesis and physical activity, resulting in obesity (Musatov et al. 2007). Conditional knockout of ERα in the VMH using *Sf1Cre* also lowers brown adipose tissue (BAT) thermogenesis in female mice and yields a mild transient weight gain in females due to increased size of gonadal fat pads (Xu et al. 2011). It should be mentioned that some of the earlier work using stereotaxic delivery of SiRNA directed against ERα to the entire VMH region showed pronounced increase in food intake (Musatov et al. 2007). However, three different genetic models generated to date using different Cre drivers, including $Esr1^{Sf1\text{-}Cre}$, $Esr1^{Nkx2\text{–}1Cre}$, and $Nkx2\text{-}1^{Sf1\text{-}Cre}$, fail to support the notion that estrogen signaling in the murine VMHvl directly controls food intake (Xu et al. 2011, Correa et al. 2015), and unpublished data H.A.I.). It is worth noting that within the VMHvl, the Cre-based recombination efficiency is substantially higher using *Nkx2-1Cre* versus *Sf1Cre*, with the former effecively eliminating all ERα in this hypothalamic subregion. Table 1 lists the phenotypes for these three different mouse models that eliminate ERα in the VMHvl.

Table 1 Comparison of mouse models that eliminate ERα in the VMHvl

Phenotype	*Nkx2-1*$^{Sf1\text{-}Cre}$	*Esr1*$^{Sf1\text{-}Cre}$	*Esr1*$^{Nkx2\text{-}1Cre}$
ERα expression	++	+	–
Tac1 expression	++	+++	+++
# of VMHvl neurons	++	+++	+++
Reproduction	Fertile	Infertile	Infertile
Body weight (chow)	Increase	Transient increase	No change
BAT thermogenesis	Normal	Lower	Lower
Food intake	Normal	Normal	Normal
Locomotion	Decreased	Trends lower	Decreased

+++ = Wild-type levels. References for the table: Xu et al. (2011), Correa et al. (2015) and unpublished data (H.A.I)

VMHvl Development

VMH projections are visible as early as embryonic day (E) 10.5 when few postmitotic neurons have been born, suggesting that formation of VMH circuitry begins at the onset of neurogenesis (Cheung et al. 2013) and also reviewed in (McClallan et al. 2006). One can follow in time the original migration from the ventricular zone to the VMHvl by BrdU labeling (Tran et al. 2003). One of the earliest molecular markers expressed throughout the VMH is the nuclear receptor steroidogenic factor 1 (SF-1, NR5A1), which appears at E9 (Ikeda et al. 2001). Although SF-1 is not required for the initial organization and migration of neurons in the developing VMH nucleus (Ikeda et al. 1995; Tran et al. 2003), this transcription factor is essential for terminal differentiation and maintenance of VMH neuronal populations (Davis et al. 2004; McClallan et al. 2006). The loss of SF-1 also results in diminished efferent projections to the amygdala (Tran et al. 2003) and altered afferent projections from the preoptic area to the VMH (Budefeld et al. 2011).

Earlier descriptive studies based on immunofluorescence staining and in situ hybridization of SF-1 protein and transcripts, respectively, supported the idea that SF-1 marks all embryonic neurons that would give rise to the VMH proper. However, comparison of the VMH neurons derived from the SF-1 lineage versus those that express SF-1 shows that the cluster of VMHvl neurons and, by association, ERα neurons in the VMH evolve into a distinct neuronal subpopulation within the VMH (Cheung et al. 2013).

Specifically, two approaches that exploit the widespread expression of SF-1 in the VMH were used to trace SF-1 expression and the major VMH axonal projections during embryonic and postnatal stages (Fig. 2b). The first relied on tandem reporters, the wheat germ agglutinin (WGA) (Braz et al. 2002) and tau-green fluorescent protein (tauGFP), knocked into the 3′-untranslated region (UTR) of the *Sf-1* (*Nr5a1*) locus. In this knockin line, referred to as *Sf-1*^{TauGFP}, WGA and GFP are under the control of intact regulatory elements and are thus coexpressed and coregulated with SF-1 expression. In the second, more standard Cre-mediated labeling line, the transgenic *Sf1:Cre* mouse was crossed with a *Z/EG* reporter mouse, referred

to as *Z/EG*$^{Sf1:Cre}$, which results in constitutive expression of eGFP (enhanced GFP) after Cre-mediated recombination (Dhillon et al. 2006). One conclusion from this work was that SF-1 neurons appear in the presumptive VMH area by E10.5 but only begin to coalesce into the conventional oval-shaped nucleus later in the development at E14.5. Even at this very early developmental stage, prominent neuronal VMH projections are evident. Further, while it has been generally assumed that SF-1 marks the entire VMH because of its early and broad expression, striking differences were observed if neurons are marked by the endogenous SF-1 promoter or instead marked by Cre recombination (Cheung et al. 2013). By directly comparing GFP^{+} labeling in all subregions of the VMH in the *Sf-1*^{TauGFP} and *Z/EG*$^{Sf1\text{-}Cre}$ lines, one can conclude that SF-1 is transiently expressed at early embryonic stages and then silenced in neurons that populate the VMHvl. These findings imply that developmental signals must exist to silence SF-1 expression in the VMHvl prior to E14.5. By this developmental point, ERα expression has already begun (Brock et al. 2015; Correa et al. 2015), but the interplay between SF-1 and ERα transcriptional response remains undefined at this juncture. Transcriptome profiling has only been done at later stages or using the entire VMH (Kurrasch et al. 2007), and the field is still awaiting data from comprehensive single cell sequencing. Ultimately, this detailed transcriptome profiling of both male and female VMHvl should provide a glimpse into the molecular complexity that exists in the VMHvl. Currently, based on different phenotypic outcomes, it is assumed that unique molecular modules will help constitute distinct neural circuits that result in different physiological endpoints. It should be noted that there are a limited number of ERα neurons in the vicinity of the VMHvl that do coexpress SF-1; this is especially true for cell bodies more dorsal to the VMHvl proper. That the VMHvl has a unique molecular signature from the other two subregions of the VMH, the central and dorsal medial, fits well with the notion that distinct SF-1-negative neurons in the VMHvl are dedicated to elaborating sex-dependent physiological and behavioral responses.

Sex Differences in Energy Expenditure

Recent studies show that aside from BAT thermogenesis, physical activity or locomotion is mediated by estrogen-responsive neurons in the VMHvl. This was discovered by manipulating a prominent developmental factor, the homeobox transcription factor Nkx2-1, which is required for proper development of several major organs, including the pancreas, lung, thyroid, and brain. NKX2-1 is also required for patterning in many brain regions including the rostroventral hypothalamus (Kimura et al. 1996; Marin et al. 2002; Shimamura and Rubenstein 1997), which gives rise to the VMH. In adult male and female mice, Nkx2-1, as with ERα, is highly restricted to the VMH_{vl} (Davis et al. 2004; Tran et al. 2003) and Fig. 2c. However, not all NKX2-1 neurons in the VMHvl express ERα. Earlier in development, NKX2-1 is expressed throughout the presumptive MBH (Marin et al. 2002; Shimamura and Rubenstein 1997; Yee et al. 2009) and appears earlier and is more broadly expressed than SF-1 as judged by immunofluorescence (Correa et al. 2015)

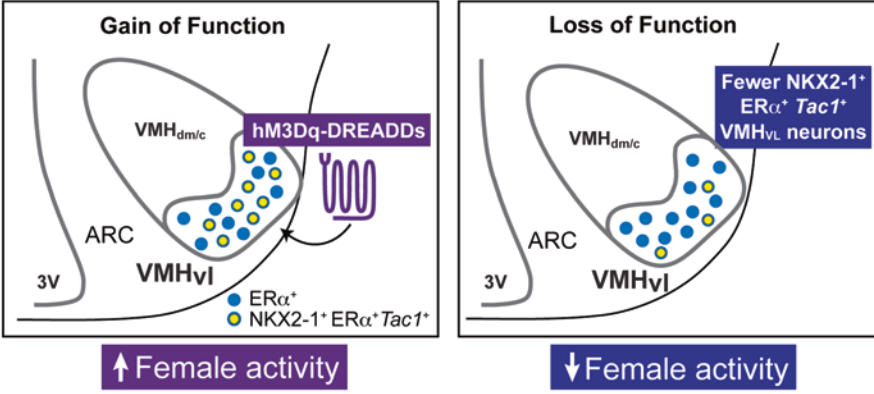


Fig. 3 A molecularly distinct subset of VMH$^{ER\alpha}$ neurons are necessary and sufficient to drive physical activity in female mice. Chemogenetic activation of VMHvl neurons increases energy expenditure via physical activity in females and requires ERα and TAC1 (left panel). In contrast, reducing the number of VMH$^{ER\alpha,TAC1}$ neurons decreases physical activity and results in female-specific obesity (right panel)

and by detailed lineage tracing using the *Nkx2-1Cre* and the reporter mouse (Salvatierra et al. 2014). Global deletion of NKX2-1 impairs the development of the VMH and other hypothalamic nuclei leading to diabetes (Sussel et al. 1998, 1999). However, if NKX2-1 is eliminated late in development (E9-10) using the *Sf1-Cre*, the VMH remains largely intact. A similar result is observed if *Synapsin-Cre* is used to delete NKX2-1 (Mastronardi et al. 2006). From these and other birthdating studies, it is concluded that NKX2-1 marks the earliest born or oldest neurons in the VMH, with the majority of these cells eventually residing in the VMHvl with ERα-expressing neurons. Further, conditional deletion of NKX2-1 in the VMHvl eliminates a sizable fraction (30 %) of ERα-expressing VMHvl neurons (Correa et al. 2015). These data are consistent with the fact that development or migration of some but not all estrogen-responsive neurons in the VMHvl depends on NKX2-1.

Ablating NKX2-1 in this VMHvl subpopulation (*Nkx2-1*$^{Sf1\text{-}Cre}$) results in a sex-specific decrease in spontaneous physical activity without affecting BAT thermogenesis or fertility, resulting in female-specific obesity independent of diet (Fig. 3 and Table 1). Recall that eliminating some but not all ERα in the VMH using the *Esr1*$^{Sf1\text{-}Cre}$ has a minimal effect on activity but does impair BAT thermogenesis and reproduction. In both models, male mice fail to show any signs of metabolic or reproductive deficits. As is true with many Cre lines, it is rare that they exhibit the temporal and spatial specificity that one would desire. In fact, this is the case for the *Sf1-Cre* transgenic line, which is active in peripheral endocrine organs as well as the VMH. Indeed, SF-1 is robustly expressed in the adrenal, the anterior pituitary, the gonads, and the spleen beginning early in development (Ikeda et al. 1994). Given that *SF1-Cre* will alter expression in the early primordial bipotential gonad and the adult ovary (Ikeda et al. 1994; Ingraham et al. 1994; Shen et al. 1994), it remains possible that the sex-dependent phenotypes described in these mouse models may partially result from a disruption of feedback loops in the hypothalamic-pituitary-gonadal axis.

To circumvent the limited spatial specificity of the Cre-Lox system and more definitively demonstrate the role of VMH neurons in promoting locomotion, viral vectors carrying designer receptors exclusively activated by designer drugs (DREADDs) were expressed in the VMH by stereotaxic delivery. Indeed, ERα is required for the full increase in pharmacogenetic-mediated or DREADD-induced locomotion (Correa et al. 2015). Male mice fail to show the same DREADD-induced responses with respect to locomotion (Fig. 3) but do exhibit a modest increase in oxygen consumption. Although increased oxygen consumption was not associated with higher locomotion or heat generation, we cannot exclude the possibility that VMHvl neurons play a minor role in male locomotion. Indeed, pharmacologic activation in the VMHvl induces running in male rats (Narita et al. 1993). DREADD-induced activation of locomotion in females appears much more sensitive when compared to DREADD- or ChR-induced activation of behaviors in males (Lee et al. 2014; Silva et al. 2013). Indeed, increased movement is observed in females even after unilateral or limited infection of DREADDs into VMHvl neurons. The ability to blunt DREADD-induced movement by genetic deletion of ERα, as shown here, establishes that ERα signaling is the main mediator of hormone-induced movement, as previously reported for female sexual behavior (Musatov et al. 2006, Lunahn et al. 1993). As mentioned above, hormone dependency has yet to be shown for experimentally induced male behaviors, including social fear, mating, and aggression (Lee et al. 2014, Silva et al. 2013).

One limitation with mouse models is the difficulty in showing a tight link between hormonal variation in cycling females and changes in energy expenditures. DREADD-induced locomotion in female mice appears to be insensitive to normal fluctuations in estrogen. Only after eliminating all gonadal hormones or all ERα signaling does one observe the dramatic influence of estrogen signaling on DREADD-induced locomotion. These findings are consistent with little to no effect of the estrous cycle on locomotion in mice (Kopp et al. 2006) and imply that the estrous cycle on physical activity is far stronger in rats than in mice. These and other data (Prendergast et al. 2014) also show that the assumption that the estrus cycle has a dramatic and robust effect on measured parameters in female mice is highly overstated, thus directly challenging the historical aversion to the use of both sexes.

The striking phenotypic differences in reproduction and metabolism observed between *Nkx2-1*$^{Sf\text{-}1\text{-}Cre}$ and *Esr1*$^{\text{Sf-1-Cre}}$ mouse models are instructive for dissecting out the complex and coordinated metabolic and reproductive functions of the VMHvl in females. One would like to define the signaling events and targets of ERα in the VMHvl that ultimately drive sex-dependent physiological endpoints. Within the VMHvl, there is a subpopulation of NKX2-1-positive VMHvl neurons coexpressing ERα and *Tac1*, which appears important for female activity (Fig. 3), is enriched in females compared to males, and is largely absent in *Nkx2-1*$^{Sf1\text{-}Cre}$ mutant females. *Tac1* is enriched in the VMHvl, as previously reported for rats (Dornan et al. 1990). Eliminating ERα neurons results in diminished *Tac1* transcripts in *Nkx2-1*$^{Sf1\text{-}Cre}$ mice, suggesting that this neuropeptide might participate in mediating female-specific physiology. However, Tac1 is not directly regulated by ERα (Correa et al. 2015). It is possible that these ERα^{+}, *Tac1*$^{+}$ VMH neurons may project to MPOA neurons, which have been linked to estrogen-induced running in rats (Spiteri et al. 2012;

Fahrbach et al. 1985). Other VMH projections relevant to locomotion might include those to the subthalamic and mesencephalic locomotor regions (Cheung et al. 2012), areas that when activated increase controlled movement in rats (Skinner and Garcia-rill 1984) or when lesioned in humans lead to deficits in locomotion (Hathout and Bhidayasiri 2005). Given that the neuropeptide-encoding gene Tachykinin 1 (Tac1) is associated with estrogen-responsive VMHvl neurons and is enriched in females raises the question as to its role in mediating sex-dependent behaviors. Unfortunately, while mice deleted globally for Tac1, as well as its receptor (NK-1), exhibit improved glucose homeostasis (Karagiannides et al. 2011a) and resistance to diet-induced obesity (Karagiannides et al. 2011b), both studies only report data from male mice. Nonetheless, this might suggest that neurokinin A (formerly substance P), encoded by Tac1, normally counteracts estrogen, opposing a negative energy state.

Excitatory Activity in VMH Neurons and Sex Differences

Nearly all VMH neurons express two markers, steroidogenic factor 1 (SF-1 encoded by *Nr5a1*) and vesicular glutamate transporter 2 (VGLUT2 encoded by *Slc17a6*). The prominent expression of *Vglut2* in the VMH (Ziegler et al. 2002; Fremeau et al. 2001) suggests that excitatory, glutamatergic neurotransmission mediates multiple aspects of VMH function, including those associated with the sexually dimorphic VMHvl (Fig. 4). In both males and females, expression of the glutamate decarboxylase (*Gad67*) that marks inhibitory neurons is for the most part completely excluded from the entire VMH as well as the VMHvl. Prior studies might predict that disrupting VMH excitatory neurotransmission would alter food intake and susceptibility to diet-induced obesity, especially given the established glutamatergic connections between the VMH and other metabolic brain centers, such as the arcuate nucleus (Fu and Van Den Pol 2008; Sternson et al. 2005). The initial work by Tong et al. examined this question by generating the VMH knockout of *Vglut2* using *Sf1-Cre*

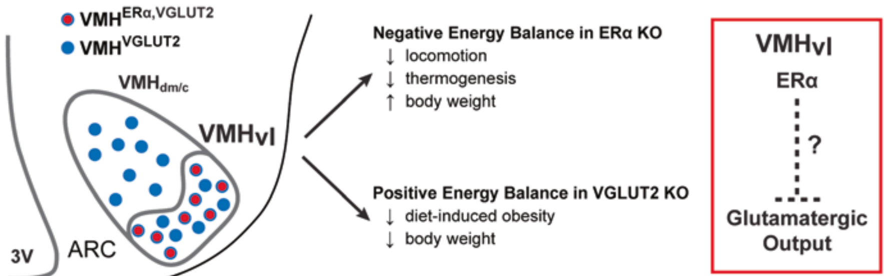


Fig. 4 Silencing glutamatergic VMH neurons promotes negative energy balance in females. Summary of the relative distribution of VMH^{VGLUT2} and $VMH^{ER\alpha,VGLUT2}$ neurons and the metabolic consequences resulting from genetic knockout of either ERα or VGLUT2. Opposing metabolic phenotypes observed in mutant female mice following deletion of ERα or VGLUT2 in the VMH suggest that ERα signaling reduces rather than enhances glutamaterigic output from the VMHvl

(*Vglut2*$^{Sf1\text{-}Cre}$), which for future reference was done in a mixed genetic background (Tong et al. 2007). Despite the fact that the VMHvl should be targeted using this approach, no sex-dependent metabolic changes were noted in their published work. However, they did detect lowered serum glucose in the fasted but not fed state in both sexes, suggesting that the VMH excitatory output is needed for the counterregulatory response to hypoglycemia. This was recently reexamined in a pure C57BL/6 background (Cheung et al. 2015): a strain with increased DIO induced weight gain and hyperglycemia (Montgomery et al. 2013; Collins et al. 2004). In this pure strain setting, sex differences emerged. When compared to *Vglut2*$^{fl/fl}$ controls, weight gain in *Vglut2*$^{Sf1\text{-}Cre}$ females was notably lower when placed on high-fat diet (HFD) at 10 weeks of age, whereas C57BL/6 mutant males showed no body weight differences (Cheung et al. 2015). Consistent with the female-specific resistance to DIO, glucose homeostasis was improved in *Vglut2*$^{Sf1\text{-}Cre}$ females as measured by an intraperitoneal glucose tolerance test (IP GTT). This result is somewhat at odds with the report that *Vglut2*$^{Sf1\text{-}Cre}$ males and females are heavier when fed a high-fat, high-sucrose diet (Tong et al. 2007), perhaps reflecting strain and dietary differences.

The reduced body weight in the female *Vglut2*$^{Sf1\text{-}Cre}$ mice, whose origins remain unclear, mimics the metabolic consequence of elevated estrogen signaling. In other words, the loss of excitatory output from the VMHvl has a negative effect on energy balance, rather than a positive effect. This result undermines the simple assumption that estrogen signaling potentiates VMH neurotransmitter output, suggesting instead that ERα signaling inhibits circuits that otherwise promote energy storage (Fig. 4). Because this brain region is tightly linked to reproductive behavior (Ogawa et al. 1998), one might speculate that in females, regulation of glutamatergic VMH neurons by estrogen maximizes fuel reserves in states of overnutrition (HFD), to ultimately improve reproductive fitness in times of undernutrition.

Interestingly, behavioral sex differences are also apparent after the loss of all excitatory output. Indeed, while both male and female *Vglut2*$^{Sf1\text{-}Cre}$ mice exhibit less anxiety in standard assays such as open field or elevated maze, mutant *Vglut2*$^{Sf1\text{-}Cre}$ males do exhibit greater exploratory drive (Cheung et al. 2015). As predicted from prior literature cited above, male resident *Vglut2*$^{Sf1\text{-}Cre}$ mice are less aggressive and attack far less frequently than *Vglut2*$^{fl/fl}$ controls. In summary, the phenotypes exhibited by *Vglut2*$^{Sf1\text{-}Cre}$ mice are consistent with emerging evidence that the VMHvl regulates sex-dependent metabolic responses and social behaviors. The ability to specifically target the excitatory output of different molecular modules in the VMHvl will be important to dissect and map the circuitry that controls male and female metabolic and behavioral endpoints.

Future Directions

One of the most critical and pressing questions raised by current data is how steroid signaling regulates the behavioral or physiological outputs between male and female ERα neurons. Thus, while it is clear in murine models that estrogen signaling

impacts energy expenditure in females, connecting the dots between estrogen and neuronal output remains obscure. This dependency on steroid signaling is perhaps the largest and most important difference between the male and female VMHvl. As mentioned above, to fully understand how these sex-dependent endpoints are established within different VMHvl modules requires the application of newer methods that allow a more granular view and finer genetic manipulation of the VMHvl. This task could be made more challenging if key factors in estrogen-responsive VMHvl neurons needed for neuronal output are not themselves direct downstream targets of ERα. Lastly, we have yet to define how well new findings in rodent models translate to humans, which is especially important if we are to appreciate the full metabolic benefits of estrogen in women's health.

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Part II
Role of Estrogens in Metabolic Homeostasis

Menopause, Estrogens, and Glucose Homeostasis in Women

Franck Mauvais-Jarvis

Abstract Randomized trials suggest that menopausal hormone therapy (MHT) prevents type 2 diabetes. Still, the mechanisms of these antidiabetic effects are a matter of controversy. This chapter provides an analysis of epidemiological and clinical evidence and proposes a mechanism for the effect of menopause and MHT on type 2 diabetes development and prevention. It discusses the beneficial role of estradiol on glucose homeostasis that is lost at menopause and improved by MHT, which delays type 2 diabetes. This chapter aims to reconcile differences among studies of the effect of menopause and MHT formulations on type 2 diabetes and argues that discrepancies arise from physiological differences in methods used to assess glucose homeostasis.

Introduction

Large randomized controlled trials have suggested that menopausal hormone therapy (MHT) reduces the incidence of type 2 diabetes in women (Espeland et al. 1998; Kanaya et al. 2003; Manson et al. 2013; Margolis et al. 2004; Salpeter et al. 2006). Surprisingly, however, the mechanisms of these findings are poorly understood. The purpose of this chapter is to integrate epidemiological and clinical evidence regarding the effect of menopause and MHT on type 2 diabetes risk in women. I discuss the mechanisms by which menopause and MHT affect pancreatic β-cell function and insulin sensitivity in women. This review focuses on studies in women, as animal studies have been reviewed elsewhere (Tiano and Mauvais-Jarvis 2012; Mauvais-Jarvis et al. 2013). The effect of selective estrogen receptor modulators on glucose homeostasis and diabetes in postmenopausal women has also been extensively reviewed (Xu et al. 2015).

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Does Menopause Alter Glucose Homeostasis?

Despite accumulated evidence in rodent models indicating that estrogens are beneficial to glucose homeostasis and prevent diabetes (Tiano and Mauvais-Jarvis 2012; Mauvais-Jarvis et al. 2013), the exact effect of menopause on dysglycemia in women is unclear. In fact, basic researchers utilize ovariectomy in rodents as a model of acute menopause. In contrast, natural menopause in women is a complex phenomenon including a progressive ovarian failure, a relative androgen excess, aging, increased central adiposity, and decreased physical activity (Santen et al. 2010). We will focus on menopausal alterations that directly alter glucose homeostasis.

Unfortunately, studies using accurate measures of glucose homeostasis to assess the direct effect of menopause on insulin resistance are limited. Using the intravenous glucose tolerance test (IVGTT) in a large cohort of women and after adjustment for age and BMI, a study observed a significant decrease in non-insulin-dependent glucose uptake (glucose effectiveness) in postmenopausal women, while insulin sensitivity was actually increased (Walton et al. 1993). In other studies using the euglycemic, hyperinsulinemic clamp – the gold standard to assess systemic insulin action – investigators found no difference in insulin action between pre- and postmenopausal women of similar age after adjustment (Muscelli et al. 2009; Toth et al. 2000). Therefore, it is possible that menopause alters the ability of glucose to promote its own disposal in an insulin-independent manner which is not detected in the steady-state conditions of the euglycemic, hyperinsulinemic clamp.

Studies of the effects of menopause on insulin secretion in women are scarce but suggest that menopause promotes β-cell dysfunction (Walton et al. 1993). However, because hepatic insulin clearance is decreased in postmenopausal women, circulating insulin concentrations during a glucose challenge are unchanged compared to those in premenopausal women. This suggests that menopause alters β-cells in ways that are not detected by clinical measurement of glucose and insulin levels and are revealed only during dynamic testing and using C-peptide measurements (Mauvais-Jarvis et al. 2017). Further studies are needed to clarify the impact of estrogen on islet function in women.

The importance of endogenous estrogens in preventing diabetes in women is highlighted by two studies. The InterAct study, a prospective case-cohort study with a follow-up of over 10 years, reported that early menopause, leading to prolonged E2 deficiency, is associated with a greater risk of type 2 diabetes (Brand et al. 2013). More recently, a prospective cohort study from the population-based Rotterdam Study with a follow-up of over 9 years also concluded that early onset of natural menopause is an independent marker for type 2 diabetes in postmenopausal women (Muka et al. 2017).

Effect of MHT on Diabetic Prevention

Available studies have not been designed to address the effect of MHT on diabetes prevention as a primary endpoint, and the existing data are not optimal to reveal the effect of estrogens on type 2 diabetes prevention. In a meta-analysis of over a

hundred randomized trials comparing MHT to placebo or no treatment in women without diabetes, MHT was associated with a decrease in fasting glucose and insulin levels leading to an improvement in insulin resistance, calculated using the homeostasis model assessment of insulin resistance (HOMA-IR) (Salpeter et al. 2006). The same meta-analysis reported a 30 % reduction in incident diabetes. The Postmenopausal Estrogen/Progestin Interventions (PEPI) study was the first large randomized placebo-controlled trial (RCT) to evaluate the effect of conjugated equine estrogens (CE) with or without progestogen on incident diabetes in postmenopausal women during a 3-year follow-up period. PEPI reported a significant decrease in fasting glucose and insulin levels in women assigned to MHT, suggesting improved insulin sensitivity (Espeland et al. 1998). The Heart and Estrogen/Progestin Replacement Study (HERS) examined the effect of MHT or placebo on the incidence of diabetes in postmenopausal women with coronary artery disease over 4 years (Kanaya et al. 2003). The incidence of diabetes was reduced by 35 % in the MHT group, mainly due to the fact that women on MHT maintained a lower fasting glucose level than women in the placebo group. The Women's Health Initiative (WHI) Estrogen Plus Progestin Trial (CE/MPA) is the largest and most recent RCT of postmenopausal women free of diabetes at baseline (Margolis et al. 2004). The WHI looked at self-reports of incident diabetes treated with antidiabetic agents. One year into the trial, the active treatment group showed lower fasting glucose and insulin and decreased HOMA-IR, compared with the placebo group. After an average of 6 years of follow-up, there was a 20 % relative reduction in incident-treated diabetes (Margolis et al. 2004; Manson et al. 2013). In HERS and WHI, a post-randomization adjustment revealed that the antidiabetic effects of MHT were independent from the reduction in BMI, suggesting that MHT has a direct beneficial impact on diabetes prevention, independent from fat reduction. In the WHI, women using CE alone also exhibited a decreased diabetes risk (Bonds et al. 2006; Manson et al. 2013). Consistent with the efficacy of CE therapy in reducing the risk of diabetes, in the extended post-intervention of a 13-year follow-up of the two WHI trials, the diabetes risk reductions dissipated (Manson et al. 2013). These RCTs confirm results from large observational studies. For example, the Nurses' Health Study (NHS) reported that current users of MHT showed a 20 % reduction of incident diabetes compared with past users and women who had never used MHT (Manson et al. 1992). Similarly, the prospective French cohort of the Etude Epidémiologique de Femmes de la Mutuelle Générale de l'Education Nationale (E3N), one of the largest observational studies in postmenopausal women (De Lauzon-Guillain et al. 2009), reported that during a 10-year follow-up, incident diabetes was 25 % lower among MHT users than among women who had never used MHT. Further, a cohort study of early postmenopausal women revealed a 69 % reduction in the incidence of diabetes in women who received MHT, compared to nonusers (Pentti et al. 2009). Obviously, a large RCT addressing the effect of MHT on diabetes prevention as a primary endpoint would be necessary in order to draw any definitive conclusions.

In the PEPI trial, MHT increased post-challenge glucose concentrations despite decreasing fasting glucose (Espeland et al. 1998). This effect of MHT was also observed in nondiabetic postmenopausal women of the Women's HOPE RCT trial

(Lobo et al. 2001) and the cross-sectional Rancho Bernardo Study (Kim and Barrett-Connor 2006). As this effect on post-challenge glucose is observed whether or not a progestogen is taken, it is likely due to CE. This slight impairment in glucose tolerance does not alter chronic glycemic control, since the major RCTs have reported that MHT was associated with both a decreased incidence of diabetes (Kanaya et al. 2003; Margolis et al. 2004; Salpeter et al. 2006) and a decrease in HbA1c (Hodis et al. 2001).

MHT has also been shown to improve glycemic control in diabetic postmenopausal women and reduced the HOMA-IR in women with diabetes by an average of 36 %, a greater reduction than that observed in postmenopausal women without known diabetes (Salpeter et al. 2006). A quarter of women in HERS were diabetic, and although the study was not powered enough to be conclusive, the trend was consistent with a beneficial effect of MHT on fasting glucose in diabetic women (Kanaya et al. 2003). In two placebo-controlled, randomized, crossover trials of oral CE or E2 treatment in postmenopausal women with type 2 diabetes, estrogens reduced fasting glucose, HbA1c, and insulin resistance without affecting postprandial glucose (Friday et al. 2001; Andersson et al. 1997). Similarly, in an RCT of oral E2 in postmenopausal women with type 2 diabetes, E2 produced a decrease in HbA1c (Brussaard et al. 1997) and increased hepatic insulin sensitivity. Therefore, the evidence is consistent with a beneficial effect of MHT on glycemic control in diabetic women.

The comparison of the effects of different routes of administration of estrogens (oral vs transdermal) on glucose homeostasis and diabetes has been recently reviewed (Mauvais-Jarvis et al. 2013).

Effect of MHTs on Insulin Secretion and Sensitivity

Studies suggest that MHT decreases visceral fat (reviewed in Santen et al. 2010), which contributes to MHT's antidiabetic action. However, in RCT, the antidiabetic effect observed in women assigned to MHT was independent from the reported reduction in BMI and waist circumference (Kanaya et al. 2003; Margolis et al. 2004). Similar observations were made in large observational studies including the NHS and E3N (Manson et al. 1992; De Lauzon-Guillain et al. 2009). Therefore, available evidence suggests that estrogens improve glucose homeostasis by directly enhancing insulin sensitivity or insulin secretion (Mauvais-Jarvis et al. 2013).

MHT Improvement in Insulin Sensitivity

A meta-analysis (Salpeter et al. 2006) suggested that MHT improves insulin sensitivity based on the HOMA-IR, which integrates fasting glucose and insulin. However, in contrast with this finding, studies that have assessed the effects of MHT

by measuring systemic insulin action during a euglycemic, hyperinsulinemic clamp have surprisingly showed no beneficial effect of MHT on insulin action, and as a result, the Endocrine Society released a statement concluding that MHT improves glucose homeostasis independently of insulin sensitivity (Santen et al. 2010). However, the observation that estrogen therapy shows no improvement in systemic insulin action using the euglycemic, hyperinsulinemic clamp conflicts with studies showing improvement in HOMA-IR and prevention of diabetes.

One potential explanation for these discrepancies is that euglycemic, hyperinsulinemic clamp studies were not designed to assess the "window of opportunity" of MHT, and there is significant age variability in most studies. Recently, a study observed that E2 increased systemic insulin action in early postmenopausal women (<6 years of menopause) but decreased it in late postmenopausal women (>10 years of menopause) (Pereira et al. 2015). This suggests that E2's beneficial effects on insulin sensitivity are dependent on time since menopause.

Another possible explanation is that estrogens may improve glucose homeostasis independently of systemic insulin action as measured by the euglycemic, hyperinsulinemic clamp which only explores the insulin-dependent fraction of glucose uptake (Mauvais-Jarvis et al. 2002; Mauvais-Jarvis 2013). It is possible that estrogen therapy enhances non-insulin-dependent glucose uptake or glucose effectiveness in women in ways that are not detected using the steady-state condition of the euglycemic, hyperinsulinemic clamp (Mauvais-Jarvis et al. 2017). New studies are needed to unravel the insulin-dependent and insulin-independent mechanisms of action of MHT.

MHT Improvement in Insulin Secretion

The beneficial effects of estrogen therapy on pancreatic β-cell function have been recently reviewed (Tiano and Mauvais-Jarvis 2012; Mauvais-Jarvis 2016). In rodent and human islets, estrogens promote survival and maintain islet function in the presence of multiple pro-apoptotic insults (Tiano and Mauvais-Jarvis 2012). Importantly, estrogen treatment also protects cultured human islet survival and function in the presence of diabetic injuries, including pro-inflammatory cytokines, oxidative stress, high glucose, and lipotoxicity (Tiano and Mauvais-Jarvis 2012). Estrogens also maintain the function of human islets transplanted into immunodeficient diabetic mice in vivo (Liu et al. 2013). Given this background, it is likely that estrogen therapy also has beneficial effects on islet β-cell function and insulin secretion in postmenopausal women, a notion supported by several studies. These studies have assessed β-cell function using IV or oral glucose challenge followed by mathematical modeling of plasma glucose, insulin, and C-peptide concentrations. Overall, studies of β-cell function in postmenopausal women are consistent with a beneficial effect of MHT increasing glucose-stimulated insulin secretion (C-peptide) and enhancing hepatic insulin clearance (C-peptide to insulin ratio), so that the insulin levels are unchanged, but liver insulin action is enhanced by insulin extraction

(Cagnacci et al. 1992; Godsland et al. 1993; Godsland et al. 2004; Paoletti et al. 2002; Spencer et al. 2000).

In summary, there is a favorable influence of estrogen on β-cell insulin secretion and hepatic insulin clearance, resulting in unchanged glucose homeostasis. These effects are lost at menopause and can be restored by MHT.

Conclusions and Future Directions

Evidence presented in this chapter argues for a beneficial role of a physiological window of endogenous estradiol on glucose homeostasis that is lost at menopause and predisposes to type 2 diabetes. MHT improves insulin secretion, glucose effectiveness, and insulin sensitivity. The improvement in insulin sensitivity is detectable using clinical indices like the HOMA-IR and the IV or oral glucose challenge, but is not detectable in the steady-state condition of the euglycemic, hyperinsulinemic clamp. Still, in RCTs, the use of MHT reduces the incidence of type 2 diabetes in women. Because of its complex balance of risks and benefits, however, MHT is not FDA approved for the prevention of type 2 diabetes in women.

The challenge with estrogens is their adverse effects when used long term for chronic disease prevention. Future therapeutic avenues lie in estrogen ligands that will provide the beneficial effect of estrogens in the brain, bone, cardiovascular system, and metabolic tissues, without the deleterious effects of estrogens on the breast and uterus and without the risk of VTE (Xu et al. 2015). These are critical future directions that must be explored in order to move the field forward and lay the foundation for improved therapies for menopause and diabetes.

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Role of Estrogens in the Regulation of Liver Lipid Metabolism

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Abstract Before menopause, women are protected from atherosclerotic heart disease associated with obesity relative to men. Sex hormones have been proposed as a mechanism that differentiates this risk. In this review, we discuss the literature around how the endogenous sex hormones and hormone treatment approaches after menopause regulate fatty acid, triglyceride, and cholesterol metabolism to influence cardiovascular risk.

The important regulatory functions of estrogen signaling pathways with regard to lipid metabolism have been in part obscured by clinical trials with hormone treatment of women after menopause, due to different formulations, routes of delivery, and pairings with progestins. Oral hormone treatment with several estrogen preparations increases VLDL triglyceride production. Progestins oppose this effect by stimulating VLDL clearance in both humans and animals. Transdermal estradiol preparations do not increase VLDL production or serum triglycerides.

Many aspects of sex differences in atherosclerotic heart disease risk are influenced by the distributed actions of estrogens in the muscle, adipose, and liver. In humans, 17β-estradiol (E2) is the predominant circulating estrogen and signals through estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and G-protein-coupled estrogen receptor (GPER). Over 1000 human liver genes display a sex bias in their expression, and the top biological pathways are in lipid metabolism and

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genes related to cardiovascular disease. Many of these genes display variation depending on estrus cycling in the mouse. Future directions will likely rely on targeting estrogens to specific tissues or specific aspects of the signaling pathways in order to recapitulate the protective physiology of premenopause therapeutically after menopause.

Introduction

Before menopause, women are protected from atherosclerotic heart disease associated with obesity relative to men. Women have a nearly decade-long delay in first myocardial infarction compared to men (Freedman et al. 2004; Lloyd-Jones et al. 2009). Furthermore, at any given age, women have about half the risk of cardiovascular disease relative to men (Roger et al. 2011; Wilmot et al. 2015). Sex hormones have been proposed as a mechanism that differentiates the differential risk of cardiovascular disease in men versus women. In women, the ovaries produce estrogens and progesterone, which are the predominant female sex hormones. Therefore, naturally cycling estrogen has been proposed to be protective against atherosclerotic cardiovascular disease. This view is supported by the increase in cardiovascular disease risk in women seen after menopause, which involves a natural decline in ovarian hormone production. Estrogens have effects in many organ systems that contribute to cardiovascular risk vs. protection, including regulation of liver lipid metabolism and serum lipoprotein levels. The liver is an important site where fatty acid, triglyceride, and cholesterol metabolism are coordinated to meet metabolic needs in normal physiology, and this coordinated metabolism goes awry with obesity. In this chapter, we review the complex effects of estrogens on regulation of plasma lipids and liver lipid metabolism in humans and animal models.

Many aspects of sex differences in atherosclerotic heart disease risk are influenced by the distributed actions of estrogens in muscle, adipose, and liver. In response to obesity, both men and women have increased free fatty acid (FA) release into blood. In response to increased FA delivery to the liver with obesity, circulating FA are packaged into triglyceride (TG)-rich very-low-density lipoprotein (VLDL) particles by the liver. Obesity is associated with increased production of VLDL-TG particles by the liver to a greater degree in men than in women (Reaven and Bernstein 1978; Mittendorfer et al. 2003). This is, in part, due to enhanced FA clearance by muscle (Frias et al. 2001; Clegg et al. 2017; Ribas et al. 2016), resulting in less FA delivery to the liver to drive VLDL-TG production. It is also known that in response to FA delivery to the liver that women secrete VLDL particles that are more TG rich (Magkos et al. 2007b), which would help the liver export liver TG and prevent liver fat accumulation with obesity. Production of more TG-rich VLDL is matched with accelerated VLDL-TG clearance rates in women (Matthan et al. 2008), which collectively contribute to lower plasma VLDL-TG levels with obesity in women. Estrogens exert regulatory control in nearly every step of these control points of lipid metabolism.

Mechanisms of Estrogen Signaling in the Liver

In humans, 17β-estradiol (E2) is the predominant circulating estrogen and is made by the ovaries and circulates as an endocrine hormone transported in plasma by sex hormone-binding globulin, where it passively diffuses across the cell membrane into target tissues. Tissues may also make estrogens locally from androgenic precursors where it acts in a paracrine manner. This is established in breast cancer cells and in the male reproductive tract but is less clear in tissues such as the liver (Pasqualini et al. 1996; Qian et al. 2001). The uterus is the classic estrogen-responsive target tissue since estrogen increases proliferation of the uterine lining. Thus, uterine mass can serve as a proxy for total body estrogen levels in animal studies. Many other tissues are responsive to estrogen action in vivo. In a transgenic mouse model designed to detect estrogen signaling, the liver was actually the most responsive to E2 (Ciana et al. 2003).

Estrogens can mediate their biologic effects in the liver through a number of mechanisms. The classic mechanism of E2 action involves E2 binding to the steroid nuclear hormone receptors, estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ). ERα and ERβ have the classic features of steroid hormone receptors – an activation function 1 (AF1) domain, a ligand-binding domain, a DNA-binding domain, and an activation function 2 (AF2) domain (Osborne and Schiff 2005). When unbound to ligand, ERα and ERβ are retained in the cytosol by association to heat shock protein 90 (Hsp90) complexes. Estrogens binding to either ERα or ERβ cause a conformational change that promotes dissociation from Hsp90, dimerization, and translocation into the nucleus. Once in the nucleus, ERα and ERβ bind to genomic locations based on sequence recognition of the DNA-binding domain (Osborne and Schiff 2005). These genomic sequences are commonly referred to as estrogen response elements (EREs) and are often characterized by an inverted repeat separated by three nucleotides (5′ AGGTCAnnnTGACCT 3′). These EREs are commonly found in the promoter or enhancer regions of liver genes whose transcription is regulated by estrogens. Over 1,000 human liver genes display a sex bias in their expression (Zhang et al. 2011). The top biological pathways are in lipid metabolism and genes related to CHD (Zhang et al. 2011). Genetic analysis of 100 mouse strains revealed important diet and sex interactions in the development of insulin resistance. Some of the most prominent genes discovered were involved in TG metabolism and FA oxidation and were sexually dimorphic (Parks et al. 2015). Additionally, chromatin immunoprecipitation assay revealed 43 of the lipid genes are transcriptionally regulated by ERα (Gao et al. 2008). In the mouse, scores of liver genes involved in TG and cholesterol metabolism vary with the 4-day estrous cycle of the mouse in an ERα-dependent manner (Villa et al. 2012), demonstrating a tight coordination of liver lipid metabolism with reproductive needs.

In addition to binding to genomic locations with by recognition of EREs, ERα and ERβ can bind to genomic locations indirectly, via protein-protein binding

with other transcription factors (Osborne and Schiff 2005). For instance, ERα interacts with the c-rel subunit of NFκB, preventing NFκB from promoting IL-6 expression (Galien and Garcia 1997). ERα can either coactivate or corepress Fos-/Jun-mediated transcription depending on the presence of ligand (Paech et al. 1997). In the liver ERα serves as a co-regulator to repress IL-1 beta gene transcription (Galien and Garcia 1997). Thus, ERα and ERβ can promote or inhibit gene transcription depending on the transcriptional machinery available at a particular genomic location.

An additional aspect of ERα regulation of liver lipid metabolism occurs by modifying signaling and is transcriptionally independent (Park et al. 2011). Estrogens can also alter cell signaling via binding to receptors localized to the plasma membrane. ERα and ERβ have been shown to localize to the plasma membrane (Bjornstrom and Sjoberg 2005; Marino et al. 2006; Levin 2009). Membrane localization is achieved through palmitoylation of a serine residue and association with caveolin-1 (Cav-1) (Levin 2009). Membrane ERα and ERβ signal through the ERK 1/2 and the PI3K pathways (Bjornstrom and Sjoberg 2005; Levin 2009; Marino et al. 2006). After removal of ovarian hormones by ovariectomy, the benefits of the ERα agonist propylpyrazoletriol (PPT) with regard to liver lipid metabolism can largely be restored by membrane-localized ERα (Pedram et al. 2013).

In addition to membrane-localized ERα and ERβ, estrogens can signal through another cell surface receptor, G-protein-coupled estrogen receptor (GPER, also called Gpr30), which is expressed in multiple tissues including liver (Sharma et al. 2017; Nilsson et al. 2011; Owman et al. 1996). E2 binding to GPER initiates two signaling cascades – one results in increases in cyclic AMP (cAMP), and the other results in increases in intracellular Ca^{2+} (Nilsson et al. 2011). Some of the signaling mediated by GPER also involves activation of epidermal growth factor receptor (EGFR) (Nilsson et al. 2011). Although GPER is most well characterized for its ability to regulate cell signaling, GPER-activated cell signaling may also regulate gene expression since E2 treatment has been shown to alter gene expression in ERα/ERβ double-knockout mice (Lindberg et al. 2002). Whole-body deletion of GPER accelerates atherosclerosis and increases LDL cholesterol levels in mice fed an atherogenic diet (Meyer et al. 2014). It is unclear though whether the LDL cholesterol increases seen in GPER null mice are due to hepatic effects or due to indirect changes due to loss of GPER in other tissues. Thus, the current literature supports a robust role of estrogen signaling through ERα to impact glucose and TG metabolism, both membrane and transcriptional effects. The relative contributions of estrogen signaling through ERα, ERβ, or GPER with regard to liver gene expression and cardiovascular risk still warrant further investigation.

Hormone Treatment and the Risk of Cardiovascular Disease in Postmenopausal Women

The important regulatory functions of estrogen signaling pathways with regard to lipid metabolism have been in part obscured by the conflicting results of clinical trials with hormone treatment of women after menopause. These controversies arise in part from the different formulations of estrogen used for treatment of women after menopause, different routes of administration, and different pairings with progestins. The estrogen hypothesis suggests that the higher level of estrogen in women before menopause protects against cardiovascular disease. In support of this, postmenopausal women have increased risk of cardiovascular disease compared to premenopausal women (Colditz et al. 1987; Hu et al. 1999; Kannel et al. 1976; van der Schouw et al. 1996). A number of prospective studies conducted in the 1970s through the 1990s suggested that hormone treatment, typically with conjugated estrogens, improved risk of cardiovascular disease (Burch et al. 1974; Bush et al. 1983; Criqui et al. 1988; Croft and Hannaford 1989; Grady et al. 1992; Grodstein and Stampfer 1995; Hammond et al. 1979; Henderson et al. 1991; Hernandez Avila et al. 1990; Petitti et al. 1987; Stampfer et al. 1985; Sullivan et al. 1990; Wilson et al. 1985; Wolf et al. 1991). Prospective studies, while informative, are subject to various sources of bias. One potential source of bias in prospective studies of estrogen treatment has been labeled the "healthy woman" bias (Aguilar-Salinas et al. 2002), a form of selection bias. This source of bias is due to active seeking of medical care. According to this model, women more willing to be on hormone treatment are also more likely to monitor their health and take other medications to treat other diseases, thus enriching for a population that is healthier overall.

To more definitively determine the effect of hormone treatment on risk of cardiovascular disease, a number of randomized controlled trials were aimed to experimentally determine whether hormone therapy could prevent cardiovascular disease in postmenopausal women. Two of the largest randomized controlled trials, conducted in 1990s, were the Women's Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Study (HERS) (Hulley et al. 1998; Manson et al. 2003). Hormone treatment consisted of conjugated estrogens and progestin if the women had an intact uterus or conjugated estrogens alone if the women had a prior hysterectomy. The WHI trial enrolled over 16,000 postmenopausal women and monitored cardiovascular disease outcomes over an average of 5.6 years. The HERS trial enrolled over 2,700 women and monitored cardiovascular disease outcomes over an average of 6.8 years (Grady et al. 2002). Despite the improvement in cholesterol and diabetes risk factors, hormone treatment did not improve cardiovascular disease in the HERS trial. In the WHI trial, hormone treatment actually worsened cardiovascular disease risk. The increased cardiovascular risk associated with hormone treatment in the WHI was the worst in women who had been assigned to hormone treatment over 10 years after the onset of menopause. This led to the development of the "timing hypothesis," which suggests that hormone treatment is

most beneficial if initiated soon after menopause and potentially harmful if initiated late (>10 year) in menopause.

The Early versus Late Intervention Trial with Estradiol (ELITE) study was designed to test the timing hypothesis (Hodis et al. 2016). The ELITE study enrolled over 600 postmenopausal women and randomized them to treatment with placebo or oral estradiol plus vaginal progesterone for 10 days per cycle. Women in the ELITE trial were stratified into two groups – one group of women were considered in early menopause if menopause occurred in the last 6 years, and the other group of women were considered in late menopause if menopause occurred at least 10 years prior to enrollment in the study. Women were followed 5 years, and carotid intima medial thickness (CIMT) and coronary artery calcium (CAC) score were used as indices of atherosclerosis. Estradiol treatment reduced the progression of CIMT in the early menopause group but failed to delay atherosclerosis in the late menopause group. This result supports timing hypothesis of estrogen treatment. Coronary atherosclerosis was approximated using CAC score, but this measure was added late to the ELITE trial. Oral estradiol did not alter coronary atherosclerosis in either the early or late menopause group. It is unclear whether the failure to detect a difference in coronary atherosclerosis was due to insufficient power or the ineffectiveness of oral estradiol to reduce coronary atherosclerosis. A post hoc analysis of recently postmenopausal women (age 50–59) in the WHI supported that treatment with conjugated estrogens reduced coronary atherosclerosis as measured by coronary calcium imaging (Manson et al. 2007). Interestingly, hormone treatment increased plasma TGs in ELITE, in agreement with the WHI and HERS trial. This may suggest that increases in TGs with hormone treatment may mitigate other improvements in plasma lipids with regard to risk of coronary heart disease. Further work will be needed to confirm whether treatment with estrogen formulations improve cardiovascular outcomes in addition to the improvements in CIMT in women beginning treatment soon after menopause and how this is balanced by a potential worsening of dyslipidemia with different hormone treatment approaches.

In addition to cardiovascular outcomes, two smaller, related randomized controlled trials found somewhat divergent results regarding hormone treatment on measures of coronary atherosclerosis. In the Women's Estrogen–Progestin Lipid-Lowering Hormone Atherosclerosis Regression Trial (WELL-HART), 226 postmenopausal women with an average age of 63.5 with known coronary disease were randomized to receive placebo, micronized E2, or micronized E2 and progesterone (Hodis et al. 2003). In the WELL-HART study, neither micronized E2 nor micronized E2 and progesterone prevented progression of coronary atherosclerosis. In the Estrogen in the Prevention of Atherosclerosis Trial (EPAT), 222 healthy postmenopausal women with an average age of 62.2 without preexisting coronary disease were randomized to receive micronized E2 or placebo (Hodis et al. 2001). Contrary to the WELL-HART study, micronized E2 treatment reduced the rate of subclinical atherosclerosis of the carotid artery in the EPAT study. The differences in the WELL-HART and EPAT studies raise several possibilities regarding hormone treatment and cardiovascular disease. Firstly, estrogen alone may be required to prevent cardiovascular disease. Secondly, hormone treatment may be effective in prevention

of atherosclerosis but may be ineffective at reversing established atherosclerosis in postmenopausal women. Thirdly, estrogen therapy may perhaps have more potent effects on preventing or reversing carotid artery atherosclerosis than on atherosclerosis in coronary arteries since the EPAT and ELITE trials both showed that estrogen therapy reduced progression of carotid atherosclerosis.

The cause of the TG-rich dyslipidemia with hormone treatment of postmenopausal women has been controversial. Normal cyclic variations in estrogen levels through the menstrual cycle do not impact VLDL-TG or VLDL-apoB kinetics or concentrations (Magkos et al. 2006). Neither cycling hormones nor hormone treatment with E2 alone or in combination alters FA concentration or fluxes to the liver to a greater degree than day-to-day variation (Jensen et al. 1994; Magkos et al. 2007a). By contrast, estrogens seem to have a significant impact on liver VLDL production that depends on the route of delivery. Oral delivery of micronized estradiol increased VLDL production rates by 80%, which was greater than conjugated estrogen, whereas transdermal estradiol had no effect on VLDL production rates in this study (Walsh et al. 1991). Another study with oral ethinyl estradiol increased VLDL-apoB production over 100% (Schaefer et al. 1983), which is a similar result found to earlier studies with conjugated equine estrogens (Glueck et al. 1975). That estrogens are the major driver of hypertriglyceridemia with oral hormone treatment of postmenopausal women is further supported in that progestins oppose the effect of estrogens, by stimulating VLDL clearance in both humans and animals (Kissebah et al. 1973; Kim and Kalkhoff 1975) and reviewed in (Magkos and Mittendorfer 2009).

Relative to oral estrogens, transdermal estradiol has less potent effects on lowering LDL cholesterol and increasing HDL cholesterol (Baksu et al. 2007; Chen et al. 2001; Sanada et al. 2004; Strandberg et al. 2003; Zegura et al. 2006). Additionally, transdermal estradiol treatment does not seem to increase plasma TGs when compared to oral estrogen formulations (Baksu et al. 2007; Chen et al. 2001; O'Sullivan et al. 1998; Sanada et al. 2004; Strandberg et al. 2003; Zegura et al. 2006). In fact, most studies demonstrate that transdermal estradiol actually reduces plasma TGs (Baksu et al. 2007; Chen et al. 2001; Zegura et al. 2006), but this was not consistently demonstrated in all trials. In a small study examining VLDL-TG kinetics, transdermal estradiol was shown to reduce plasma TG by increasing the rate of VLDL-TG clearance without affecting VLDL-TG production (Smith et al. 2014). Since transdermal estradiol seems to have less potent effects on plasma lipid metabolism compared to oral estrogen formulations, the liver is likely responsible for most of estrogen's effects on increasing VLDL-TGs in the blood after menopause.

Lack of Estrogen Signaling Promotes Liver TG Accumulation and Leads to Hepatic Insulin Resistance

The converse implication of estrogen-mediated reductions in FA delivery to the liver and estrogen-mediated increases in VLDL-TG export is that deficiency of estrogen, with antagonist, after menopause, or in experimental models, leads to liver

fat accumulation. Tamoxifen (TMX) is an antiestrogen drug used for the treatment of hormone-sensitive breast cancer. One side effect of TMX is the development of nonalcoholic fatty liver disease and steatohepatitis (Nishino et al. 2003; Murata et al. 2000; Oien et al. 1999). As women transition to menopause, the risk of nonalcoholic fatty liver disease increases (Ryu et al. 2015; Yang et al. 2014). Aging is a natural risk factor for NAFLD, which may confound the impact of menopause on risk of NAFLD. However, some younger women have their ovaries surgically removed for medical reasons. In women undergoing surgical menopause, the risk of NAFLD is increased nearly twofold (Matsuo et al. 2016). Furthermore, in postmenopausal women, hormone treatment reduced plasma levels of liver enzymes, a marker of liver damage in NAFLD (McKenzie et al. 2006). Thus, absence of ovarian hormones leads to an increased risk of NAFLD that is at least partially reversible with estrogen treatment in postmenopausal women.

The impact of reductions in estrogen signaling on liver lipid metabolism has been studied extensively in rodent models. Ovariectomy, or surgical removal of ovaries, in rodents leads to an accumulation of liver triglyceride content (Barrera et al. 2014; Cote et al. 2012; de Oliveira et al. 2016; Paquette et al. 2007; Rogers et al. 2009), similar to that seen in postmenopausal women. Additionally, a chemical model of menopause created by administration of 4-vinylcyclohexene diepoxide (VCD), which depletes primordial follicles, creates insulin resistance, fatty liver, and dyslipidemia (Romero-Aleshire et al. 2009). In addition to estrogen deficiency causing steatosis in rodent models, estrogen replacement reduces steatosis (Barrera et al. 2014; Bryzgalova et al. 2008; Villa et al. 2012; Camporez et al. 2013; Chambliss et al. 2016; Palmisano et al. 2016; Wang et al. 2015; Zhu et al. 2013; Kim et al. 2014).

Estrogens and the Physiologic Regulation of Liver Lipid Metabolism Through ER Alpha

The physiologic reason that estrogen regulates to liver TG metabolism may be connected to an evolutionarily conserved need to coordinate nutritional status with reproduction. Insects, birds, and fish all have increased transport of TG from the liver to facilitate egg development through estrogen-like pathways (Davis 1997). To define tissue-specific contributions of estrogen signaling, several groups have created hepatocyte-specific ERα knockout mice to define the effects of estrogen signaling specifically through liver ERα. Della Torre and colleagues demonstrated a requirement for liver ERα in mediating amino acid regulation of the reproductive cycle (Della Torre et al. 2011). Using a mouse model with ERα deficiency in hepatocytes, we demonstrated that the ability of estrogens to reduce liver steatosis is lost in with deletion of liver ERα, suggesting that estrogens are acting directly in the liver to reduce TG content through ERα (Palmisano et al. 2016; Zhu et al. 2013; Villa et al. 2012). As expected, loss of liver ERα results in increased expression of

lipid synthesis genes (Bryzgalova et al. 2006), loss of estrogen regulation of target genes (Palmisano et al. 2016; Zhu et al. 2013), and impaired estrogen regulation of other lipid metabolic target genes (Della Torre et al. 2016). One proposed mechanism for ERα regulation of lipid synthesis targets involves estrogen-ERα regulation of the nuclear receptor Small Heterodimer Partner (SHP), a target gene of ERα (Palmisano et al. 2016; Wang et al. 2015). Additionally, estrogen-ERα regulation of liver lipid metabolism has been proposed to act via microRNA mir-125b (Zhang et al. 2015). Additional work is needed to establish the clinical relevance of these proposed targets in humans and to develop therapies that can recapitulate the lipid-lowering effect of estrogen in the liver to reduce the clinical burden of NAFLD.

In female mice, estradiol treatment at the time of ovariectomy blocks the effects of hyperinsulinemia with regard to lipid metabolism, leading to a suppression of de novo lipogenesis and maintenance of hepatic VLDL production (Zhu et al. 2013). The net effect of E2 treatment was to reduce liver TG and diacylglycerol content and to improve insulin action with regard to glucose metabolism. In female mice lacking the liver ERα, E2 following ovariectomy limits adiposity but fails to improve insulin sensitivity, fails to limit liver DAG, and fails to prevent insulin suppression of VLDL production. E2 administration at the time of ovariectomy and high-fat diet feeding significantly decreases liver TG and DAG content compared to ovariectomy and sham mice by limiting ^{14}C-glycerol deposition into liver triglycerides and diacylglycerol, combined with maintaining the efficiency of triglyceride export from the liver in the setting of hyperinsulinemia (Camporez et al. 2013; Zhu et al. 2013).

The effects of liver estrogen signaling to improve liver insulin sensitivity, suppress lipogenesis, and promote VLDL output from the liver were independent of estrogen's ability to regulate body weight as E2-treated hepatocyte knockout mice after ovariectomy were lean due to intact estrogen signaling in CNS and other tissues, yet E2 treatment failed to regulate liver metabolism (Zhu et al. 2013). Additionally, paired feeding in mouse studies shows direct effects of E2 protecting from fatty liver that are independent on its effects in the CNS to reduce food intake (Bryzgalova et al. 2008).

Estrogen Signaling Limits Liver Fat Accumulation by Reducing de novo Lipogenesis in the Liver

The mechanisms by which estrogen signaling protects against hepatic steatosis include reductions in de novo lipogenesis, as reported by different laboratories. Using a combination of chromatin immunoprecipitation and tiled microarrays (ChIP-on-chip) approach, Gao et al. identified binding regions of ERα to DNA in intact chromatin in the liver (Gao et al. 2008). This analysis revealed 19 gene ontology (GO) categories including lipid biosynthesis (GO 0008610) and fatty acid metabolism (GO 0006520) which are significantly enriched for genes that had ERα recruited to their promoter after 2 h of estradiol treatment (Gao et al. 2008).

Conventional ChIP followed by qPCR shows binding of ERα to promoter regions of lipogenesis genes including STAT3 and SHP which are consistently increased after treatment with estradiol or ERα agonist (Gao et al. 2008). This report is consistent with their previous observation that E2 treatment promotes ERα binding to STAT3 promoter and STAT3-Tyr phosphorylation, which subsequently suppresses Fasn, Scd1, Acaa1, and Gpam expression in the liver in ob/ob mice (Gao et al. 2006). Estradiol treatment in female mice with intact ovary suppresses FASN and SCD-1 in the liver (Bryzgalova et al. 2008). The authors also reported that E2 treatment decreases HGP by suppressing G-6-P expression (Bryzgalova et al. 2008).

E2 treatment suppresses liver lipogenesis by maintaining ACC phosphorylation as has been reported in studies from our laboratory and others (Cole et al. 2010; Zhang et al. 2013; Zhu et al. 2013, 2014). This mechanism likely contributes to the correction of pathway-selective insulin resistance in the liver by E2 treatment. This is due to the observation that insulin suppresses ACC phosphorylation during hyperinsulinemic clamp to promote lipogenesis, which action is diminished after E2 treatment (Zhu et al. 2013, 2014). ACC phosphorylation is regulated by AMPKα phosphorylation in the liver (Tuazon et al. 2015). Estradiol induces signal transduction through ERα, which localizes to both the plasma membrane and nucleus. A study by Pedram et al. shows that activation of liver estrogen signaling by ERα agonist PPT promotes AMPK phosphorylation in WT mice and transgenic mice expressing only the ligand-binding domain of ERα exclusively at the plasma membrane but not in ERα knockout mice. This study shows that gene expression changes mediated by membrane-localized ERα result in important metabolic effects independent of nuclear ERα (Pedram et al. 2013). In this study, phosphorylation of AMPK by activation of ERα is associated with phosphorylation of ACC. In addition, activation of membrane ERα also counteracts insulin's action to promote the mRNA of lipogenesis gene Srebf1 (Pedram et al. 2013). Additionally, oral CE and the SERM and BZA also promote AMPK phosphorylation via ERα in the liver after ovariectomy (Kim et al. 2014). In this study by Kim et al., oral CE and BZA reduce hepatic FAS expression and FAS activity and are associated with a decrease in liver TG accumulation in female after OVX. This appears to be mediated in part by inducing CEACAM1 expression and phosphorylation, which triggers CEACAM1 binding to and downregulation of FAS activity in liver (Kim et al. 2014).

E2 treatment also likely promotes FA oxidation in liver. Levels of mRNA for CPT-1, a protein to transport fatty acid into mitochondrial for β-oxidation, are induced with E2 treatment. Increased oxygen consumption and liver ATP production associated with changes in UCP2 expression in the liver were reported in E2 treatment after ovariectomy, indicating increased fatty acid oxidation in the liver in those mice (Camporez et al. 2013). Additionally, E2 and CE increase production of FGF21 by the liver which may also increase hepatic FA oxidation (Kim et al. 2014).

Lipotoxicity due to the accumulation of lipid in hepatocytes leads to hepatic insulin resistance with regard to glucose metabolism. Insulin's action to suppress hepatic glucose production (HGP) during hyperinsulinemic-euglycemic clamp is blunted after ovariectomy female mice compared to sham controls after a short term of high-fat diet feeding, although liver TG and DAG are not significantly increased

with this duration of diet (Camporez et al. 2013). The blunted insulin action is associated with decreased insulin signaling, indicated by phosphorylation of AKT, in the liver (Camporez et al. 2013). Without challenged by high-fat feeding, HGP is blunted by 20% in global ERα KO mice compared to WT controls, which is also associated with diminished insulin signaling response during hyperinsulinemic-euglycemic clamp (Ribas et al. 2010). In unconscious mice with no differences in glucose disposal, decreased glucose infusion rate (GIR) during clamp is attributed to increased HGP in ERα-deficient mice compared to WT controls (Bryzgalova et al. 2006). Furthermore, we found that ovariectomy with high-fat diet feeding results in liver TG and DAG accumulation, decreased GIR and increased HGP, and diminished hepatic insulin signaling during hyperinsulinemia (Zhu et al. 2013).

Estrogen Regulation of Factors Indirectly Affecting Liver Lipid Metabolism

While estrogens have been proposed to directly regulate a number of pathways involved in cardiovascular disease directly, especially in the liver, estrogen activity in a number of other tissues may contribute indirectly to plasma lipid responses to estrogens and liver lipid metabolism by estrogens. One of the original hypotheses purported to explain male-female differences in cardiovascular disease relates to body fat distribution differences between men and women. With obesity, women have more subcutaneous fat, whereas men have more visceral fat. Additionally, body fat distribution changes in women from a more subcutaneous distribution to a more visceral distribution of fat with menopause (Svendsen et al. 1995). Since body fat distribution, as measured by waist-to-hip ratio, predicts risk of cardiovascular disease (Canoy et al. 2007; Yusuf et al. 2005), women may have lower risk of cardiovascular disease due to a more favorable body fat distribution. The hypothesis that body fat distribution contributes to risk of cardiovascular disease was put forth by Vague in 1947 (Vague 1947). Experimental evidence to prove this hypothesis would take many decades, but two large prospective studies confirmed that body fat distribution did indeed predict risk of future cardiovascular disease (Canoy et al. 2007; Yusuf et al. 2005). Exercise and weight loss can reduce waist-to-hip ratio and reduce risk of cardiovascular disease, but long-term weight loss in obese patients remains a clinical challenge due to weight regain. Pharmacologic agents that modify body fat distribution are not currently available. Furthermore, a pooled meta-analysis found that waist-to-hip ratio contributed to cardiovascular risk similarly between men and women (de Koning et al. 2007). Therefore, it is important to understand other factors that may explain how women have lower risk of cardiovascular disease relative to men.

Some of estrogen's protective effects in the liver are likely indirectly due to estrogen signaling adipose tissue to limit the release of serum FA in response to insulin, and in skeletal muscle to promote FA oxidation, thereby limiting FA deliv-

ery to the liver. In fasting, about 75% of the lipid that ends up in VLDL is from FA delivered to the liver by lipolysis and only ~4% from lipogenesis. With feeding, 43% still come from adipose, and 25% are from diet, either from chylomicrons or spillover from the plasma FA pool (Barrows and Parks 2006; Barrows et al. 2005). Thus, FA flux to the liver is a much more important driver of fatty liver and dyslipidemia than insulin-driven de novo lipogenesis (reviewed in Otero et al. 2014). In this regard, it is important that female humans and rodents are protected against FA-mediated insulin resistance in muscle and whole body in response to increased delivery of FA experimentally (Frias et al. 2001; Hevener et al. 2002). Female mice with muscle-specific ERα knockout have insulin resistance and muscle lipid accumulation due to abnormal mitochondrial function (Ribas et al. 2016).

In response to increased FA flux, the liver oxidizes or re-esterifies FA. Some of this FA results in diacylglycerol accumulation, which activates PKCs and causes impaired glucose metabolism (Jornayvaz et al. 2011). The FA that is made into TG is matched with increased VLDL-TG secretion during fasting. Beyond physiologically normal liver fat (~5%) however, VLDL export is maximized, and liver fat accumulation ensues (Fabbrini et al. 2009). Thus, the dyslipidemia and glucose abnormalities of obesity result in part from impaired coordination of the adipose, muscle, and liver with regard to the flux of FA, and estrogen signaling pathways exert regulatory control on several of these key steps as reviewed above.

Mouse-Human Differences in Estrogen-Regulated Liver Lipid Metabolism and Implications for Disease

Studies with hormone treatment after menopause suggest that estrogen treatment reduces plasma glucose and insulin levels. These reduced insulin and glucose levels ultimately were associated with lower incidence of impaired glucose tolerance and type-2 diabetes (Bonds et al. 2006; Espeland et al. 1998; Ferrara et al. 2001; Rossi et al. 2004; Zhang et al. 2002). Thus, estrogen lowers risk of type-2 diabetes, a negative risk factor for coronary heart disease. Despite improvements in a number of risk factors, hormone treatment in postmenopausal women had certain negative effects on risk of cardiovascular disease. Hormone treatment increased plasma TGs in the WHI, HERS, and ELITE trials along with a number of prospective studies (Hodis et al. 2003; Hsia et al. 2006; Hulley et al. 1998; Barrett-Connor et al. 1997; Trial 1995; Wiegratz et al. 1998; Walsh et al. 1991; Schaefer et al. 1983). Several studies have demonstrated that this increase in plasma TGs is due to increased VLDL production with estrogen treatment approaches (Walsh et al. 1991; Schaefer et al. 1983; Glueck et al. 1975). The progestin component of hormone treatment accelerates TG clearance and thus likely does not contribute to hypertriglyceridemia (Kissebah et al. 1973). Additionally, only oral CE increases serum TG, likely because of high first-pass metabolism, which is minimized by transdermal E2. Mechanisms responsible for this increase in VLDL-TG production with oral CE have been hampered

since mouse models do not recapitulate the increase in plasma TGs in response to E2 treatment (Bourassa et al. 1996; Camporez et al. 2013; Marsh et al. 1999; Zhu et al. 2013).

Cholesteryl ester transfer protein (CETP) is a 74 kD glycoprotein that is expressed in the liver and adipose and is secreted into the circulation where it shuttles TG and cholesteryl esters (CE) between lipoproteins. With obesity, TGs are elevated in VLDL. CETP shuttles these TGs into HDL particles, which destabilizes binding of the HDL scaffold protein ApoA1, leading to increased HDL clearance and low HDL cholesterol levels. Although CETP has been mostly studied in association with HDL cholesterol levels, the expression pattern of CETP suggests an important role of CETP the metabolic adaptation to obesity. CETP is highly expressed in the liver and adipose, tissues that mediate glucose and TG metabolism (Jiang et al. 1991). CETP activity varies as much as six- to eightfold in human studies (Tato et al. 1995; de Vries et al. 2005). Cholesterol feeding, insulin, and ovarian hormones, all lead to significant changes in CETP activity (Arii et al. 1997; Marotti et al. 1993; Johansson et al. 2012). Our recent work demonstrated that CETP is required for mice to increase plasma TGs in response to E2 treatment after ovariectomy (Palmisano et al. 2016). CETP is a secreted plasma protein that shuttles TG and cholesteryl ester between plasma lipoproteins. Mice naturally lack CETP. Transgenic expression of CETP in mice results a similar hypertriglyceridemic effect of estrogen in female mice (Palmisano et al. 2016). We demonstrated that CETP expression creates estrogen gain of function for several pathways involved in liver lipid metabolism. For instance, E2 treatment increases in the activity of protein disulfide isomerase (PDI), a protein involved in the lipidation of VLDL only in CETP mice. Additionally, we found that estrogen-mediated increases in VLDL-TG production were eliminated with hepatocyte-specific deletion of the nuclear receptor small heterodimer partner (SHP) but not ERα (Palmisano et al. 2016). Since increased plasma TGs are associated with increased risk of cardiovascular disease, the increase in plasma TGs caused by estrogen may mitigate some of the beneficial aspects of estrogen treatment on cardiovascular disease risk. A better understanding of the CETP-SHP-PDI pathway regulating estrogen-mediated increases in VLDL production may lead to therapeutic strategies that alleviate the hypertriglyceridemic effect of estrogen treatment approaches.

Selective Estrogen Delivery Approaches and Liver Lipid Metabolism

Experimental models have demonstrated therapeutic value of targeting estrogen toward specific tissues or toward specific aspects of estrogen signaling pathways. An estrogen dendrimer conjugate (EDC) comprised of estradiol (E2) molecules linked to a poly(amido)amine dendrimer selectively activates nonnuclear ER and in mice. This EDC compound does not have activity to promote uterus growth and

does not promote breast cancer growth but does blunt liver fat accumulation with obesity, suggesting that the nonnuclear effects of estrogen are critical with regard to regulation of hepatic fat content (Chambliss et al. 2016; Chambliss et al. 2010). Interesting targeting nonnuclear estrogen with EDC did not prevent atherosclerosis in apoE null mice, which estradiol did (Chambliss et al. 2016).

Treatment of female mice after ovariectomy with bazedoxifene (BZA) alone, or in combination with low-dose estradiol or conjugated estrogen, each prevent weight gain with high-fat feeding after ovariectomy (Kim et al. 2014). The BZA also prevents fatty liver accumulation after ovariectomy, but not to as great a degree as conjugated estrogens (Barrera et al. 2014; Kim et al. 2014). Interestingly compared to conjugated estrogens and E2, BZA is a much more potent inducer of several pathways which may mediate estrogen's protective effects with regard to liver fat metabolism, FGF15, and SHP (Kim et al. 2014).

Additionally, targeting estradiol to metabolic tissues by conjugating GLP-1 to estradiol prevents weight gain and glucose metabolism in obese mice. These actions are not mediated by the GLP-1 component alone. Like the EDC compound, GLP-1 conjugated estradiol does not promote growth of the uterus or MCF-7 breast cancer cells (Finan et al. 2012).

Estrogen Regulation of Liver Cholesterol Uptake and Reverse Cholesterol Transport

Estrogen is proposed to protect against atherosclerosis via its role in reverse cholesterol transport. Reverse cholesterol transport (RCT) is the process of cholesterol removal from peripheral tissues and delivered into the feces, either by direct excretion into bile or conversion to bile acids and subsequent secretion into bile (reviewed elsewhere Rosenson et al. 2012). Estrogen's role in the initial steps of the RCT pathway is controversial in humans. Two studies by the same group support that plasma from women has greater cholesterol efflux capacity (Badeau et al. 2009) or similar cholesterol efflux capacity relative to men (Badeau et al. 2013). In premenopausal women, the concentration of estrogen in plasma is not associated with cholesterol efflux capacity (Badeau et al. 2013). In premenopausal women, polycystic ovary syndrome (PCOS), which is a state of low estrogen, is associated with reduced cholesterol efflux capacity (Roe et al. 2014). The estrogen deficiency of menopause, however, increases the cholesterol efflux capacity of HDL relative to premenopausal women, likely because of increased VLDL-TG levels after menopause (El Khoudary et al. 2016). In postmenopausal women, hormone replacement therapy effectively increases cholesterol efflux capacity of HDL (Ulloa et al. 2002). Thus, estrogen has been shown to have some effects on cholesterol efflux capacity, but there is no consistent relationship between estrogen enhancing and impairing this initial step in RCT based on the literature in humans.

Estrogen signaling pathways have a more firmly established role promoting the later aspects of RCT through action in the liver. Liver estrogen signaling through ERα has been shown to regulate hepatic cholesterol uptake and the efflux capacity of HDL from macrophages during the proestrus period when estrogen levels are high (Della Torre et al. 2016). The role of sex and estrogen on later stages in RCT is not well studied in humans, since no available methodologies exist yet to quantify RCT in humans. Our recent work demonstrates that female mice have increased total body RCT compared to males fed a Western diet (Zhu, submitted). We also found that liver deletion of ERα impaired total body RCT in female mice, suggesting that liver ERα is required for females to enhance total body RCT (Zhu, submitted). E2 and PPT treatment of mice both promotes liver secretion of cholesterol into bile and is prevented by concurrent treatment with an ERα antagonist (Wang et al. 2004).

Regulation of Hepatic Cholesterol Biosynthesis by Estrogens

A functional estrogen-responsive element has been identified within the promoter of the HMG-CoA reductase gene, one of the first steps of liver cholesterol synthesis. In peripheral tissues such as breast ductal epithelium and uterine endometrium, E2 promotes HMG-CoA reductase gene promoter activity and transcription to increase cholesterol biosynthesis for cell proliferation (Di Croce et al. 1999). However, E2 treatment reduces free cholesterol content in hepatocytes (Semenkovich and Ostlund 1987). Animal studies show that HMG-CoA reductase protein levels are lower in female or E2-treated male rats than in adult untreated males, which is regulated through nuclear SREBP2 activity (De Marinis et al. 2008). In line with this observation, Pedram et al. reported that the ERα agonist PPT suppresses HMG-CoA reductase expression and hepatic cholesterol content accompanied by decreased expression of *srebf2* (Pedram et al. 2013).

Regulation of Hepatic Cholesterol Uptake by Estrogens

To maintain efficient HDL-mediated cellular cholesterol efflux from foam cells, cholesterol and cholesteryl esters in HDL particles are either removed by the liver through the scavenger receptor class B member I (SR-BI) pathway or transferred via cholesteryl ester transfer protein (CETP) to apoB-containing particles in the blood and subsequently cleared through LDLR or remnant receptor pathways. Estradiol promotes liver secretion of cholesterol into bile, and this is prevented by concurrent treatment with an ERα antagonist (Wang et al. 2004). In ovariectomized rats, estrogen deficiency downregulates expression of a number of enzymes involved in bile acid synthesis (Liao et al. 2015). In mice, natural variation in estrogen cycling is associated with changes in expression of bile acid synthesis gene expression

(Della Torre et al. 2016). Additionally, estrogen enhances synthesis of bile acids, which is also preventable with concurrent treatment with an ERα antagonist (Wang et al. 2006). Furthermore, E2 fails to promote bile secretion in mice lacking ERα (Wang et al. 2004), suggesting that estrogen acts through ERα to promote bile secretion. The mechanism of estrogen-mediated increases in bile acid synthesis gene expression seems to require liver ERα (Della Torre et al. 2016; Yamamoto et al. 2006).

LDLR

Impaired regulation of several steps in RCT can associate with increased CHD risk (Rader and Tall 2012), and hepatic LDLR and SR-BI play critical roles in RCT. The activity of low-density lipoprotein (LDL) receptors in the liver constitutes a major mechanism by which dietary and hormonal agents may regulate plasma cholesterol levels (Rudling et al. 1992). Ethinyl estradiol treatment promotes clearance of β-VLDL and LDL in human subjects with type III hyperlipidemia (Kushwaha et al. 1977). An in vitro study showed that estradiol increases cell surface LDLR activity in human hepatoma cells but not in human fibroblasts (Kushwaha et al. 1977). LDL bound to LDLR is stimulated by ethinyl estradiol in a dose- and time-dependent manner, and this binding is decreased by a pretreatment of LDL in the media (Kushwaha et al. 1977). In rats, pharmaceutical doses of estrogens stimulate hepatic LDLR mRNA and protein levels, which are accompanied by a markedly increased clearance of plasma LDL concomitant with a decrease in plasma cholesterol (Ma et al. 1986; Cooper et al. 1987; Windler et al. 1980; Di Croce et al. 1996).

Although the LDLR promoter does not contain a classical estrogen-responsive element, observations that LDLR mRNA is stimulated by estrogen in vivo and in human hepatoma cells suggest an alternative mechanism of estrogen-regulated expression of this gene. Using human hepatoma cells that transiently express functional ERα and LDLR promoter constructs, Li et al. demonstrated that E2 promotes LDLR promoter activity mediated by the Sp1 binding to the promoter (Li et al. 2001). Consistently, we observed that LDLR protein levels from liver tissue were decreased in hepatic ERα knockout female and male mice (unpublished data). Both tyrosine kinase (TK) and protein kinase C (PKC) signaling pathways are activated by E2 in vivo and in hepatoma cells (Marino et al. 1998; Marino et al. 2001). A LDLR promoter construct was transfected in human hepatoma cells overexpressing ERα, and the promoter activity was analyzed in the absence and presence of TK and PKC inhibitors (Distefano et al. 2002). This study demonstrated that basal transcription of LDLR gene depends on PKC activity, and TK activity is required for the induction of LDLR gene expression by E2 (Distefano et al. 2002).

Indirect effects of estrogens on hepatic LDLR expression have also been reported. Estrogen may promote LDLR through the action of growth hormone, which as supported by the observation that E2 fails to stimulate hepatic LDLR expression and to decrease plasma cholesterol when given to hypophysectomized rats (Steinberg et al. 1967; Rudling et al. 1992). Reduction of free cholesterol content in human hepatoma cells by estradiol treatment has been shown to be an important mechanism for maintaining LDLR activity by lowering intracellular cholesterol (Semenkovich and

Ostlund 1987). LDLR protein levels are increased by E2 in a dose-dependent manner in human hepatic HuH7 cells; however, LDLR mRNA does not increase unless E2 doses are high, suggesting a posttranslational regulation of LDLR by estrogen (Starr et al. 2015). Additionally, estrogen also likely promotes LDLR-mediated cholesterol uptake through modifying PCSK9 activity because E2 treatment fails to increase LDLR in PCSK9 knockout HuH7 cells (Starr et al. 2015).

SR-BI Stimulation of HDL cholesterol uptake in the liver promotes reverse cholesterol transport and reduces atherosclerosis in mice (Arai et al. 1999; Zhang et al. 2005; Rader et al. 2009). Several groups have found that estrogens upregulate mRNA expression of SR-BI, the HDL receptor, and promotes HDL cholesterol uptake in peripheral tissues (Lopez and McLean 2006; Fukata et al. 2014). Therefore, estrogen regulation of HDL uptake by the liver may contribute to the sex difference in cardiovascular risk. Stimulation of this pathway may be a potential therapeutic target for preventing cardiovascular disease.

Estrogens Protect Against Fatty Liver and Hepatic Insulin Resistance in Males

Males also express estrogen receptors in many tissues, and aromatase catalyzes the conversion of androgens to estrogens. In humans, loss of function mutations in ERα or aromatase genes associates with glucose intolerance, hyperglycemia, and hyperinsulinemia (Maffei et al. 2004; Rochira et al. 2007; Smith et al. 1994). Aromatization of testosterone to estradiol is responsible for the increase in libido and prevention of visceral adiposity associated with testosterone treatment in men (Finkelstein et al. 2013). Hepatic steatosis has been reported in ERα-deficient male mice but not in ERβ-deficient male mice (Ohlsson et al. 2000). The aromatase knockout (ArKO) mice exhibit a striking accumulation of lipid droplets in the liver accompanied by increased expression of lipogenic genes such as FASN and SCD-1 (Jones et al. 2000, 2001; Chow et al. 2011). Estradiol treatment or agonist specific to ERα in ArKO male mice suppresses FASN gene expression and reverses liver fat accumulation (Chow et al. 2011; Jones et al. 2000). Antiestrogen treatment of TMX in male mice induces TG accumulation in the liver by activation of fatty acid synthesis (Cole et al. 2010). In line with this, the incorporation of ^{3}H-oleate into TG in hepatocytes was increased after TMX treatment (Cole et al. 2010). In male rats, high-fat diet induced the fatty liver which is improved by E2 treatment and associated with the downregulation of lipogenesis by ACC phosphorylation with E2 (Zhang et al. 2013). In line with the observation for hepatocyte treated with TMX, E2 treatment decreased the incorporation of ^{14}C labeling from the lipid-containing phase when de novo lipogenesis in hepatocytes was determined using a ^{14}C-labeled acetate method (Zhang et al. 2013) The authors also reported similar changes in gene expression in men with liver steatosis (Zhang et al. 2013).

In male mice, deletion of hepatic ERα promotes liver TG and DAG content after high-fat diet feeding, which corresponds with dysregulation of insulin-stimulated ACC phosphorylation and DGAT1/2 protein expression (Zhu et al. 2013; Zhu et al. 2014). Our studies with E2 treatment of mice after OVX demonstrated that estrogen signaling through hepatic ERα helps prevent insulin resistance associated with high-fat diet feeding in males. Thus, augmenting hepatic estrogen signaling through ERα may lessen the impact of obesity on diabetes and cardiovascular risk in both sexes.

Conclusions and Future Directions

Efforts to understand how women are protected from cardiovascular disease relative to men have led to the discovery that estrogens regulate a number of steps in liver lipid metabolism. Treatment of postmenopausal women with various estrogen formulations does not wholly restore the protection from cardiovascular disease seen in premenopausal women. Estrogen therapy and hormone treatment approaches can protect against fatty liver, insulin resistance, and diabetes but do not conclusively protect from cardiovascular disease. The hypertriglyceridemic effect of exogenous oral estrogen therapy may mitigate some of the other cardioprotective benefits of estrogens. A deeper understanding of the mechanisms of estrogen signaling pathways will likely yield specific targets governing estrogen's effect on lipid metabolism. Future directions will likely rely on targeting estrogens to specific tissues or specific aspects of the signaling pathways in order to recapitulate the protective physiology of premenopause therapeutically after menopause. Furthermore, estrogen signaling pathways in the liver are protective against insulin resistance in males; thus the pathways identified in studying sex differences have potential therapeutic significance in both men and women with regard to obesity associated with cardiovascular risk.

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The Role of Skeletal Muscle Estrogen Receptors in Metabolic Homeostasis and Insulin Sensitivity

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Abstract Women in the modern era are challenged with facing menopausal symptoms as well as heightened disease risk associated with increasing adiposity and metabolic dysfunction for up to three decades of life. Treatment strategies to combat metabolic dysfunction and associated pathologies have been hampered by our lack of understanding regarding the biological causes of these clinical conditions and our incomplete understanding regarding the effects of estrogens and the tissue-specific functions and molecular actions of its receptors. In this chapter we provide evidence supporting a critical and protective role for skeletal muscle estrogen receptor α in the maintenance of metabolic homeostasis and insulin sensitivity. Studies identifying the critical ER-regulated pathways essential for disease prevention will lay the important foundation for the rational design of novel therapeutic strategies to improve the health of women while limiting secondary complications that have plagued traditional hormone replacement interventions.

Overview: Menopause, Sex Hormones, and Metabolic Disease

The National Vital Statistics report from the Centers for Disease Control and Prevention indicates that life expectancy has increased for white females from 48 years in 1900 to 80.9 years in 2009, with a noted gender difference of almost 5 years compared to male counterparts. Considering that menopause occurs on average at age 51 (National Institutes of Health, NIA www.nia.nih.gov), women in the modern era are challenged with facing menopausal symptoms as well as heightened disease risk associated with increasing adiposity and metabolic dysfunction for up to three decades of life. Arming women with knowledge about the health consequences of ovarian failure and furthering our understanding of the biological actions of estrogens in nonreproductive tissues have become critical endeavors if we

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wish to improve the health of women around the world. Although many researchers and clinicians have focused on the impact of replacement estrogens to ameliorate clinical symptoms and provide protective health benefit, an incomplete understanding of hormone action, estrogen receptor distribution, and function has contributed to our continued confusion and failure to advance therapeutic strategies to combat chronic disease-associated pathologies.

Regarding the benefits of exogenous hormone replacement therapy (HRT) on diabetes risk after menopause, large randomized clinical trials of postmenopausal estrogen-based HRT compared with placebo, as well as prospective cohort studies, have shown reductions in fasting glucose, insulin, and incidence of new-onset T2D (Bonds et al. 2006; Kanaya et al. 2003; Margolis et al. 2004; Szmuilowicz et al. 2009; Pentti et al. 2009; Mauvais-Jarvis et al. 2017). Meta-analyses indicate a 30% lower relative risk [RR 0.7 (CI, 0.6–0.9)] of new-onset T2DM in postmenopausal women following HRT compared with placebo (Salpeter et al. 2006). The mechanism by which HRT reduces T2D incidence in postmenopausal women is not yet known; however, molecular studies in rodents indicate that this protective effect may be achieved in part as a consequence of estrogen-induced insulin sensitization. Considering that 75–85% of insulin-stimulated glucose disposal is into skeletal muscle and since skeletal muscle typically represents 30–40% of total body mass, we have focused our efforts in understanding the effects of estradiol/ERα action in this tissue.

In this chapter we will present studies related to the biological actions of estrogen receptors in skeletal muscle in controlling glucose homeostasis and insulin sensitivity, as insulin resistance and metabolic dysfunction are identified as major underpinnings involved in the pathobiology of many chronic diseases (not just obesity and type 2 diabetes) that plague our society today. We will present basic research suggesting that the estrogen receptor (ER), specifically the α form of the receptor, is an important target to combat metabolic dysfunction.

Molecular Mechanisms of Estrogen Receptor (ER) Action

Early studies in reproductive tissues investigating the actions of estradiol led to the paradigm of classical nuclear ERs as ligand-activated transcription factors (O'Malley 1971). Although ERs exist in two main forms, α and β, which have multiple splice variants of unknown function, ERs exhibit tissue specificity in expression and function (Nilsson et al. 2001). The classical, or genomic mechanism of ER action, describes a scenario whereby ligand-activated ER dissociates from its chaperone and binds as a dimer either directly to estrogen response elements (ERE) in target gene promoters or indirectly to AP-1 or SP-1 response elements through association with other transcription factors, tethering the activated ER to DNA (Safe and Kim 2008) (Fig. 1). Following DNA binding, ER dimers interact with basal transcription factors leading to activation or repression of target gene expression. Overlap in binding sites for E_2-liganded ERα and ERβ is observed when receptors are expressed individually; however, when both ERs are present, few sites are shared. Each ER restricts the binding site occupancy of the other, with ERα typically dominating (Charn et al. 2010). Moreover, ligand-activated ERs promote transcription in a

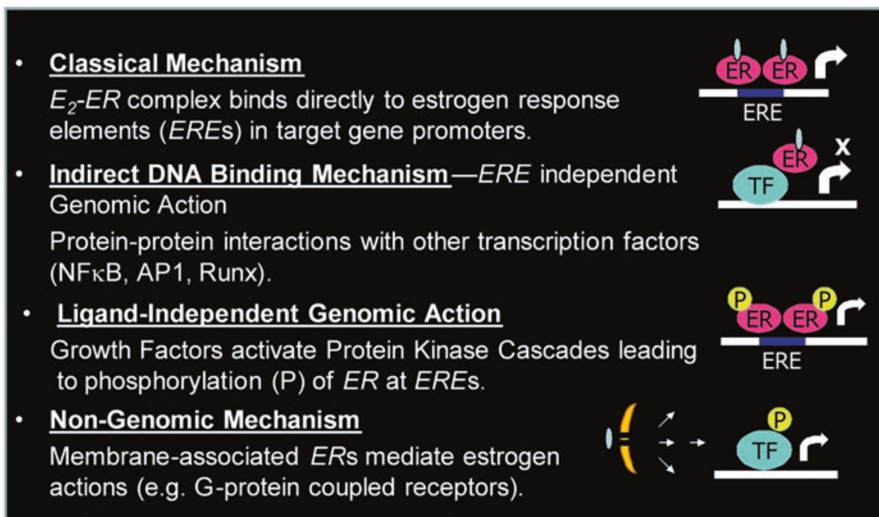

Fig. 1 Molecular actions of ERα to activate or repress target genes by classical, DNA binding, or non-genomic actions. *ERE* estrogen response element in target gene promoters, *P* phosphorylation, *TF* transcription factor

cyclic fashion. The repeated cycling of the receptor complex on- and off-target promoters in the presence of continuous E_2 stimulation may represent a mechanism of continuous sensing and adaptation to the external hormonal milieu to yield the appropriate transcriptional response (Shang et al. 2000).

In addition to classical signaling, E_2-ERα can act within seconds to minutes via extranuclear and membrane-associated forms of the receptor (Hammes and Levin 2007). Membrane-associated receptors are localized to caveolae where they congregate with other signaling molecules, including G proteins, growth factor receptors, tyrosine kinases (Src), linker proteins (MNAR), and orphan G-protein-coupled receptors (GPCRs) (Levin 2015). In a variety of cell types, membrane and extranuclear pools of ERs activate protein kinases that phosphorylate transcription factors to promote their nuclear translocation and transcriptional action (Hammes and Levin 2007; Tiano and Mauvais-Jarvis 2012). The G-protein-coupled estrogen receptor (GPER), or GPR30, has been reported to respond to E_2; however, its role as an ER is still controversial. Although emerging evidence in murine muscle cells shows diverse distribution of GPR30 in the nucleus, mitochondria, and cytoplasm (Ronda and Boland 2016), functional aspects of this receptor in vivo remain unclear; thus, GPR30 will not be discussed in this chapter. Although it is thought that reproductive functions are almost exclusively mediated via classical nuclear ERs acting as ligand-activated transcription factors, a large component of ER actions related to energy metabolism are thought to also involve extranuclear ERs (Liu and Mauvais-Jarvis 2010). More recently an emerging theme in the field is that for many targets, nuclear and nonnuclear signaling must collaborate to achieve the full biological action of estradiol (Pedram et al. 2016). Although non-genomic signaling is

supported for specific cell types under defined conditions, scientific dissection of these pathways remains challenging; thus a central question in the field pertaining to the tissue-specific sites of action and the molecular mechanisms by which ERα selectively activates or represses target genes still persists.

Estrogen Action, Metabolic Function, and Insulin Sensitivity Reduced whole-body ERα expression or impaired ERα function due to genetic alteration (including genetic variants) has been linked with increased prevalence of specific features of the metabolic syndrome including insulin resistance and obesity in both male and female humans and rodents (Smith et al. 1994; Okura et al. 2003a, b, c; Nilsson et al. 2007; Deng et al. 2000; Casazza et al. 2010: Yamada et al. 2002). Since obesity is a prominent phenotype observed in estrogen- or ERα-deficient rodent models, the specific role of ERα in adipocytes and the phenotypic outcomes of obesity as a consequence of adipose-specific ERα deletion in mice are currently under investigation by several laboratories around the world. Whether the obesity phenotype observed in the whole-body Esr1$^{-/-}$ mice or women harboring an *ESR1* polymorphisms is explained by impaired ERα action in adipose tissue specifically or as a result of a secondary phenotype of ERα impairment in other metabolic tissues requires resolution.

Insulin resistance is a central disorder in the pathogenesis of obesity and type 2 diabetes and is a defining feature of the metabolic syndrome, a clustering of metabolic abnormalities including obesity, hypertension, glucose intolerance, and dyslipidemia (DeFronzo et al. 1992; Miranda et al. 2005). Metabolic dysfunction and a clustering of these abnormalities are worrisome as this clinical distinction is now thought to impact nearly a quarter of the US population and drives increased risk of numerous chronic disease states including diabetes, cardiovascular disease, neurodegeneration, and certain forms of cancer (Cornier et al. 2008; Alberti et al. 2009). Normally cycling premenopausal women show enhanced insulin sensitivity compared to men when sensitivity is normalized to lean mass, since women have a reduced lean body mass compared to men. Improved insulin sensitivity and protection against factors promoting insulin resistance are likely underpinnings of reduced type 2 diabetes incidence observed for premenopausal women compared with men (Park et al. 2003; Yki-Jarvinen 1984). Case in point, although a 40–50% reduction in insulin-mediated glucose disposal is consistently observed in males following high-fat feeding (Hevener et al. 2007; Choi et al. 2007), E_2-replete females, humans and rodents, are typically protected against a high-fat diet and acute fatty acid-induced insulin resistance (Hevener et al. 2002; Frias et al. 2001).

In remarkable contrast to the metabolic protection seen in normally cycling premenopausal women, following menopause or OVX, a precipitous decline in insulin sensitivity coincides with a dramatic increase in fat mass and elevated circulating inflammatory markers, LDL, triglycerides, and fatty acids. OVX mice and rats become insulin resistant, show impaired exercise-stimulated glucose disposal into muscle (Campbell and Febbraio 2002), and are more susceptible to the deleterious effects of high-fat diet or lipid oversupply, and these physiological consequences of OVX are prevented by restoration of circulating estradiol or ERα-specific agonist

within a physiological concentration (Stubbins et al. 2011; Hamilton et al. 2016; Gorres et al. 2011).

Although chronic administration of E_2 is shown to improve insulin sensitivity in rodents, the acute action of E_2 to promote insulin-stimulated glucose uptake into muscle remains disputed, this despite consistent observations of E_2-induced activation of Akt and AMP-activated protein kinase (AMPK) (Rogers et al. 2009; Gorres et al. 2011). Furthermore, although administration of intravenous conjugated estrogens and E_2 to postmenopausal women or OVX rats, respectively, elicited a significant increase in glucose disposal during hyperinsulinemic-euglycemic clamp studies (Van Pelt et al. 2003; Alonso et al. 2010), ex vivo treatment of skeletal muscle with E_2 failed to recapitulate the same increase in insulin-stimulated glucose disposal (Rogers et al. 2009). It could be that methodological issues have clouded this relationship since only superphysiological insulin concentrations have been tested thus far, potentially masking the true effects of estradiol on insulin action at physiological doses. This ex vivo observation by Rogers et al. is also in contrast to short-term estradiol effects on insulin action in myotubes from postmenopausal women and age-matched men (Salehzadeh et al. 2011), where the in vivo findings for enhancement of insulin action by E_2 are recapitulated in human primary cells in culture.

Similar to findings for ovarian failure in women and rodents, a reduction in circulating estrogens resulting from rare inactivating mutations of the Cyp19 (aromatase) or experimental deletion in Cyp19 in mice confers an obesity-insulin resistance phenotype (Rochira et al. 2007; Jones et al. 2000; Guercio et al. 2009; Takeda et al. 2003; Maffei et al. 2004, 2007; Jones et al. 2007; Morishima et al. 1995; Smith et al. 1994). The physiological and genetic evidence argues that E_2 and ER favor insulin sensitivity in rodents and humans of both sexes when E_2 is maintained within a tight physiological concentration. Indeed, replacement or augmentation of E_2 to supraphysiological levels or over-stimulation of ERs is thought to induce insulin resistance secondary to hyperinsulinemia and or a reduction in total GLUT4 expression in muscle (Nadal et al. 2009; Barros et al. 2008). In fact, two studies have reported that in postmenopausal women, higher plasma levels of E_2 were prospectively associated with increased risk of developing T2D (Ding et al. 2007; Kalyani et al. 2009). Clearly, additional studies in rodents and humans using a dose-response strategy are necessary to better understand the interplay of steroid hormones including E_2, testosterone, and progesterone on the regulation of metabolism and insulin action in glucoregulatory tissues. Thus, many questions remain: does E_2 enhance skeletal muscle insulin sensitivity and at what minimal pharmacological dose, and what are the critical tissues of E_2 action that confer protection against nutrient-induced insulin resistance?

Estrogen Receptors: Structure and Function

The cellular effects of estrogens are mediated by two ERs: ESR1 (this is the gene that encodes ERα) and ESR2 (or ERβ). ESR1 was identified in 1958 (Jensen et al. 2010), and ESR2 was first identified in the rat prostate and ovary in 1996 (Kuiper

et al. 1996). Different splice variants of each receptor have been identified, and each exhibits distinct tissue expression patterns and functions (Nilsson et al. 2001; Jia et al. 2015). With the exception of the ERαD3 isoform, all the other ERs are composed of six functional domains, from A to F, which contain the NH2-terminal domain (NTD), the DNA-binding domain (DBD), and the COOH-terminal ligand-binding domain (LBD). Two regions, named activation functions (AFs), have been identified as crucial for the transcriptional response of the ERs; the first one is localized at the NTD, while the second one in the LBD (Arnal et al. 2013). *ESR1* and *ESR2* are structurally different in the ligand-binding pockets, and this knowledge was responsible for driving the development of receptor-specific selective ligands (Jia et al. 2015), since ERs act as ligand-mediated transcriptional factors (Jia et al. 2015; Liao 1975; O'Malley 1971; Chen et al. 2004). Although ERs were the first of the nuclear superfamily to be cloned (Green et al. 1986), the tissue-specific gene targets and mechanisms of action, including activation and repression of genes involved in the integrative regulation of metabolic health, are an area of intense investigation.

*ERα/*ESR1 *and Its Role in Regulating Whole-Body Metabolism*

ESR1 is broadly expressed in the central nervous system and in peripheral tissues including adipose, skeletal muscle, liver, and immune cells (Kuiper et al. 1997). Both women and men as well as male and female mice carrying *ESR1* variants develop features of the metabolic syndrome including obesity, glucose intolerance, and insulin resistance, and clustering of these metabolic abnormalities increases disease risk (heart disease, type 2 diabetes, and certain forms of cancer) (Heine et al. 2000; Okura et al. 2003a; Smith et al. 1994; Ribas et al. 2010b). Of translational relevance, total body ERα knockout (KO) mice (ERαKO) recapitulate a similarly remarkable metabolic dysfunction compared to that observed in a male human subject with a rare inactivating receptor mutation or subjects with genetic polymorphisms in the receptor (Heine et al. 2000; Okura et al. 2003a; Smith et al. 1994). Not only do these mice have increased adiposity caused by reductions in energy expenditure, but they also exhibit glucose intolerance and insulin resistance, thus demonstrating the critical role for *ESR1* in regulating energy and metabolic homeostasis (Heine et al. 2000; Bryzgalova et al. 2006; Ribas et al. 2010b). The integration of central and peripheral *ESR1* action as well as the interaction of ERα and sex chromosome action remains to be defined; however, the tissue dissection approach to studying ERα using mice with conditional deletion alleles has allowed the research community the opportunity to delineate unique aspects of ERα biology in a tissue and sex-specific context.

Recently, observational findings indicate that *ESR1* expression levels are reduced in muscle from women with the metabolic syndrome and that natural variation in muscle *ESR1* expression in women is inversely correlated with adiposity and fasting

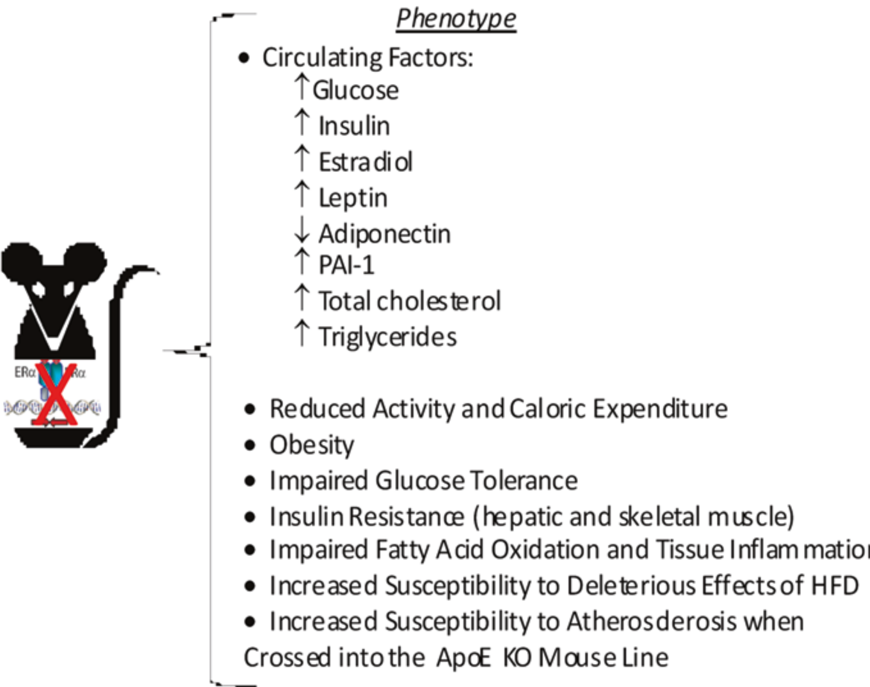

Fig. 2 The impact of whole-body deletion of ERα on metabolic phenotypes and atherosclerosis lesion development (Ribas et al. 2010b; Cooke et al. 2001; Heine et al. 2000; Villablanca et al. 2004; Bryzgalova et al. 2006; Couse et al. 1995)

insulin, markers of metabolic health (i.e., low muscle *ESR1* expression levels are associated with metabolic dysfunction and increased adiposity) (Ribas et al. 2016). Remarkably similar findings were observed across numerous strains of inbred female mice as well as in genetically obese animals. Collectively these data suggest that maintenance of ERα expression or activation of muscle *ESR1* could serve as an effective means to improve metabolic health and combat diseases associated with metabolic dysfunction (Ribas et al. 2016). Although these strong correlative findings suggest a relationship between muscle ERα expression levels and metabolic health, few studies have tested the direct role of muscle ERs on metabolism and insulin action.

To date, several laboratories have characterized the whole-body ERαKO mouse (Fig. 2) (Heine et al. 2000; Couse and Korach 1999; Ohlsson et al. 2000; Ribas et al. 2010b); however, the complex phenotypes of this mouse models have only prompted many additional questions including delineation of the tissues responsible for conferring the insulin resistance-obesity phenotype. Does obesity arise from a loss of ERα within adipocytes specifically or can it be driven as a secondary phenotype resulting from a loss of ERα in brain, skeletal muscle, liver, or even select immune

cells? Furthermore, does a loss of ERα specifically from myocytes drive the skeletal muscle insulin resistance, or does this ERαKO phenotype arise from/exacerbated by increased adiposity and altered adipokine/cytokine secretion?

Although two forms of the receptor are expressed in many of the glucoregulatory tissues, ERα is expressed at much higher abundance than ERβ or GPR30, as ERβ and GPR30 transcript is nearly undetectable in muscle from human and rodents (Salehzadeh et al. 2011; Wiik et al. 2005; Baltgalvis et al. 2010; Ribas et al. 2016). Consistent with these observations, homozygous deletion of ERβ failed to produce insulin resistance (Ohlsson et al. 2000) in contrast to the marked skeletal muscle insulin resistance observed in ERαKO animals (Fig. 2) (Ribas et al. 2010b; Riant et al. 2009). The underlying mechanism contributing to impaired insulin action in muscle of ERαKO animals remains disputed. Findings reported by Bryzgalova et al. (2006) suggest reduced total GLUT4 levels in muscle as an underlying cause for the ERαKO insulin resistance phenotype; however, these findings were not supported by Ribas et al. (2010b). Furthermore, despite maintenance of GLUT4 mRNA and protein, Ribas et al. reported more dramatic skeletal muscle insulin resistance in ERαKO mice than Bryzgalova et al. Hevener and colleagues suggest that the skeletal muscle insulin resistance observed in ERαKO mice is predominantly a consequence of direct ERα deletion effects on insulin action and a secondary impact of inflammation on proximal insulin signaling in muscle. This hypothesis was later confirmed using muscle-conditional deletion alleles studied in vivo, ex vivo, and in vitro.

Indeed, in muscle-specific ERα knockout mice, and myotubes with ERα knockdown, no alteration in GLUT4 mRNA or protein in skeletal muscle was observed despite reduced insulin-stimulated glucose disposal into the muscle during clamp studies. Findings in the muscle-specific ERα mouse are consistent with those of whole-body ERα mice (Ribas et al. 2010a). Furthermore, additional studies by Barros et al. (2006, 2008) assessing GLUT4 expression in response to ovariectomy with/without E_2 supplementation are in conflict with other studies of similar design (Campbell et al. 2003; Alonso et al. 2009; Salehzadeh et al. 2011; Baltgalvis et al. 2010; Hansen et al. 1996). Given the lack of consensus ERE in the GLUT4 promoter (Barros and Gustafsson 2011) and absence of confirmatory findings in cellular reporter and chromatin immunoprecipitation assays, the regulation of GLUT4 expression by ERα requires further investigation. GLUT4 is regulated by several redundant transcriptional pathways (Murgia et al. 2009; Zorzano et al. 2005). Considering that total GLUT 4 transcript and protein are not reduced in humans or rodents in the context of insulin resistance, obesity and type 2 diabetes, or between men and women (Fu et al. 2009; Hoeg et al. 2009), it is likely that in the absence of ERα, other transcription factors compensate to maintain GLUT4 levels (Garvey et al. 1992, 1998; Banks et al. 1992; Brozinick et al. 1993, 1994; Hevener et al. 2000). This is not to say that ERα is not involved in the exercise-stimulated increase in GLUT4 observed following training (Fu et al. 2009; Dela et al. 1994; Rodnick

et al. 1990), given the concomitant increase in ERα expression observed in muscle of exercise-trained humans and mice (Wiik et al. 2005; Lemoine et al. 2002a, b).

Myocyte enhancer factor 2 (MEF2) expression and a functional MEF2 element in the GLUT4 promoter are critical for GLUT4 gene expression (Mora and Pessin 2000). Furthermore, reciprocal regulation between ERα and MEF2 can be observed in cardiomyocytes via ERα interaction with class II HDAC (van Rooij et al. 2010). Despite complex transcriptional signal integration in the regulation of GLUT4 expression (Moreno et al. 2003; Gan et al. 2011; Oshel et al. 2000; Smith et al. 2008; Zorzano et al. 2005; Murgia et al. 2009), it is conceivable that elevated ERα action could promote increased GLUT4 transcription via heightened protein tethering with MEF2 on the GLUT4 promoter or by indirect action via AMPK (Gong et al. 2011; Rogers et al. 2009). It is important to note that transcriptional activity of the GLUT4 promoter is quite low under basal conditions and other ovarian hormones, e.g., progesterone, are shown to play an antagonistic role in the regulation of GLUT4 expression (Campbell and Febbraio 2002). These issues as well as the dose of E_2 administration during interventional studies (presumably off-target effects of superphysiological doses of E_2 are deleterious to metabolism), the age and hormone status of the human subjects and rodents used, are important considerations when interpreting the literature.

Considering the varying roles that muscle and adipose tissue play in controlling whole-body metabolic homeostasis, it is likely that the interplay of transcriptional regulators of GLUT4 vary markedly between tissues. Taken together, these data would suggest a potential role for ERα as an enhancer of GLUT4 transcription in muscle under certain conditions, but not necessarily obligatory in the direct regulation of GLUT4 expression under basal conditions.

Collectively, work by Ribas et al. suggests that the skeletal muscle insulin resistance observed in whole-body ERαKO mice and animals with a muscle-specific deletion of ERα is predominantly the result of impaired insulin signal transduction (Ribas et al. 2010a). A role for ERα in the regulation of proximal insulin signal transduction has been suggested previously as E_2 administration to insulin-resistant rodents increases insulin receptor substrate (IRS)-1 abundance and insulin-stimulated tyrosine phosphorylation as well as phosphorylation of Akt at activation site Ser473 (Ordonez et al. 2008; Riant et al. 2009). Akt serves many functions in myocytes including ERα-induced regulation of myogenic differentiation (Galluzzo et al. 2009), suppression of muscle-atrophy ubiquitin ligases via FOXO1 inhibition (Stitt et al. 2004), and induction of genes associated with myocellular proliferation (Galluzzo et al. 2009; Enns et al. 2008; Enns and Tiidus 2008; Thomas et al. 2010; Kamanga-Sollo et al. 2010). In breast cancer cell lines, endothelial cells and cortical neurons, ERα-specific binding and activation of PI3kinase, as well as suppression of the tumor suppressor and PI3kinase inhibitory protein, PTEN, are well established (Lee et al. 2005; Noh et al. 2011; Simoncini et al. 2000, 2002; Mannella and Brinton 2006); however, studies on this direct interaction are limited in skeletal muscle. Additionally, E_2 acting via ERα is also shown to promote phosphorylation of p38 MAPK (Ronda et al. 2010a, b), and transduction of a signaling cascade is

shown to enhance GLUT4 intrinsic activity and glucose uptake (Niu et al. 2003; Furtado et al. 2002; Sweeney et al. 1999). Furthermore, ERα activation of Akt and MAPK pathways is thought to underlie E_2-mediated protection of muscle against age-induced sarcopenia (Sorensen et al. 2001; Leger et al. 2008; Tiidus 2000; Messier et al. 2011; Chen et al. 2005; Sipila et al. 2001; Teixeira et al. 2003), exercise-induced muscle damage (Dieli-Conwright et al. 2009; Sotiriadou et al. 2006; Tiidus 2000; Thomas et al. 2010), and myocyte apoptosis in the face of a variety of cellular perturbations (Boland et al. 2008; Vasconsuelo et al. 2008; Wang et al. 2010; McLoughlin et al. 2009). Thus, ERα stimulation of muscle growth and insulin sensitivity via these pathways is reasonable to posit.

In contrast to the protective actions of ERα, there is evidence that ERβ may promote insulin resistance in skeletal muscle when the ERβ-ERα is elevated. In OVX mice, while ERα-specific activation by PPT improves muscle insulin action (Gorres et al. 2011), conversely, ligand-specific activation of ERβ by DPN fails to improve muscle insulin action (Gorres et al. 2011). Furthermore, ovariectomy of hyperestrogenic female ER-deficient mice (suppresses E_2 action through the remaining ERβ) improves glucose tolerance and insulin sensitivity (Naaz et al. 2002). Moreover, administration of an ERβ-selective agonist to male E_2-deficient ArKO mice decreases glucose uptake (Barros et al. 2006). Finally, evidence indicates that ERβ-deficiency protects against diet-induced insulin resistance in male mice by increasing PPARγ signaling in adipocytes, which indirectly improves skeletal muscle insulin action by promoting lipid accumulation in adipose tissue and not muscle (Foryst-Ludwig et al. 2008).

ERα and Skeletal Muscle Fatty Acid Metabolism and Inflammation

Normally cycling premenopausal women are protected against acute lipid-induced insulin resistance compared with estrogen-deficient women and men (Hoeg et al. 2011; Frias et al. 2001). Furthermore, muscles from premenopausal women show enhanced insulin sensitivity despite 47% higher triglyceride content compared with age-matched men (Hoeg et al. 2009). This is consistent with a reduced respiratory quotient and greater reliance on fatty acids oxidation as a fuel source in women (Cortright and Koves 2000). These data indicate interesting similarities between E_2 replete women and exercise trained subjects including elevated muscle ERα expression (Wiik et al. 2005; Lemoine et al. 2002a, b), heightened insulin sensitivity (Brozinick et al. 1993), elevated muscle lipid tolerance (Amati et al. 2011), and enhanced oxidative capacity (Maher et al. 2010b; Turcotte et al. 1992). Consistent with the reported effects of E_2 on metabolism, estrogen supplementation is shown to enhance lipid oxidation in vivo in men during acute endurance exercise (Hamadeh et al. 2005) and palmitate oxidation in myotubes from male subjects ex vivo (Salehzadeh et al. 2011). The effect of E_2 to increase the expression of fatty acid transport protein FAT/CD36 and FABP as well as transcription factors and key enzymes that regulate oxidative metabolism (Fu et al. 2009; Campbell et al. 2003; Maher et al. 2010a) likely underlies these observations in male subjects. Moreover, E_2 treatment reduced HFD-induced insulin resistance in skeletal muscle by 50% (assessed by hyperinsulinemic-euglycemic clamp) in an ERα-dependent manner

(Riant et al. 2009). The mechanistic link between the accumulation of lipid intermediates, activation of inflammatory signaling cascades, and impaired insulin action is shown in myocytes and rodent muscle, and indeed these factors are observed concurrently in obese, type 2 diabetic subjects (Wellen and Hotamisligil 2005; Itani et al. 2002; Adams et al. 2004; Yang et al. 2009a), as well as muscle from whole-body and muscle-specific ERαKO mice (Ribas et al. 2010b). Bioactive lipid intermediates including DAG and ceramides are believed to activate stress kinases including IKKβ, c-Jun-N-terminal kinase (JNK), and certain nPKCs (Summers 2006; Holland et al. 2007a, b; Itani et al. 2002). Indeed, muscle from normal chow-fed whole-body ERαKO mice showed heightened inflammatory signaling as reflected by markedly increased JNK phosphorylation and TNFα transcript (Ribas et al. 2010b). In addition to the marked increase in bioactive lipid intermediates found in ERαKO muscle, the production of radical oxygen species and the possible ERα derepression of selective inflammatory targets within the nucleus are likely mediators of heightened muscle inflammation.

In addition, exercise and E_2 are shown to rapidly stimulate AMPK phosphorylation in both muscle and myotubes (D'Eon et al. 2008; Rogers et al. 2009). AMPK is considered a central regulator of many cellular processes including growth, mitochondrial biogenesis, and oxidative metabolism (Mihaylova and Shaw 2011; Hardie 2011). Comparable to the effects of E_2, the ERα-selective agonist PPT stimulates AMPK phosphorylation in muscle of ovariectomized female rats (Gorres et al. 2011), while OVX or whole-body ERα deletion is associated with reduced skeletal muscle levels of phosphorylated AMPK (Kim et al. 2010; Ribas et al. 2010b). Recent evidence from Lipovka et al. shows that ERα but not β directly binds the βγ-subunit domain of AMPK α (Lipovka et al. 2015). Muscle PPARα, PPARδ, and UCP2 expression are also reduced in whole-body ERαKO mice suggesting that E_2 acting via ERα is essential in the regulation of a coordinated program regulating oxidative metabolism. Interestingly, although the phenotype of impaired muscle fatty oxidation was recapitulated in the muscle-specific ERαKO mice (MERKO), no alteration in basal p-AMPK, PPARα, PPARδ, or UCP2 was observed (Ribas et al. 2010a), thus suggesting that these specific alterations in muscle gene expression are secondary to the loss of ERα in other metabolic tissues (e.g., CNS, adipose tissue, or liver).

The Role of Muscle ERα in the Regulation of Mitochondrial Function

Despite some of these model differences in gene and protein expression, skeletal muscle insulin resistance and bioactive lipid accumulation were surprisingly similar between ERαKO and MERKO animals. Triacylglycerol, diacylglycerol, and ceramides were all elevated significantly in muscle from female mice lacking ERα globally or specifically in muscle (Ribas et al. 2016). Consistent with these

observations, oxygen consumption rates in C2C12 myotubes with ERα knockdown were reduced significantly. In addition, mitochondria from muscle cells depleted of ERα produced high levels of reactive oxygen species (ROS) thus promoting cellular oxidative stress. Additional analyses of mitochondrial morphology and function corroborated a defect in respiratory complex 1 activity in MERKO muscle (Ribas et al. 2016).

This defect in mitochondrial function was paralleled by a reduction in expression of the only mammalian mitochondrial (mt) DNA polymerase, *Polg1,* in MERKO muscle as well as murine myotubes with *Esr1* knockdown. Additionally, heavy water labeling of newly synthesized mtDNA showed a reduction in the rate of mtDNA replication, this functionally supporting an impact of the reduction in *Polg1* expression in MERKO mouse muscle (Ribas et al. 2016). Further mechanistic studies showed that estradiol and ERα-selective ligand treatment induced *Polg1* expression in muscle cells; however, ligand was ineffective to induce gene expression when the receptor was absent. Considering the presence of a consensus ERE in the *Polg1* promoter, ongoing studies in the Hevener laboratory will delineate the mechanism(s) by which ERα regulates mtDNA replication via *Polg1*.

mtDNA replication is intimately linked with mitochondrial remodeling by a process known as fission (Lewis et al. 2016). The Hevener laboratory has shown that treatment of murine myotubes with ERα agonists also promotes this mitochondrial morphological alteration inducing the severing of a mitochondrion into two daughter organelles via scission achieved by high-order dynamin-related protein 1 oligomers. Interestingly, although ERα activation promotes mitochondrial fission, it appears to achieve this shift in mitochondrial architecture by a coordinated enzymatic regulation of the mitochondrial fission activator calcineurin and the calcineurin inhibitor Rcan1 (Ribas et al. 2016). Supporting the role of ERα in the regulation of mitochondrial morphology, we observed that mitochondria from both female and male MERKO mouse muscle were enlarged, elongated, and hyperfused thus suggesting a reduction in fission-fusion dynamics. Internally consistent with the morphological data obtained by transmission electron microscopy, analysis of mitochondrial dynamics signaling showed reduced fission signaling by Drp1 (including increased phosphorylation at the inhibitory Ser^{637} site and reduced total Drp1 protein on the outer mitochondrial membrane) as well as increased abundance of the inner and outer mitochondrial membrane fusion proteins OPA1 and Mfn2 (Ribas et al. 2016) (Fig. 3). Of interest, Ribas et al. observed marked increase in expression of the mitochondrial fission inhibitor Rcan1 in myotubes with Esr1-KD, female MERKO muscle, and muscle from women displaying clinical features of the metabolic syndrome. Ribas et al. overexpressed Rcan1 in myotubes using lentivirus and showed that *Rcan1* expression elevated to levels seen in MERKO mouse muscle impaired insulin action (Ribas et al. 2016). Moreover, recent unpublished data from the Hevener laboratory shows that chronic impairment in muscle mitochondrial fission promotes skeletal muscle insulin resistance. Therefore we hypothesize both a reduction in the direct effects of ERα on insulin signaling and indirect effects of

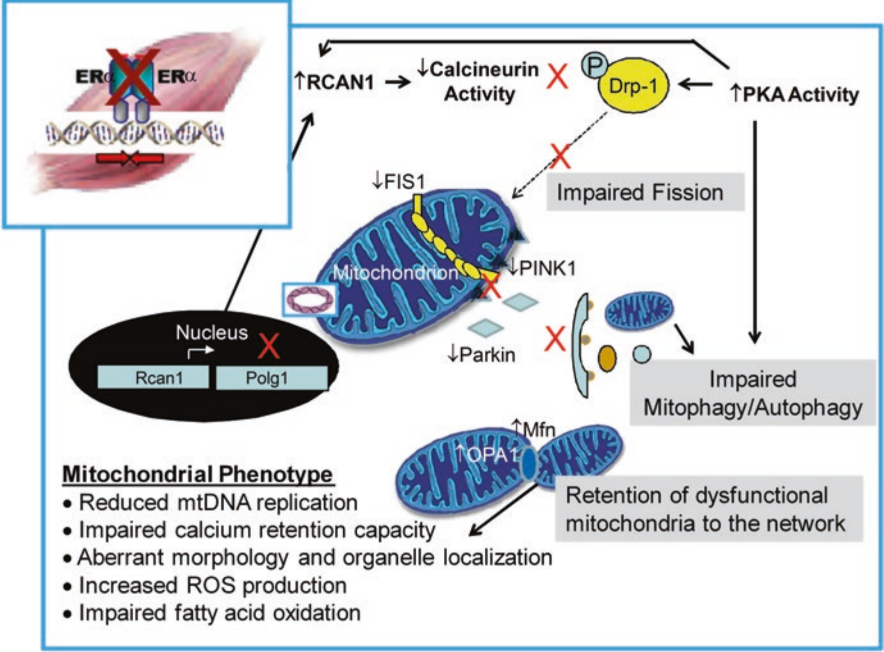

Fig. 3 The impact of ERα deletion on mitochondrial function and dynamics. Skeletal muscle-specific ERα deletion reduced mitochondrial DNA replication and impaired muscle oxidative metabolism, despite maintenance of mtDNA copy number. Increased PKA and reduced calcineurin activity levels promoted elongated, hyperfused mitochondria in MERKO muscle. The morphological changes coupled with an imbalanced PKA-calcineurin axis blunted mitochondrial fission signaling through DRP1 and FIS1 and impaired macroautophagy, both processes critical for mitochondrial turnover, mitophagy. In addition to defects in mitochondrial fission, unopposed fusion of the outer and inner mitochondrial membranes was permitted by increased expression of mitochondrial-specific fusion proteins, Mfn 2 and OPA1. Collectively, the retention of damaged mitochondria to the network was paralleled by increased ROS production, inflammation, and insulin resistance in skeletal muscle from MERKO mice. Collectively, findings implicate a critical role for ERα in the maintenance of muscle mitochondrial and metabolic health (Ribas et al. 2016)

ERα on insulin action, mediated via mitochondrial dysfunction, contribute to the development of global insulin resistance and metabolic dysfunction (Fig. 4).

In light of the observation that Rcan1 was only induced in female MERKO muscle and not in males, despite a similar impairment in fission signaling in both sexes of MERKO mice, the Hevener laboratory has initiated additional studies to flesh out the sex-specific mechanisms that underlie altered mitochondrial dynamics and function in the absence of ERα. These studies are viewed to be of translational importance since it is well known that sex is an important biological variable contributing to differences in disease incidence and mechanisms of pathobiology.

Follow-up studies in the Hevener laboratory in MERKO mice, as well as new studies in muscle-specific *Polg1*, Parkin (*Park2*), and Drp1 (*Dnml1*) KO animals, and mice with muscle-specific ERα overexpression, will allow us to determine if the

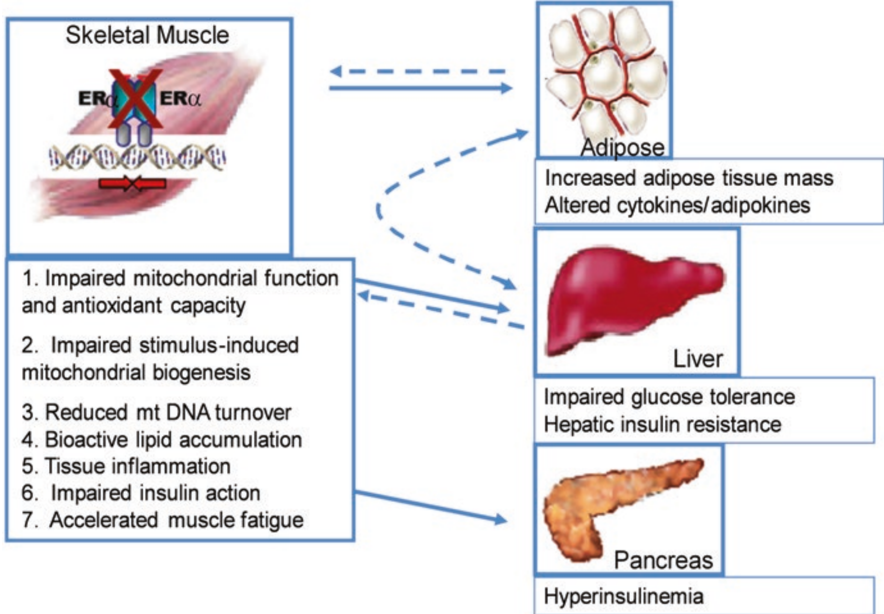

Fig. 4 The impact of skeletal muscle-specific ERα deletion on whole-body insulin sensitivity and adiposity in female mice. Skeletal muscle-specific deletion of ERα promotes muscle insulin resistance and secondary phenotypes of glucose intolerance, hepatic insulin resistance, and increased adiposity (Ribas et al. 2016)

stall in mtDNA replication observed in muscle from MERKO mice is a primary defect driving altered muscle mitochondrial metabolism and defective insulin action. Secondly, we will learn whether the impairment in mitochondrial quality control and turnover seen in MERKO muscle is a consequence or causal of the stall in DNA replication and contributory or resultant of insulin resistance. Since most of the ERα target genes identified to date have been determined in cancer cells in culture, it will be important to identify cell-specific ERα target genes as tissue dissection studies of ERα progress. The use of broad transcriptomic, proteomic, and metabolomic approaches coupled with more targeted chromatin immunoprecipitation analyses in rodents harboring conditional ERα deletion alleles will allow for the identification of novel ERα target genes as well as reveal new mechanism(s) controlling metabolic function and insulin action signaling nodes specifically in the muscle. In our view, these mechanistic interrogations will lead to the development of novel ERα ligands that can be harnessed for therapeutic exploitation to combat metabolic disease without generated off-target effects that have plagued traditional hormone replacement strategies.

Collectively findings in the muscle-specific ERαKO, MERKO, mouse model support the notion that ERα is critical for maintaining fatty acid oxidation in skeletal muscle by mechanisms including the regulation of (1) cellular fatty acid transport, 2) activation of intermediary signaling critical for shifting substrate metabolism,

(3) transcriptional regulators of fatty acid metabolism, (4) and mitochondrial DNA replication, function, and quality control. Thus, ERα expression in skeletal muscle may be a central regulator of adiposity by indirect action as MERKO mice fully recapitulated the obesity phenotype observed in the whole-body ERαKO.

Moreover, E_2 treatment reduced HFD-induced insulin resistance in skeletal muscle by 50% (assessed by hyperinsulinemic-euglycemic clamp) in an ERα-dependent manner (Riant et al. 2009). The mechanistic link between the accumulation of lipid intermediates, activation of inflammatory signaling cascades, and impaired insulin action is shown in myocytes and rodent muscle, and indeed these factors are observed concurrently in obese, type 2 diabetic subjects (Wellen and Hotamisligil 2005; Itani et al. 2002; Adams et al. 2004; Yang et al. 2009a), as well as muscle from whole-body and muscle-specific ERαKO mice (Ribas et al. 2010b). Bioactive lipid intermediates including DAG and ceramides are believed to activate stress kinases including IKKβ, c-Jun-N-terminal kinase (JNK), and certain nPKCs (Summers 2006; Holland et al. 2007a, b; Itani et al. 2002). Indeed, muscle from normal chow-fed whole-body ERαKO mice showed heightened inflammatory signaling as reflected by markedly increased JNK phosphorylation and TNFα transcript (Ribas et al. 2010b). In addition to the marked increase in bioactive lipid intermediates found in ERαKO muscle, the production of radical oxygen species and the possible ERα derepression of selective inflammatory targets within the nucleus are likely mediators of heightened muscle inflammation.

Markers of inflammation and oxidative stress are elevated in rodent models of obesity and in patients with type 2 diabetes (Hotamisligil 2008; Donath and Shoelson 2011). Myotubes and skeletal muscle with ERα deletion showed a marked reduction in Gpx3 expression, a primary antioxidant enzyme that scavenges hydrogen peroxide and diminishes oxidative stress (Baltgalvis et al. 2010; Ribas et al. 2010b). In contrast, E_2 replacement in OVX animals led to a marked increase in Gpx3 expression in skeletal muscle (Baltgalvis et al. 2010). Considering that Gpx3 expression levels in skeletal muscle are elevated in females compared to males (Borras et al. 2003), are reduced in T2DM patients (Chung et al. 2009), and are associated with insulin resistance and metabolic dysfunction (Chung et al. 2009), and the gene is now identified as a causal candidate for obesity (Yang et al. 2009b), additional work studying the direct role of estrogen action in the regulation of antioxidant enzymes including Gpx3 appears warranted. Although reductions in mitochondrial number and function have been implicated in the pathobiology of insulin resistance (Patti et al. 2003; Befroy et al. 2007; Morino et al. 2005; Petersen et al. 2004), and gender dimorphisms in mitochondrial biology have been described (Gomez-Perez et al. 2008), whether E_2/ERα preserves insulin action by maintenance of mitochondrial integrity including control of oxidant production remains unknown. Emerging unpublished findings from the Hevener laboratory indicate that skeletal muscle ERα is critical for the maintenance of mitochondrial function and quality control including the turnover of damaged organelles by mitophagy. The mechanism(s) underlying these findings remain incompletely understood.

Conclusions and Perspectives

In recent years, novel molecular targets have emerged offering the prospect of pharmacological intervention to restore metabolic homeostasis and insulin action, as well as ameliorate complications associated with diabetes and obesity. The inherent beauty of using estrogens or ER agonists as therapeutic agents is underscored by decades of research and in-depth knowledge related to biological/clinical efficacy and toxicity profiles obtained by in vivo studies in preclinical models and humans. Estrogens are shown to promote energy homeostasis, improve body fat distribution, and diminish insulin resistance, β-cell dysfunction and inflammation. The challenge with estrogens, however, is their relatively narrow therapeutic index when used chronically. Thus, the translation of the basic advances in diabetes and obesity treatment described in this review, although successful in rodents, is problematic when extending to clinical practice. However, 10 years after the WHI concluded that the risks of hormone therapy outweighed its benefits, reevaluation of the WHI findings and determination that the risks of breast cancer, coronary heart disease, stroke, and pulmonary embolism with estrogen-progestin treatment were overstated prompted a position statement by the North American Menopause Society stating that HRT has a role in short-term treatment of menopausal symptoms (*The 2012 hormone therapy position statement of: The North American Menopause Society* 2012). Thus, considering the new and more positive light estradiol is receiving by clinical experts in the field, it will be important to determine whether short-term treatment with HRT during early menopause offers protection against metabolic dysfunction and insulin resistance.

Additionally, it is imperative that we determine how to modulate the specific ER-mediated mechanisms and pathways involved in energy balance and glucose homeostasis and develop estrogen mimetics that initiate specific cellular events that produce metabolic benefit without unwanted side effects. This could be achieved possibly by fusion peptides (Clemmensen et al. 2016; Vogel et al. 2016) or through novel SERMs that retain the beneficial metabolic effects of E_2 in desired tissues including skeletal muscle, while exerting antagonist action in breast and uterus. With regard to whole-body metabolism, obesity, and insulin sensitivity, future studies should focus on identifying the critical nodes of ERα-mediated metabolic crosstalk between all glucoregulatory tissues as these integrative networks may reveal new pharmacological targets for therapeutic exploitation. Lastly, a major limitation in our understanding and interpretation of E_2-ER action is the lack of information regarding the contribution of extranuclear vs. nuclear ER actions, as well as ligand vs. non-ligand-mediated functions of estrogen receptors in controlling key regulatory metabolic nodes in insulin-responsive tissues. Delineation of these pathways will be critical in moving the field forward and advancing therapeutic strategies to improve metabolic function and overall women's health.

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Estrogens and Body Weight Regulation in Men

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Abstract Our understanding of the metabolic roles of sex steroids in men has evolved substantially over recent decades. Whereas testosterone once was believed to contribute to metabolic risk in men, the importance of adequate androgen exposure for the maintenance of metabolic health has been demonstrated unequivocally. A growing body of evidence now also supports a critical role for estrogens in metabolic regulation in men. Recent data from clinical intervention studies indicate that estradiol may be a stronger determinant of adiposity than testosterone in men, and even short-term estradiol deprivation contributes to fat mass accrual. The following chapter will outline findings to date regarding the mechanisms, whereby estrogens contribute to the regulation of body weight and adiposity in men. It will present emergent clinical data as well as preclinical findings that reveal mechanistic insights into estrogen-mediated regulation of body composition. Findings in both males and females will be reviewed, to draw comparisons and to highlight knowledge gaps regarding estrogen action specifically in males. Finally, the clinical relevance of estrogen exposure in men will be discussed, particularly in the context of a rising global prevalence of obesity and expanding clinical use of sex steroid-based therapies in men.

Introduction

Obesity has become a global epidemic. Overweight and obesity have an estimated global prevalence of over 2 billion people, surpassing that of undernutrition for the first time in history (Popkin et al. 2012). Although women are at higher risk for obesity than men, men are more likely to experience obesity-related disorders including insulin resistance, type 2 diabetes mellitus (T2DM), nonalcoholic fatty liver disease, and cardiovascular disease (Kautzky-Willer and Handisurya 2009). Thus, identifying risk factors for the development of obesity and its associated

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complications specifically in men has been a growing area of interest. Over the past two decades, sex steroid deprivation in men has garnered increased attention as a key risk factor for metabolic disease. Thus, men with physiologic hypogonadism or those undergoing androgen deprivation treatment (ADT) for prostate cancer are at higher risk for the incident development of obesity and associated metabolic disorders including insulin resistance, nonalcoholic fatty liver disease, and T2DM (Ding et al. 2006; Hamilton et al. 2011; Keating et al. 2012). A contributory, causal role for testosterone deprivation in these metabolic disorders is supported by clinical data demonstrating that exogenous testosterone therapy reverses the increased adiposity evident in hypogonadal men and may improve insulin sensitivity in hypogonadal men with T2DM (Bhasin et al. 2003, 2010; Isidori et al. 2005; Jones et al. 2011).

Importantly, both clinical and preclinical data indicate that the beneficial metabolic effects of testosterone in men are not solely androgen mediated. Testosterone can undergo conversion to 17β-estradiol by the enzyme aromatase, and estradiol deficiency now appears a key facet of the metabolic risk conferred by either physiologic or iatrogenic hypogonadism. This recognition of the metabolic importance of estrogens in men was initially generated when rare syndromes of congenital estrogen deficiency in men were first reported about two decades ago (Jones et al. 2006). These clinical observations were followed by a succession of animal models demonstrating the critical, protective roles of estrogens in regulating body weight, adiposity, and glucose homeostasis in male as well as female mice. Notably, male mice with genetic deletion of estrogen receptor-α (ERα) or aromatase exhibit a more pronounced phenotype of obesity and metabolic dysregulation than do male mice with disruption of androgen receptor (AR) signaling (Fan et al. 2005; Heine et al. 2000; Jones et al. 2000). These preclinical findings more recently have been corroborated by clinical intervention studies in men that have suggested a stronger effect of estradiol than testosterone in suppressing fat mass accrual in men. Greater understanding of the mechanisms by which estrogens regulate body weight and body composition in men is essential. These mechanistic insights will be critical for (1) formulating optimal strategies for sex steroid replacement in hypogonadal men, (2) avoiding potential harm of sex steroid-based interventions in clinical practice, and (3) developing novel interventions for the prevention and treatment of obesity in men.

Estrogen Production and Metabolism

In men, ~15% of circulating estrogens derive directly from testicular production with the remainder generated from androgens through peripheral activity of the enzyme aromatase (Hemsell et al. 1974). The predominant form of circulating estrogen in men is 17β-estradiol, which is generated by aromatization of testosterone. Estrone is also found in circulation and formed from the aromatization of androstenedione. Estrogens can mediate effects through multiple pathways, with

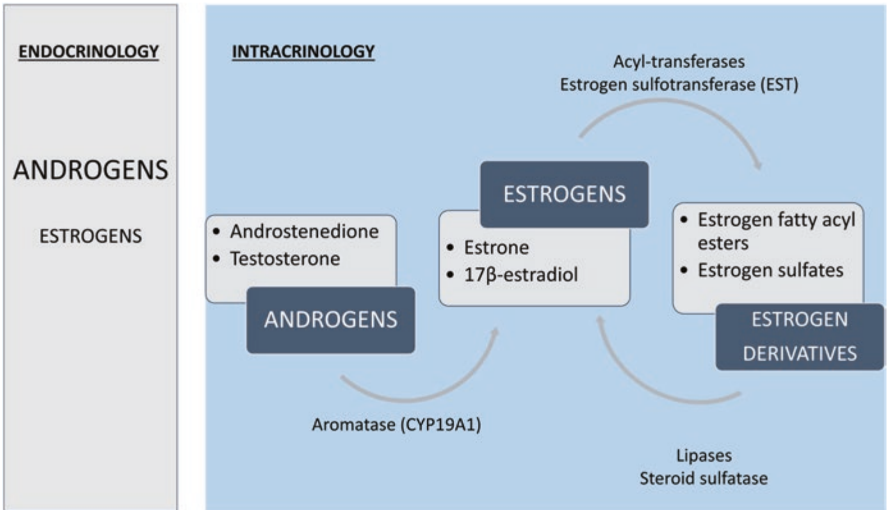

Fig. 1 Extensive regulation of estrogen production and metabolism within peripheral tissues is enabled by local expression of aromatase, which converts androgens to estrogens. Estrogens further can be converted to estrogen sulfates and estrogen fatty acyl esters through the activity of estrogen sulfotransferase and acyltransferases, respectively. Finally, these estrogen derivatives can be converted back to parent estrogens by steroid sulfatase and lipase activity

both genomic and non-genomic effects conferred through binding of its canonical receptors ERα and ERβ. Estrogens also can signal through a membrane-bound G-protein-coupled receptor (GPER). Further, ERα and ERβ can mediate both genomic and non-genomic ligand-independent effects (Foryst-Ludwig and Kintscher 2010). Of note, all three receptors have been implicated in the regulation of body weight and adiposity in both males and females (Cooke et al. 2001; Davis et al. 2014; Heine et al. 2000). Adipose tissue in particular is rich in estrogens and other sex steroids, with markedly higher concentrations of both estrogens and androgens than are found in serum (Deslypere et al. 1985).

Importantly, circulating estrogen levels are not the sole determinant of tissue estrogen levels, highlighting the distinction between endocrinology (hormones released into circulation) and intracrinology, the cell-specific regulation of sex steroid metabolism (Fig. 1). Indeed, several clinical studies have demonstrated dissociations between circulating and intra-adipose estrogen levels, including studies with male subjects (Blankenstein et al. 1992; Bélanger et al. 2006; Deslypere et al. 1985; Wang et al. 2013). Aromatase is expressed broadly throughout central and peripheral tissues including brain and adipose tissue and skeletal muscle; therefore, through local aromatase activity in key metabolic tissues, estrogen production is regulated in tissue-specific fashion (Matsumine et al. 1986; Simpson 2004). Estrogen metabolism also is locally regulated, predominantly through the enzyme estrogen sulfotransferase (EST), which inactivates 17β-estradiol through sulfoconjugation.

Estrogens alternatively can undergo conversion to fatty acyl esters mediated by acyl-transferases. Estrogen fatty acyl esters are found in both serum and peripheral tissues, as are estrogen sulfates. Further, estrogen fatty acyl esters and estrogen sulfates can undergo conversion to their bioactive counterparts through lipase and steroid sulfatase activity, respectively. Adipose tissue is particularly enriched in estrogen fatty acyl esters and consequently has an extensive buffering system that enables local regulation of estrogen production and metabolism. Notably, in a study of obese men, 17β-estradiol fatty acyl ester concentrations did correlate in serum and fat (Wang et al. 2013), possibly indicating that serum estrogen levels influence stored estrogen content in adipose tissue, but conversion to bioactive forms is locally regulated. These findings highlight the limitations of circulating levels as an index of tissue-specific estrogen regulation and underscore the need to carefully delineate tissue-specific pathways of estrogen metabolism and signaling in order to fully define estrogen-mediated mechanisms of body weight regulation in men.

Genetic Mutations in Men and Mice

In the 1990s, an initial series of cases were published detailing men with rare genetic mutations in the genes encoding aromatase or ERα that led to partial or complete loss of estrogen signaling (Carani et al. 1997; Morishima et al. 1995; Simpson 1998; Smith et al. 1994). Although initially reported as predominantly a skeletal phenotype, congenital estrogen deficiency soon was identified as a syndrome of metabolic perturbations characterized by increased central adiposity, insulin resistance, and nonalcoholic fatty liver disease (Maffei et al. 2007). Further, in the case of aromatase deficiency, these metabolic disturbances could be reversed with exogenous estradiol but not testosterone treatment (Maffei et al. 2007).

The development of genetically modified mice used to model estrogen deficiency provided further, compelling evidence of the metabolic importance of estrogen signaling in males. These preclinical models also provided new insights into the mechanisms whereby estrogens contribute to energy balance and body weight regulation in male as well as female mice. Thus, a striking metabolic phenotype first was observed for both male and female mice with global ERα deficiency. ERα-deficient male mice exhibited increased adiposity that became more pronounced with age, such that older mice had more than double the white adipose tissue mass of wild-type controls without differences in brown fat mass (Cooke et al. 2001; Heine et al. 2000; Ohlsson et al. 2000). This phenotype was ascribed to reduced energy expenditure in ERα-deficient mice rather than differential food intake (Heine et al. 2000). Male mice with adipocyte-specific ERα deficiency similarly exhibited significant increases in white adipose tissue mass, suggesting that loss of ERα signaling specifically within adipose tissue leads to changes in energy metabolism that favor increased adiposity.

In contrast to models of ERα deficiency, male mice with global ERβ deficiency exhibited comparable body weight and insulin sensitivity to wild-type mice on a

chow diet (Ohlsson et al. 2000). When female ERβ-deficient mice were exposed to a high-fat diet, however, they exhibited greater adiposity but less insulin resistance than wild-type controls, a phenotype that was ascribed to enhanced PPARϒ activation (Foryst-Ludwig et al. 2008). To date, a parallel interaction between high-fat feeding and ERβ deficiency has not been established for male mice.

Similar to male mice with abrogated ERα signaling, male mice with aromatase deficiency were shown to exhibit increased adiposity (Jones et al. 2001). In these mice, increased fat mass was attributable to reductions in spontaneous physical activity and glucose oxidation and found in association with insulin resistance and hepatic steatosis (Jones et al. 2000; Takeda et al. 2003). Liver from aromatase-deficient male mice showed increased expression of genes involved in fatty acid synthesis, and steatosis was reversed with administration of either 17β-estradiol or an ERα but not ERβ agonist (Cooke et al. 2001). In a second model of male mice with aromatase deficiency, hyperglycemia was attributed specifically to hepatic insulin resistance and increased gluconeogenesis (Van Sinderen et al. 2014). This liver phenotype again was reversed with exogenous estradiol. In female aromatase-deficient mice, adipose tissue exhibited increased expression of lipoprotein lipase (Lpl), consistent with increased fatty acid uptake, though this finding has not yet been corroborated in male mice (Misso et al. 2003).

More recently, potential metabolic roles of the membrane estrogen receptor GPER (GPR30) have been supported through genetic models. Male mice with global GPER deficiency showed greater body weight and fat mass accrual than wild-type controls. Increased adiposity was found in association with greater adipocyte size and ascribed to reduced energy expenditure rather than differential food intake (Davis et al. 2014). Notably, obesity due to GPER deficiency evolved earlier in male than female mice (Davis et al. 2014).

Central Mechanisms of Estrogen-Mediated Body Weight Regulation

Estrogens could regulate body weight in men through numerous mechanisms in both central and peripheral tissues (Fig. 2). Extensive work has demonstrated that central estradiol signaling plays key roles in the regulation of appetite, energy expenditure, and body weight (Brown and Clegg 2010; Mauvais-Jarvis et al. 2013). Aromatase is broadly expressed in the brain, but expression is particularly enriched in the hypothalamus, the principal regulatory site of appetite and energy expenditure as well as reproductive behavior (Abdelgadir et al. 1994; Roselli et al. 2009). The hypothalamus also has abundant ER expression, particularly in the arcuate, paraventricular, and ventromedial nuclei, with ERα expression generally higher than ERβ (Brown and Clegg 2010; Merchenthaler et al. 2004; Simerly et al. 1990). The central nervous system further is a site of de novo estrogen generation, as

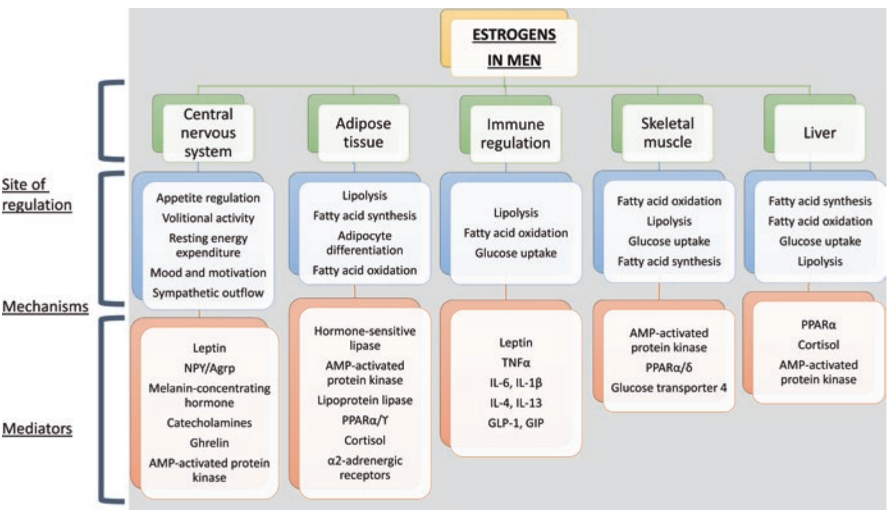

Fig. 2 Estrogens influence body weight and body composition in men through myriad pathways in key metabolic tissues

astrocytes and neurons have all the requisite enzymes to synthesize estradiol from cholesterol (Gillies and McArthur 2010).

Estradiol is well recognized as a critical regulator of both food intake and energy expenditure in females, with extensive research demonstrating its signaling effects particularly in the arcuate and ventromedial nuclei of the hypothalamus (Mauvais-Jarvis et al. 2013). Although most work to date has focused on the central mechanisms whereby estradiol regulates body weight in females, considerable evidence supports a parallel role for estradiol in males, as well. Aromatase expression in most brain regions is comparable in men and women (Stoffel-Wagner et al. 1999), and hypothalamic ER expression is comparable in male and female rodents, including within both the arcuate and paraventricular nuclei (Brown et al. 1992; Chakraborty et al. 2008).

Preclinical findings in males suggest an interaction between central estradiol and leptin signaling, as has been well established in females. The anorexigenic effects of leptin are more pronounced in females than in males, a phenomenon that has been ascribed to estradiol-mediated sensitization to leptin signaling. Supporting this idea, intact male rats did not exhibit changes in food intake after central leptin administration. However, a significant reduction in food intake after leptin administration was evident in orchiectomized male rats treated with subcutaneous estradiol (Clegg et al. 2006). Although increased leptin sensitivity was seen in males only with pharmacological estradiol treatment, these observations do not exclude a leptin-sensitizing effect of estradiol at physiological levels in males; thus, additional

work is needed to determine whether diminished leptin sensitivity is found in males with selective estradiol deprivation. Further supporting a dynamic interaction between estradiol and leptin in males, both males and females exhibited ERα upregulation in the arcuate nucleus of the hypothalamus in the setting of leptin deficiency (Chakraborty et al. 2008). Importantly, leptin is a critical regulator not only of energy intake but also energy expenditure. Thus, leptin also increases energy expenditure, in part through enhanced activity of the sympathetic nervous system (Morton and Schwartz 2011).

However, the anorexigenic effects of estradiol in males clearly are not mediated solely through leptin, as genetically leptin-deficient obese male mice exhibited diminished food intake and body weight loss after estradiol treatment (Gao et al. 2007). This phenomenon was ascribed to estradiol-mediated stimulation of POMC neurons in the arcuate nucleus through a leptin-independent, ERα-STAT3 pathway. The anorexigenic effects of supraphysiologic estradiol administration in males have been ascribed to other pathways, as well. Thus, in male rats, reductions in food intake after estradiol treatment were thought to be due in part to inhibition of the orexigenic neuropeptide melanin-concentrating hormone (MCH) (Mystkowski et al. 2000). Estradiol-mediated regulation of MCH may reflect an indirect interaction; although ERα did not co-localize with MCH neurons in male rats, both neuronal populations were found in abundance within the lateral hypothalamic area (Muschamp and Hull 2007). Nonetheless, estradiol-induced hypophagia has been observed in both MCH- and leptin-deficient male mice, and the hypophagic effect became more pronounced when mice were exposed to a high-fat diet (Tritos et al. 2004). Additional postulated effects of estradiol-mediated hypophagia in males include regulation of cannabinoid and ghrelin signaling. Thus, estradiol has been shown to downregulate cannabinoid receptor expression in the hypothalamus and attenuate cannabinoid-induced hyperphagia (Kellert et al. 2009; Riebe et al. 2010). Further, 17β-estradiol was found to inhibit the hyperphagic effect of central administration of the orexigenic hormone ghrelin in male rats (Clegg et al. 2007).

Notably, animal data do not uniformly demonstrate that estradiol-mediated effects on appetite regulation are similar in males and females with comparable degrees of estradiol exposure. A clear sexual dimorphism in hypothalamic estradiol signaling was illustrated by an experimental model that examined in parallel female sheep and castrated male sheep administered exogenous estradiol. Females and estradiol-supplemented males showed pronounced differences in the expression of hypothalamic genes implicated in appetite regulation in response to both light cycle and food restriction (Archer et al. 2004). A sexual dimorphism in the role of hypothalamic ERα signaling also has been demonstrated in mice, as ERα silencing in the ventromedial nucleus of the hypothalamus led to body weight gain in female but not male mice (Frank et al. 2014). Similarly, knockdown of ERα in POMC neurons in the arcuate nucleus of the hypothalamus promoted increased food intake and body weight gain exclusively in female mice (Xu et al. 2011).

In addition to the direct regulation of appetite through hypothalamic signaling, estradiol also could mediate changes in energy balance through indirect effects on

mood and motivation in men. Thus, these affective effects could produce changes in appetite and volitional activity that influence overall energy balance and, consequently, body weight. In male rats subject to orchiectomy, testosterone replacement conferred changes in behavior indicative of antianxiety and antidepressant effects, but these behavioral changes were abrogated with concurrent administration of an aromatase inhibitor (Carrier et al. 2015). Lower serum 17β-estradiol levels correlated with more depressive symptoms in a cohort of older men (Castanho et al. 2014), as well as in a population of men with obesity (Monteagudo et al. 2016).

Interestingly, in male mice fed a diet high in phytoestrogens, decreased adiposity was found despite increased food intake (Cederroth et al. 2007). This phenotype was ascribed in part to increased voluntary activity and resting energy expenditure consequent to changes in hypothalamic neuropeptides. Hypothalamic gene expression was notable for increased mRNA expression of the orexigenic orexin A and MCH but reduced mRNA expression of agouti-related peptide (Agrp) (Cederroth et al. 2007). Whereas increases in orexin A and MCH expression were thought to underlie the increased food intake in these animals, increased basal metabolic rate, lipid oxidation, and volitional activity were ascribed to reduced Agrp expression. Of note, in immortalized hypothalamic neurons treated with 17β-estradiol, ERα-mediated signaling reduced whereas ERβ-mediated signaling augmented Agrp expression (Titolo et al. 2006). An ERα-dependent increase in voluntary activity manifest as wheel running also was seen in male mice subject to orchiectomy followed by estradiol treatment (Ogawa et al. 2003).

Thus, findings to date support a role for central estradiol signaling in the regulation of appetite and energy expenditure in males. However, most findings are predicated on models that expose males to supraphysiologic doses of exogenous estradiol. These models underscore the importance of not viewing males simply as estrogen-deficient females and the corresponding need for continued investigation of physiologic estrogen signaling specifically in males. Indeed, some experimental models argue against a potent role for estradiol in appetite regulation at physiologic levels in males. Thus, additional work is necessary to better discriminate between concentration-dependent effects and true sexual dimorphisms in centrally mediated effects of estradiol in body weight regulation.

Adipose-Specific Mechanisms of Estrogen-Mediated Body Weight Regulation

Among peripheral metabolic tissues, adipose tissue is particularly enriched in both aromatase and ER expression. Adipose tissue aromatase is found predominantly in preadipocytes and is part of the pro-adipogenic program coordinated by glucocorticoid signaling (Simpson 2004). Similar to the CNS, adipose tissue generally exhibits higher expression of ERα than ERβ, and expression levels are comparable in men and women (Pedersen et al. 2001). ERα has been identified in both mature

adipocytes and preadipocytes, whereas ERβ was found exclusively in mature adipocytes (Dieudonné et al. 2004). The regulation of ER in adipose tissue is partially dependent on estradiol and exhibits both cell type specificity and sexual dimorphisms. Whereas 17β-estradiol upregulated expression of both ERα and ERβ in adipocytes harvested from women, it selectively upregulated ERα expression in adipocytes from men (Dieudonné et al. 2004). Further, ERα expression in preadipocytes was not estradiol-responsive in cells from either sex.

Preclinical and clinical data collectively support an overall anti-obesogenic role for estradiol action in adipose tissue. Fat mass accrual occurs through adipocyte hypertrophy as well as adipocyte hyperplasia, the generation of new adipocytes through preadipocyte differentiation. Adipocyte hypertrophy results from progressive accumulation of lipid within the cell, either through lipogenesis or uptake of extracellular lipid. One described mechanism by which estradiol can suppress adiposity is inhibition of Lpl, which hydrolyzes triglycerides and thereby releases free fatty acids for cellular uptake. Estradiol has been shown to inhibit Lpl activity in women and suppress Lpl expression in cultured 3T3-L1 cells, an immortalized cell line that can be induced to differentiate into cells that phenotypically resemble adipocytes (Homma et al. 2000; Price et al. 1998). Increased Lpl-mediated fatty acid uptake also has been proposed as a primary mechanism underlying the increased adiposity seen in aromatase-deficient mice though this was proposed on findings limited to female mice (Misso et al. 2003). Illustrating the dose dependency of estrogen-mediated effects, high-dose estradiol treatment inhibited Lpl protein expression in adipocytes isolated from women, whereas the lowest treatment dose enhanced Lpl expression (Palin et al. 2003). Again, however, parallel dose-dependent responses in adipocytes harvested from men have not been determined.

Although orchiectomy has been used extensively in rodent studies to investigate the metabolic effects of gonadal steroids in males, few studies to date have carefully discriminated between androgen- and estrogen-mediated outcomes. In a recent study, male mice were subject to orchiectomy with testosterone replacement with or without an aromatase inhibitor. Orchiectomy led to increases in adiposity in conjunction with increased adipose tissue expression of Lpl and the lipogenic genes fatty acid synthase and sterol regulatory element-binding protein-1 (SREBP-1). These changes in both fat mass and gene expression were fully reversed by testosterone replacement (Holland et al. 2016). Adipose tissue expression of Lpl and fatty acid synthase appeared at least partially suppressed by estrogen exposure, whereas SREBP-1 expression exhibited more pronounced estrogen-mediated inhibition. Thus, treatment with testosterone and an aromatase inhibitor led to an intermediate phenotype, with less adiposity than seen in orchiectomized animals but persistent increases in fat mass and lipogenic gene expression relative to animals administered testosterone replacement alone (Holland et al. 2016). Diminished expression of lipogenic genes regulated by SREBP-1 also was seen in vitro in cultured murine adipocytes treated with 17β-estradiol (D'Eon et al. 2005). Further supporting an anti-obesogenic role for estrogen in males, male mice fed a diet enriched in phytoestrogens exhibited reduced adiposity, with enhanced AMP-activated protein

kinase (AMPK) signaling in adipose tissue (Cederroth et al. 2008). As AMPK promotes fatty acid β-oxidation and suppresses lipogenesis, its regulation by estrogens may be another mechanism whereby estrogen favors lipid utilization over lipid uptake, synthesis, and storage. Collectively, these preclinical findings indicate that adequate androgen and estrogen exposure are likely both required to restrain adiposity in males.

Estradiol signaling in males has been shown to contribute to the regulation of preadipocyte proliferation and differentiation, central facets of fat mass accrual. Again, sexual dimorphisms are evident in the effects of estradiol on adipogenesis. Thus, 17β-estradiol increased preadipocyte proliferation and enhanced differentiation in cells harvested from female but not male rats. In mature adipocytes from rats of both sexes, however, estradiol treatment upregulated expression of PPARϒ, the central transcriptional regulator of adipocyte differentiation (Dieudonne et al. 2000). In a clinical study, 17β-estradiol increased proliferation of preadipocytes harvested from both men and women, although the rate of proliferation was significantly faster in preadipocytes from women (Anderson et al. 2001). One mechanism underlying estradiol's role in adipogenesis may be regulation of glucocorticoid metabolism. Although glucocorticoids induce aromatase expression and estrogen production during preadipocyte differentiation, this may reflect a key role for estradiol in the restraint of adipogenesis through negative feedback regulation of glucocorticoid signaling. Thus, in male mice, exogenous 17β-estradiol administration reduced body weight gain in a model of diet-induced obesity. This lower adiposity occurred in association with reduced adipose tissue mRNA expression and activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), the enzyme that generates cortisol from cortisone (Dakin et al. 2015). These findings led the authors to propose that inhibition of cortisol generation and its associated pro-adipogenic effects is a primary mechanism through which estradiol suppresses fat accrual in males (Dakin et al. 2015). Supporting this conclusion, treatment with 17β-estradiol rapidly inhibited 11β-HSD activity in cultured 3T3-L1 adipocytes; interestingly, this effect did not appear dependent on ER signaling (Tagawa et al. 2009).

Estradiol further has been implicated in the regulation of adipocyte lipolysis, another mechanism by which it can serve as a critical determinant of the balance between lipid storage and mobilization. Both pro- and anti-lipolytic roles for estradiol have been reported. Thus, upregulation of α2-adrenergic receptors was found after estradiol treatment of subcutaneous adipocytes from women, suggesting inhibition of lipolysis (Pedersen et al. 2004). In apparent contrast, however, 17β-estradiol treatment amplified catecholamine-induced lipolysis in murine adipocytes (D'Eon et al. 2005). Consistent with both seemingly discordant findings, testosterone treatment was shown to simultaneously upregulate both β- and α2-adrenergic receptors in adipocytes harvested from castrated male hamsters (Giudicelli et al. 1993). Whether these respective effects were androgen or estrogen dependent was not established. In subcutaneous adipocytes isolated from women, estradiol also was shown to exert pro-lipolytic effects through dose-dependent stimulation of hormone-sensitive lipase (Palin et al. 2003). It remains to be established whether estradiol confers similar effects in adipocytes isolated from men.

Estrogen concentrations are regulated within adipose tissue not only through local production by aromatase but also estrogen metabolism mediated primarily by EST. The role of EST in body weight and fat mass regulation has been examined, and interestingly, EST exhibits sexually dimorphic expression and may exert divergent effects on adipogenesis in rodents and humans. EST expression was identified in white adipose tissue from male but not female rodents, and EST deficiency in male mice led to increases in epididymal fat mass with larger adipocyte size (Khor et al. 2008). These findings are consistent with in vitro data demonstrating that EST expression declined with the differentiation of both 3T3-L1 cells and primary mouse adipocytes, whereas EST overexpression inhibited 3T3-L1 cell differentiation (Wada et al. 2011). In humans, EST expression initially was detected in subcutaneous adipose tissue from obese men and women and subsequently in adipose tissue from nonobese women, as well (Ahima et al. 2011; Ihunnah et al. 2014). In striking contrast to preclinical findings, EST overexpression in human adipose tissue stem cells promoted adipocyte differentiation in association with increased expression of lipogenic genes, whereas differentiation was inhibited by EST knockdown. Further, these effects were shown to be ER dependent, indicating that they were conferred specifically through the enzymatic activity of EST that leads to inactivation of estradiol (Ihunnah et al. 2014). The authors also showed a positive correlation between adipose tissue EST expression and BMI. Critically, however, these findings were generated with adipose stem cells isolated exclusively from women and need corroboration in men, as well.

Other Peripheral Mechanisms of Estrogen-Mediated Body Weight Regulation

Although aromatase and ER expression are particularly enriched in adipose tissue, estrogen signaling in other peripheral metabolic tissues also may make a significant contribution to estrogen-mediated regulation of energy metabolism and body weight. In female mice, estradiol was found to downregulate expression of lipogenic genes in both skeletal muscle and liver. Further, estradiol increased activation of AMPK and PPARδ in skeletal muscle, putative mechanisms through which estradiol appeared to cause a shift away from lipid storage and toward fatty acid oxidation (D'Eon et al. 2005). ERα deficiency in skeletal muscle led to increases in gonadal fat mass and impaired glucose tolerance in female mice, findings ascribed to dysregulated mitochondrial turnover and function (Ribas et al. 2016). Both ERα and ERβ have been shown to regulate glucose transporter 4 (GLUT4) expression in skeletal muscle as well as adipose tissue in male mice with divergent effects of the two receptor subtypes; GLUT4 expression is upregulated by ERα but downregulated by ERβ (Barros et al. 2006, 2009). Although the ERα-mediated increase in GLUT4 expression may be seen as beneficial with regard to enhanced glucose

disposal, it nonetheless also contributes to increased energy uptake in adipocytes that could manifest as fat mass accrual in the setting of positive energy balance.

An emergent area of research involves the roles of sex steroids in regulating intestinal flora. The composition of the gut microbiome has received substantial attention as a potential contributor to obesity and associated metabolic dysregulation (Khan et al. 2016). A recent mouse model offers initial evidence that sex steroid exposure may influence the composition of the gut microbiome. Thus, in male mice fed a high-fat diet, castration led to increases in visceral adiposity in association with alterations in intestinal microflora (Harada et al. 2016). Further, castration-induced increases in visceral fat were blocked with antibiotic administration. Importantly, changes in the intestinal microflora were not seen on a regular chow diet, suggesting these changes may reflect not a direct, sex steroid-mediated effect but rather an interaction between sex steroids and diet and/or excess energy intake. Further, this study did not discriminate between androgen- and estrogen-mediated effects on intestinal flora. Clinical data regarding sex steroids and the gut microbiome are scant. In a cross-sectional study that included healthy men, urinary estrogen levels exhibited a strong, positive correlation with diversity in intestinal flora and fecal β-glucuronidase activity, leading the authors to posit that the gut microbiome may regulate circulating estrogen levels (Flores et al. 2012). The possibility exists, too, that this relationship may be inverse or bidirectional. Thus, estrogen signaling in these extra-adipose sites of energy metabolism could also contribute to estrogen-mediated changes in energy balance, body weight, and adiposity in males. Critically, however, estrogens mediate dose-, context-, and sex-dependent roles, and additional work is therefore necessary to establish and characterize these estrogen-mediated effects specifically in males.

The Immunomodulatory Effects of Estradiol

A growing area of research is focused on defining how the immunomodulatory effects of estradiol might contribute to estrogen-mediated regulation of energy balance and body composition. Estradiol is known to influence immune cell function, with extensively described effects on cellular differentiation, phenotype, and function in both adaptive and innate immunity (Cunningham and Gilkeson 2011; Kovats 2012, 2015). Resident immune cells are present within adipose tissue and undergo dynamic changes in both number and phenotype during states of both positive and negative energy balance with associated adipose tissue remodeling (Gerriets and MacIver 2014; Mathis 2013; Olefsky and Glass 2010; Suganami and Ogawa 2010; Weisberg et al. 2003). Animal models demonstrate that adipose tissue immune cells – through the secretion of paracrine effectors including growth and angiogenic factors, matrix metalloproteinases, and cytokines – are critical mediators of lipid and glucose metabolism, adipocyte differentiation, and tissue remodeling (Lacasa et al. 2007; Lu et al. 2010; Lumeng et al. 2008; Spencer et al. 2010; Suganami and Ogawa 2010; Xu et al. 2003; Ye and McGuinness 2013).

Estradiol-mediated effects on immune function are highly context dependent and vary as a function of estradiol concentration and timing of exposure, the distinct microenvironment and concurrent signals, and target cell type (Straub 2007). Irrespective of the context-dependent magnitude and directionality of these estradiol-mediated effects, however, estradiol has been reproducibly shown to modulate cellular differentiation, survival, and chemokine and cytokine production in both lymphocytes and myeloid cells. Most of estradiol's immunomodulatory effects are thought to be mediated through ERα signaling, but ERβ and GPER also have been implicated in estradiol-mediated effects on immune cell function (Blasko et al. 2009; Monteiro et al. 2014; Straub 2007).

The regulation of cytokine secretion constitutes one general mechanism whereby estradiol can mediate indirect effects on energy metabolism, insulin sensitivity, and adipogenesis. Estradiol has been shown to regulate production of the predominantly macrophage-derived cytokines TNFα, IL-1β, and IL-6, with either stimulatory or inhibitory effects on secretion that depend largely on macrophage activation state. In general, at higher concentrations, these cytokines exert inhibitory effects on adipocyte differentiation and fatty acid synthesis while promoting lipolysis. Importantly, however, immune-derived mediators play complex roles in metabolic regulation and defy simple designation as good or bad for metabolic health (Wang and Ye 2015; Ye and McGuinness 2013). TNFα illustrates this principal, as its common designation as a "pro-inflammatory" or insulin resistance-promoting cytokine fails to capture the complexity and context dependence of its metabolic effects. Thus, the effects of TNFα exposure on GLUT4 expression and insulin signaling in adipocytes varied as a function of time and TNF dose (Stephens et al. 1997). Elevated circulating levels of TNFα were found in aromatase-deficient mice and thought to play a pathogenic role in the metabolic dysregulation conferred by aromatase deficiency. Strikingly, however, when TNF receptor type 1 (TNFR1) was knocked down in these mice, hepatic steatosis and insulin resistance not only failed to improve but rather were further exacerbated (Toda et al. 2010).

Estradiol-regulated cytokines other than TNF also appear to have significant metabolic roles. Evidence from both rodents and humans supports a role for IL-6 in promoting the secretion of glucagon-like peptide-1 (GLP-1), a gut-derived hormone that regulates satiety and glucose homeostasis (Kahles et al. 2014; Lebherz et al. 2016). The cytokines IL-4 and IL-13 have been implicated in the regulation of adipose tissue energy metabolism, as both were shown to increase expression of uncoupling protein 1 (UCP1) and thermogenic capacity in adipocytes (Qiu et al. 2014). Thus, through regulation of cytokine secretion alone, estradiol could mediate changes in adipocyte differentiation and energy metabolism that could substantially impact fat mass accrual. Moreover, in addition to direct effects on immune cell phenotype and function, estradiol also could modulate immune activity indirectly, through pathways involving leptin, glucocorticoids, or sympathetic nervous system function, all of which are regulated by 17β-estradiol and, in turn, influence immune cell function.

Findings in female mice further lend in vivo evidence that estradiol regulates adiposity and energy metabolism through immunomodulatory effects. Thus, ovari-

ectomy resulted in altered cytokine expression and immune cell populations in adipose tissue (Rogers et al. 2009). Lending more direct evidence to this proposed model, female mice were generated with selective ERα deficiency in either myeloid or all hematopoietic cells. In both models, ERα deficiency conferred significant increases in fat mass (Ribas et al. 2011). In mice with myeloid-specific ERα deficiency, increased fat mass was found in association with increased adipocyte size and greater tissue macrophage infiltration (Ribas et al. 2011). Thus, these findings indicate that the immunomodulatory effects of estradiol are at least partially responsible for the obesity and associated metabolic derangements seen in mice with global ERα deficiency. To date, however, parallel studies have not been performed in male mice.

Estrogens Beyond 17β-Estradiol: Estrone and 17α-Estradiol

Although 17β-estradiol is the predominant circulating estrogen in men and premenopausal women, it may not be the only relevant estrogen with regard to metabolic regulation in men. Among men enrolled in the Diabetes Prevention Program, positive associations were found between serum estrone concentrations and the incident development of T2DM (Mather et al. 2015). Another study similarly found that circulating estrone levels are associated with incident development of T2DM in men and, further, are a better predictor of diabetes development than serum estradiol levels (Jasuja et al. 2013b). Changes in serum estrone levels were shown to positively correlate with BMI, and increased prevalence of both diabetes and cardiovascular disease was found among men in the highest quintile of serum estrone levels (Jasuja et al. 2013a). Other studies have corroborated this positive association between body weight and serum estrone levels in obese men (Bélanger et al. 2006; Kley et al. 1980a, b).

This reproducible association between serum estrogen levels and adiposity in men has been ascribed to increased adipose tissue aromatase activity in the setting of fat mass accumulation (Brind et al. 1990). Rather than merely a marker of adipose tissue aromatase activity, however, estrone could mediate metabolic effects. Thus, both estrone and its fatty acyl ester oleoyl-estrone have been shown to influence body weight and adiposity in rodents as well as adipogenesis in in vitro models. Administration of oleoyl-estrone to female rats reduced fat mass through both decreases in food intake and enhanced lipolysis and fat oxidation (Sanchis et al. 1996, 1997). Similarly, oleoyl-estrone decreased food intake in male Zucker rats and produced even more marked loss of fat mass than seen in female rats (Grasa et al. 2001). The anti-adiposity effects of oleoyl-estrone were abrogated with corticosteroid treatment, suggesting inhibition of glucocorticoid signaling may be a key mechanism through which oleoyl-estrone reduces fat mass (Serrano et al. 2009). In contrast, however, elevated estrone exposure has been posited to contribute directly to increases in body weight and adiposity (Remesar et al. 1999). In acute food deprivation in rats, serum estrone levels rose whereas estrone fatty esters declined, find-

ings potentially consistent with their respective putative effects on energy conservation and utilization (Vilà et al. 1999).

Another estrogen that has been implicated in metabolic regulation is 17α-estradiol. A naturally occurring enantiomer of 17β-estradiol, 17α-estradiol is best described as a paracrine regulator in brain (Toran-Allerand et al. 2005) but more recently was found to play roles in body weight regulation and energy homeostasis. In male mice, systemic administration of 17α-estradiol led to reductions in adiposity greater than those seen with modest caloric restriction, with particular loss of visceral adipose tissue (Stout et al. 2017). Both central and peripheral mechanisms of action were supported. Thus, 17α-estradiol-treated mice exhibited lower food intake with associated changes in the expression of hypothalamic genes implicated in appetite regulation, and 3T3-L1 adipocytes showed changes in energy-sensing pathways after 17α-estradiol treatment in vitro. Male mice treated with 17α-estradiol further showed increased AMPK activation in adipose tissue but not liver or skeletal muscle (Stout et al. 2017). The regulation of 17α-estradiol production has not been elucidated, but its local production is indicated by persistent presence throughout the brain after gonadectomy and/or adrenalectomy in mice (Toran-Allerand et al. 2005). Thus, estrone and 17α-estradiol underscore the complexity of local estrogen metabolism. Careful, tissue-specific interrogation of an expanded scope of estrogens and their derivatives will therefore be essential to fully delineate the estrogen-mediated mechanisms of body weight regulation in men.

Estrogen and Obesity in Men: Too Much or Too Little?

Estradiol deficiency clearly predisposes males to increased adiposity and metabolic dysregulation. In apparent contrast, however, obesity in men has been associated with hyperestrogenemia, and, further, excessive estradiol exposure has been postulated to play an exacerbating role in the progression of obesity and attendant metabolic dysregulation. Though not uniformly, obesity in men is often characterized by a profile of low circulating androgens but elevated levels of circulating estrone and 17β-estradiol (Schneider et al. 1979; MacDonald et al. 2010). The reasons for this variable co-occurrence of obesity and hyperestrogenemia in men are not well defined but may include repeat number of a TTTA polymorphism in the aromatase gene *CYP19A1* (Hammoud et al. 2010). Although overtly elevated serum estrogen levels are not found in all men with obesity, it has been proposed that obesity also may represent a state of relative rather than absolute estrogen excess. Thus, increased peripheral aromatization of testosterone in obese men may lead to enhanced central estradiol signaling that suppresses gonadotropin production and contributes to a sustained state of hypogonadotropic hypogonadism (Mah and Wittert 2010). This serum profile of sex steroids is normalized with weight loss, as serum testosterone and gonadotropin levels rise, whereas serum estradiol levels fall subsequent to weight loss effected either through bariatric surgery or behavioral change (Armamento-Villareal et al. 2016; Corona et al. 2013; Mihalca and Fica 2014;

Pellitero et al. 2012). Thus, whether absolute or relative, estrogen excess is believed to have a bidirectional relationship with obesity in men and to contribute to progressive adiposity and metabolic dysregulation (Corona et al. 2011; Mah and Wittert 2010).

Indeed, some preclinical evidence supports pro-adipogenic effects of estradiol. In male but not female preadipocytes, 17β-estradiol induced aromatase activity, leading the authors to postulate that excess estradiol within adipose tissue may be self-propagating and contribute to progressive increases in leptin and cortisol signaling with attendant pro-adipogenic effects (Dieudonné et al. 2006). Further, enhanced conversion of testosterone to estradiol could cause a relative androgen depletion and thereby limit the anti-adipogenic effects conferred by androgens within adipose tissue (Dieudonne et al. 2000). One possible way to reconcile the apparent paradox of intra-adipose estradiol as both pro- and anti-adipogenic is to view obesity as a dynamic rather than static state, one characterized by positive energy balance and continual delivery of excess glucose and lipids to peripheral tissue. Adipose tissue remains the primary sink for this excess energy intake, but fat mass expansion in obesity becomes increasingly limited by adequacy of blood and oxygen supply as well as physical constraints. Viewed in this context, increased estradiol generation within adipose tissue could be seen as an adaptation with variable effects on adipogenesis and lipid storage that serve to both restrain adipogenesis and maintain some capacity to store the excess glucose and lipid that otherwise would contribute to ectopic fat accrual.

Importantly, even as this increased estradiol production may be adaptive, it may nevertheless confer both harmful and beneficial effects with regard to metabolic regulation. In this model, estradiol is a regulatory mediator that can exert either pro- or anti-adipogenic effects or both concurrently. With regard to adipogenesis, for example, 17β-estradiol was shown to inhibit 11β-HSD1 activity in adipocytes from male rats, thus suppressing the formation of pro-adipogenic cortisol (Tagawa et al. 2009). However, it also increases leptin expression and secretion, which, in turn, enhances 11β-HSD1 expression in preadipocytes and, moreover, promotes further estradiol generation through upregulation of aromatase (Dieudonné et al. 2006). The concentration-dependent effects of estradiol on Lpl activity further demonstrate the potential for estradiol to play divergent roles in lipid uptake by adipocytes, again indicating estradiol could either inhibit or promote lipid accumulation in adipocytes. Consequently, the net impact on adipogenesis and fat mass will be contingent on local estradiol concentrations and concurrent signals in the tissue environment as well as the total delivery of energy substrate to adipose tissue (Fig. 3). These context-dependent effects of estradiol are also illustrated by estradiol-mediated regulation of TNFα. At higher concentrations, TNFα can inhibit adipocyte differentiation, promote lipolysis, and inhibit insulin-stimulated glucose uptake, thus acting as a potent anti-adipogenic signal (Ruan et al. 2002). Therefore, estradiol-mediated suppression of TNFα production would serve to promote adipogenesis, lipid storage, and glucose uptake in adipocytes. However, these anti-adipogenic effects of TNFα are concentration dependent, as are the inhibitory effects of estradiol on TNFα generation. Thus, pro-adipogenic effects of estradiol through TNFα suppres-

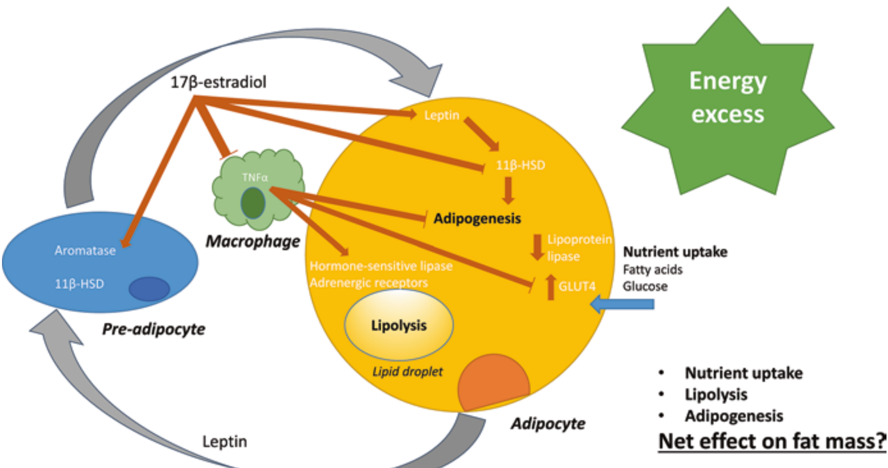

Fig. 3 Estradiol mediates myriad effects within adipose tissue and contributes to the regulation of adipocyte differentiation, nutrient uptake, and lipolysis. When viewed in isolation, these effects could be seen as either pro- or anti-obesogenic. The cumulative effect of estradiol on fat mass accrual is contingent upon context and, critically, overall energy balance

sion only would be evident in obesity or other states characterized by elevated production of both estradiol and TNFα within adipose tissue. Conversely, in a state of neutral or negative energy balance, TNFα production is generally lower, which may be one factor that allows the anti-adipogenic effects of estradiol to become manifest.

Estradiol excess therefore may not truly represent an aberrant response that promotes further fat mass accrual in the setting of obesity; rather, a more physiologically relevant model may be one of a propagated, amplified process consequent to a continued state of positive energy balance. Thus, the process may be wholly regulated and reversible, as supported by the normalization of serum estradiol levels as well as marked improvements in metabolic regulation in obese subjects after induction of negative energy balance leading to body weight reductions (Petersen et al. 2005; Viljanen et al. 2009; Wing and Group 2010). This model suggests that increased estradiol production within adipose tissue is an adaptive response to continual delivery of nutrient excess rather than a primary pathogenic driver of obesity in men. The increased estradiol production may be promoting continued – though not unrestrained – adipogenesis as well as nutrient uptake in order to handle excess lipid and glucose, thus maintaining adipose tissue as the primary reservoir for energy storage while addressing the constraints on adipose tissue expansion. An adaptive rather than pathogenic role of increased intra-adipose estradiol is further supported by clinical studies of aromatase inhibitors in obese men. Although aromatase inhibition reduces circulating estradiol levels and restores normal circulating testosterone levels, clinical intervention trials to date have failed to show any associated metabolic benefit (Burnett-Bowie et al. 2009; Loves et al. 2008, 2013).

These negative findings underscore the importance of understanding estradiol as a paracrine and intracrine mediator rather than solely assessing circulating levels (Simpson 2003).

Clinical Intervention Studies

Only over the past few years have clinical intervention studies begun to confirm preclinical evidence that estradiol contributes to body weight regulation and metabolic health in men. One small study examined the effects of testosterone replacement in obese men with low-normal baseline serum testosterone concentrations. Whereas treatment with testosterone gel led to significant reductions in adiposity, these changes were not seen when testosterone was co-administered with an aromatase inhibitor (Juang et al. 2014). In a larger study of healthy men, two subject cohorts were administered the GnRH analogue goserelin acetate to suppress endogenous sex steroid production. Simultaneously, subjects in the first cohort received either placebo gel or variable doses of add-back testosterone gel, and the second cohort of subjects received either placebo gel or testosterone gel with an aromatase inhibitor. Strikingly, whereas androgen exposure appeared to mediate changes in lean mass, estradiol rather than testosterone was found to be the primary determinant of changes in fat mass (Finkelstein et al. 2013). Subsequently, another clinical study similarly enrolled healthy, eugonadal men and rendered them medically castrate through use of the GnRH antagonist acyline. Subjects in this study variably received placebo gel, low-dose or full replacement dose testosterone gel, or full replacement dose testosterone gel with an aromatase inhibitor. In all three treatment groups rendered sex steroid deficient, significant increases in body fat mass were evident within only 4 weeks of drug treatment (Chao et al. 2016). Again, estradiol rather than testosterone deprivation exhibited a stronger correlation with the observed increases in adiposity.

Implications for Clinical Practice

Better understanding of the importance of estrogens for maintaining metabolic health in men is essential for optimal treatment of male hypogonadism and, further, for understanding the metabolic implications of sex steroid manipulation in clinical practice. Exogenous testosterone therapy in hypogonadal men restores circulating androgen levels but not fertility, prompting interest in identifying alternative therapeutic strategies. The ER antagonist clomiphene and its derivatives also have been proposed as therapeutic interventions for secondary hypogonadism in men (Wiehle et al. 2013). Clomiphene blocks estradiol-mediated gonadotropin suppression and

thereby helps restore normal testosterone production while preserving fertility (Kaminetsky et al. 2013; Kim et al. 2016; Wiehle et al. 2014). The use of aromatase inhibitors also has been proposed for men with hypogonadism associated with hyperestrogenemia, including subsets of men with late-onset and obesity-associated hypogonadotropic hypogonadism (de Boer et al. 2005; Tan et al. 2014; Zumoff et al. 2003). However, as appreciation grows of the critical metabolic roles of estrogens in men, caution may be warranted in the pursuit of such anti-estrogen-based approaches to treatment of male hypogonadism. Similarly, use of selective androgen receptor modulators for treatment of hypogonadism has been proposed (Bhattacharya et al. 2016; Thirumalai et al. 2017), but this strategy may not fully restore estradiol signaling and, therefore, fail to optimize metabolic health in hypogonadal men. Rather, selective estrogen receptor modulators may prove equally critical for improving body composition and metabolic regulation in male hypogonadism. Novel, targeted pharmacological strategies that promote ERα signaling may prove important not only for treatment of male hypogonadism, as a dual ERα/GLP-1 agonist has been developed as a potential therapeutic for metabolic disorders (Finan et al. 2012).

Another population for whom the metabolic effects of estradiol could prove highly relevant are men with prostate cancer. In the USA, prostate cancer affects 2 million men, and up to 50% of these men will undergo androgen deprivation therapy (ADT) at some point in their treatment course (Meng et al. 2002). The most common form of ADT involves GnRH analogues that confer central hypogonadism, and over the past decade, clinical evidence has compellingly demonstrated the men undergoing ADT are at substantially higher risk of increased adiposity, insulin resistance, T2DM, and cardiovascular disease than age-matched controls with or without prostate cancer (Cannata et al. 2012; Hamilton et al. 2011; Keating et al. 2006, 2012; Shahani et al. 2008). ADT-induced hypogonadism is a state of both androgen and estrogen deficiency, and the latter is now believed to contribute substantially to the metabolic dysregulation evident in men receiving GnRH analogues. Interestingly, estradiol therapy was among the ADT formulations originally used for treatment of prostate cancer (Cannata et al. 2012), and interest in estrogen-based ADT recently has been renewed as it could effectively suppress androgen production while reducing the metabolic sequelae of GnRH analogues (Phillips et al. 2014).

Finally, as efforts continue to develop an effective form of hormonal contraception for men (Ayoub et al. 2016; Zitzmann et al. 2017), these findings collectively underscore the need to carefully assess changes in estradiol exposure consequent to different contraceptive regimens. Thus, mounting evidence suggests that estradiol replacement is a pivotal facet of the treatment of male hypogonadism and, by corollary, states of estradiol deprivation – whether consequent to physiologic hypogonadism, androgen deprivation therapy, or hormonal forms of contraception – must be avoided as possible to optimize metabolic health in men.

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Estradiol Regulation of Brown Adipose Tissue Thermogenesis

Ismael González-García, Manuel Tena-Sempere, and Miguel López

Abstract Physiologically, estrogens carry out a myriad of functions, the most essential being the regulation of the reproductive axis. Currently, it is also dogmatic that estrogens play an important role modulating energy balance and metabolism. In this sense, it is well known that low estrogens levels, occurring due to ovarian insufficiency, in conditions such as menopause or ovariectomy (OVX), are associated with increased food intake and decreased energy expenditure, leading to weight gain and obesity at long term. Concerning energy expenditure, the main effect of estradiol (E2) is on brown adipose tissue (BAT) thermogenesis. Thus, acting through a peripheral or a central action, E2 activates brown fat activity and increases body temperature, which is negatively associated with body weight. Centrally, the hypothalamic AMP-activated protein kinase (AMPK) mediates the E2 action on BAT thermogenesis. In this chapter, we will summarize E2 regulation of BAT thermogenesis and how this can influence energy balance and metabolism in general.

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Introduction

Although being an enormous global health problem, obesity can be considered the simple consequence of a thermodynamic imbalance where energy intake exceeds energy expenditure. Obesity per se or through the development of other comorbidities, including cancer, cardiovascular disease, and T2D, causes thousands of deaths per year worldwide (Dietrich and Horvath 2012a, b; Clemmensen et al. 2016; Tschop et al. 2016). In fact, World Health Organization (WHO)'s latest report shows that 13% of adults globally are currently obese. Of those, 11% are men, and 15% are women, clearly demonstrating a gender dimorphism. As said above, body weight gain can be explained as a thermodynamic unbalance where the calories obtained from food are continuously higher than the calories expend by metabolism, thermogenesis, or physical activity. This naïve description, though, represents only the tip of a much complex reality, in which a homeostatic system controlling energy balance, which includes a complex amount of cross-regulation and integration mechanisms that involve signaling systems throughout the whole body (Schneeberger et al. 2014; Scott et al. 2014; Gautron et al. 2015; Lopez et al. 2016), become persistently deregulated.

Several central signals, such as neuropeptides and transmitters, as well as a variety of peripheral hormones have been identified as regulators of food intake and energy expenditure (Schneeberger et al. 2014; Scott et al. 2014; Gautron et al. 2015; Lopez et al. 2016). On the latter, substantial attention has been paid recently to reveal the roles of signals from metabolic tissues, such as the pancreas, the adipose tissue, and the gut (Scott et al. 2013; Allison and Myers 2014), where even the contribution of microbiota has been also extensively studied (Cani et al. 2016; Chevalier et al. 2015; Suarez-Zamorano et al. 2015). In spite of that, other more "classical" endocrine organs, such as the adrenals, the thyroid, and the gonads, have been long known to secrete hormones that play key roles in the modulation of metabolism and energy balance (López et al. 2013; López and Tena-Sempere 2015; Martinez-Sanchez et al. 2014). In this chapter, we will focus on estrogens, in particular on the effects of estradiol (E2) on energy expenditure, by controlling brown adipose tissue (BAT) thermogenesis.

Metabolic Effects of Estrogens

Ovarian hormones, including estrogens, such as 17β-estradiol, are pleotropic regulators of several cellular functions (López and Tena-Sempere 2015; Mauvais-Jarvis et al. 2013). Nowadays, it has become clear that estrogens are major modifiers of the energy balance (López and Tena-Sempere 2015; Mauvais-Jarvis et al. 2013; Gao and Horvath 2008; Frank et al. 2014), whose significance is reinforced by the fact that they are produced in various organs, besides the ovary, and that their levels fluctuate physiologically during the lifespan and in different pathological

conditions. Nevertheless, our understanding on the effects and key sites of actions of E2 in the control of whole-body metabolism, body weight, and energy expenditure is still incomplete.

Reduced levels of E2 after menopause or ovariectomy (OVX) are associated with hyperphagia, decreased energy expenditure, and weight gain (Mauvais-Jarvis et al. 2013; Blaustein and Wade 1976; Carr 2003; Rogers et al. 2009a; Martinez de Morentin et al. 2014; Martinez de Morentin et al. 2015). E2 replacement in those situations prevents or reverts OVX-induced obesity and metabolic alterations by decreasing energy intake and increasing energy expenditure (Mauvais-Jarvis et al. 2013; Gao and Horvath 2008; Martinez de Morentin et al. 2014, 2015; Wren 2009). Moreover, differences in the size of meals and body weight have been observed in rats throughout the estrous cycle, along with variations in endogenous E2 levels (Mauvais-Jarvis et al. 2013; Blaustein and Wade 1976; Martinez de Morentin et al. 2014; Tritos et al. 2004), as well as during pregnancy and lactation (Martinez de Morentin et al. 2015; Key et al. 2001; Garcia et al. 2003).

Estrogen receptor alpha (ERα) is the principal mediator of E2 in energy homeostasis (López and Tena-Sempere 2015; Mauvais-Jarvis et al. 2013). Pharmacological studies have revealed that while the specific ERα agonist, propylpyrazoletriol (PPT), promotes anorexia, the selective ERβ agonist, diarylpropionitrile (DPN), does not (Martinez de Morentin et al. 2014, 2015; Roesch 2006). In this sense, male and female mice with global deletion of ERα (ERα KO) show hyperphagia, hypometabolism, increased adiposity (due to both hypertrophy and hyperplasia), hyperleptinemia, and insulin resistance (Heine et al. 2000; Ohlsson et al. 2000; Geary et al. 2001). Similarly, both male and female mice with genetic ablation of the enzyme responsible for the ultimate synthesis of estrogens, aromatase (Ar KO), develop obesity (Jones et al. 2000, 2001). A similar phenotype was also reported in humans suffering aromatase deficiency (Grumbach and Auchus 1999). On the other hand, the knockout of estrogen receptor beta (ERβ) does not induce obesity or any associated metabolic alterations (Ohlsson et al. 2000).

At peripheral level, E2 controls nearly every single aspect of metabolism, such as insulin sensitivity by acting on the pancreas, liver, and skeletal muscle (Frias et al. 2001; Hevener et al. 2002; Rogers et al. 2009b; Stubbins et al. 2012), pancreatic beta cell function (Tiano and Mauvais-Jarvis 2012), and lipid metabolism (Martinez de Morentin et al. 2014; Iverius and Brunzell 1988; Gao et al. 2006; Gonzalez et al. 2012). The actions of E2 on adipose tissue are particularly important, regulating its pattern of distribution (Lovejoy and Sainsbury 2009), differentiation (Lapid et al. 2014), inflammation, and fibrosis (Davis et al. 2013; Kim et al. 2014a). Interestingly, most of these actions determine the sexual dimorphism in the control of energy homeostasis, such as the body fat pattern – which differs between women and men – or the insulin sensitivity (Mauvais-Jarvis et al. 2013; Stubbins et al. 2012; Lovejoy and Sainsbury 2009; Palmer and Clegg 2015).

Moreover, E2 acts also on the central nervous system (CNS) to regulate energy balance. ERs are widely expressed in the brain, predominantly in hypothalamic sites, such as the arcuate (ARC), ventromedial (VMH), and paraventricular (PVH) nuclei, as well as the preoptic (POA) and lateral (LHA) hypothalamic areas

(Simerly et al. 1990; Simonian and Herbison 1997; Voisin et al. 1997; Osterlund et al. 1998; Merchenthaler et al. 2004), all of them with essential functions in the modulation of energy metabolism (Schneeberger et al. 2014; Scott et al. 2014; López et al. 2007; Sohn et al. 2013). The functional importance of this distribution is sustained by the fact that central administration of E2 exerts a profound catabolic response (Martinez de Morentin et al. 2014, 2015). Genetic murine models have also established the key role of ERα in the regulation of body weight. Brain-specific deletion of ERα in male and female mice induced a larger visceral adiposity and hyperphagia associated with decreased energy expenditure and locomotor activity (Xu et al. 2011).

Numerous lines of evidence have reported that E2 has a nucleus-specific action in the hypothalamus to regulate energy homeostasis, particularly within the ARC and the VMH. Accordingly, most of the effects of estrogens on food intake are known to occur in the ARC, more specifically in proopiomelanocortin (POMC; an anorectic neuropeptide) neurons, where ERα is abundantly expressed (Simerly et al. 1990; Osterlund et al. 1998; Merchenthaler et al. 2004). Thus, selective deletion of ERα in POMC neurons increases appetite, without changes in energy expenditure or fat distribution (Xu et al. 2011). The molecular pathway mediating the action of estrogens on POMC neurons is uncertain, but convincing data have proven that E2 inhibits AMP-activated protein kinase (AMPK); a key energy sensor (Lage et al. 2008; Carling et al. 2011; Hardie et al. 2012; Schneeberger and Claret 2012), in the hypothalamus, and that genetic activation of this kinase within the ARC reverse the anorectic effect of E2 (Martinez de Morentin et al. 2014). A different and not excluding hypothesis suggests that E2 blunts the interaction between POMC neurons in the ARC with melanin-concentrating hormone (MCH) neurons in the LHA, inducing a decrease in food intake and body weight (Mystkowski et al. 2000). Worth mentioning, the influence of E2 on POMC neurons is not restricted to the regulation of gene expression or neuronal activity but modulates also synaptic plasticity. Thus, E2 exerts a strong increase in the amount of excitatory inputs to POMC neurons through a STAT3 (signal transducer and activator of transcription 3)-dependent (but leptin-independent) mechanism (Gao et al. 2007). The molecular effectors mediating these events have not been established, but inhibition of hypothalamic fatty acid synthase (FAS; which would lead to higher levels of malonyl-CoA (Lopez et al. 2008; Lage et al. 2010)) seems to play a key role (Martinez de Morentin et al. 2015). Of note, the effects of E2 on hypothalamic fatty acid metabolism and POMC neurons are absent in pregnant rats preserving the hyperphagic state (Martinez de Morentin et al. 2015). To conclude, E2 acts also in AgRP/NPY (agouti-related protein/neuropeptide Y) neurons in the ARC to modulate food intake (Martinez de Morentin et al. 2014, 2015; Shimizu et al. 2008; Pelletier et al. 2007; Santollo et al. 2007).

BAT Thermogenesis and Contribution to the Energy Balance

In a thermoneutral situation, obligatory thermogenesis (the heat generated automatically by the metabolic rate) is sufficient to conserve body temperature, without the contribution of any other homeostatic thermoregulatory mechanism (López et al. 2013; Cannon and Nedergaard 2004; Silva 2006). The lowest environmental temperature at which this happens is named thermoneutral temperature. Smaller animals, such as rodents, as a consequence of their larger surface area-to-volume ratio (which makes them prone to lose more heat), have a higher thermoneutral temperature (30 °C in mice and 28 °C in rats) than bigger species, such as humans (23 °C) (López et al. 2013; Cannon and Nedergaard 2004; Silva 2006). When ambient temperature falls below this threshold, the immediate response is to trigger heat-saving strategies, such as vasoconstriction, piloerection, rounded positions, and decreased mobility. Those mechanisms are very limited, and additional thermogenic mechanisms are promptly stimulated. This additional heat, generated on demand, is called facultative or adaptive thermogenesis (López et al. 2013; Cannon and Nedergaard 2004; Silva 2006). Shivering is the earliest and most primitive reaction to produce more heat. However, homeothermic animals have developed more efficient and long-term mechanisms of non-shivering facultative thermogenesis, which allow the expenditure of energy reserves to produce heat. In mammals, including humans, the organ responsible for facultative thermogenesis is the brown adipose tissue (BAT) (Cannon and Nedergaard 2004; Silva 2006; Nedergaard et al. 2007). In small mammals inhabiting at sub-thermoneutral temperatures, large amounts of active brown adipose tissue can be readily identified (Cannon and Nedergaard 2004; Silva 2006). In humans, while a big part of BAT rapidly atrophies in the months after birth, recent studies have proven the existence of functional brown fat depots persisting into adulthood (Nedergaard et al. 2007; Cypess et al. 2009; Marken Lichtenbelt et al. 2009; Virtanen et al. 2009). In fact, it was only during the last decade that different studies identified for the first time BAT in humans. PET and ^{18}F-fluorodeoxyglucose (^{18}F-FDG) uptake demonstrated the presence of BAT in the cervical, supraclavicular, perirenal, intercostal, and periaortic regions (Nedergaard et al. 2007; Cypess et al. 2009; Marken Lichtenbelt et al. 2009; Virtanen et al. 2009; Zingaretti et al. 2009). It is currently known that, instead of brown adipocytes, human BAT is composed by beige or brite ("brown in white") adipocytes (Wu et al. 2012; Jespersen et al. 2013); this type of cells has also being described in white adipose tissue (WAT) under some situations. This process where precursor cells placed in WAT become beige/brite cells instead of white adipocytes is called browning (Nedergaard and Cannon 2014; Contreras et al. 2015, 2016).

Morphologically, brown adipocytes contain multiple small lipid droplets and a very high number of mitochondria. In typical mitochondria, the energy resulting from the electron transport through the respiratory chain is locally stored as a proton gradient within the inner membrane of this organelle. This proton force is used by the ATP synthase to produce ATP from ADP (Futai et al. 1989; Boyer 1997; Von et al. 2009) (for extensive review). This state is imperfect, and some energy is

dissipated as heat, rather than being captured in ATP, because the leak of protons across the membrane, in the direction of the gradient, is an exergonic reaction. In the inner mitochondrial membrane of BAT's mitochondria, there exists a protein named uncoupling protein 1 (UCP1), which offers an alternative channel for the protons back into the mitochondrial matrix, circumventing ATP synthase and generating heat (Cannon and Nedergaard 2004; Silva 2006; Nicholls and Locke 1984).

Brown adipocytes have a variety of receptors to several molecules, which modulates its function. Together with estrogens (but this will be deeply explained in a section below), thyroid hormones (THs) also exert direct effects on BAT. Thyroid receptors (TR) are highly expressed in BAT, and THs are required together with NE to induce a full thermogenic response (López et al. 2013; Silva 2006; Bianco et al. 1988). The TRα1 maintains normal adrenergic response, while the TRβ1 induces UCP1 gene expression. TR deficiency produces cold intolerance and hypothermia associated with a reduction in BAT thermogenesis (Martinez De et al. 2010; Ribeiro et al. 2010). Moreover, deiodinase-2 null mice also exert a deficient adrenergic response to cold exposure (de Jesus et al. 2001; Christoffolete et al. 2004; Castillo et al. 2011).

Insulin induces in BAT a drastic change in terms of glucose uptake suggesting a possible direct stimulation of thermogenesis in brown adipocytes (Storlien et al. 1986). Insulin receptor deletion in BAT induces a decrease in the weight of BAT, and enzymes of fatty acid synthesis are decreased without dysplasia in the tissue (Guerra et al. 2001). Thus, insulin is an essential hormone for lipid accretion in BAT and therefore to BAT function (Guerra et al. 2001). Moreover, it has been shown that insulin pathway modulation can be used as a therapeutic approach to increase BAT thermogenesis and energy expenditure (Ortega-Molina et al. 2012, 2015). Phosphatidylinositol-3-kinase (PI3K), a kinase mediator of insulin pathway, is counteracted by phosphatase and tensin homologue (PTEN). Recent data showed that PTEN positively regulates a BAT-selective thermogenic program by blocking PI3K, (Ortega-Molina et al. 2012, 2015). Thus, pharmacological PI3K inhibitors increase BAT thermogenesis and whole-body energy expenditure. Adiponectin (ADPN; also called adipocyte complement-related protein (Acrp30), apM1, or adipoQ) is a protein secreted from the adipose tissue (Hu et al. 1996), placenta (Caminos et al. 2005), and cardiomyocytes (Caminos et al. 2005), among other tissues. Adiponectin decreases UCP1 expression and therefore BAT thermogenesis (Dong et al. 2013). Accordingly, deletion of ADPN induces UCP1 expression in BAT, browning of inguinal fat, and increases in body temperature (Qiao et al. 2014). Adrenal steroids, glucocorticoids, and mineralocorticoids have receptors in the BAT (Feldman 1978; Zennaro et al. 1998). Both receptors induce in adipocytes a decrease in UCP1 levels (Viengchareun et al. 2001; Soumano et al. 2000). Fibroblast growth factor 21 (FGF21), a protein secreted by the liver, is involved in several physiological functions such as glucose homeostasis, insulin sensitivity, and ketogenesis (Kharitonenkov et al. 2005). Moreover, FGF21 induces BAT thermogenesis (Hondares et al. 2010) and was found that mice lacking this protein have a defective adaptation to chronic cold exposure concomitantly with a decreasing in browning of WAT (Hondares et al. 2010).

Despite some factors can act directly on BAT, inducing or decreasing its activity and thus its thermogenic capability, the essential physiological trigger of BAT thermogenesis is the sympathetic nervous system (SNS) and central nervous system (CNS) the main controller. In the brain, several areas have been described as participants in the BAT thermogenesis, although hypothalamus plays the most relevant role (Scott et al. 2014; Contreras et al. 2015; Morrison et al. 2012; Richard et al. 2012; Bellefontaine and Elias 2014; López et al. 2016; Cornejo et al. 2016). In this sense, the ARC, DMH, PVH, and VMH nuclei, as well as in the LHA and POA, are hypothalamic areas that modulate thermoregulation of brown fat (Scott et al. 2014; Contreras et al. 2015; Morrison et al. 2012; Richard et al. 2012; Bellefontaine and Elias 2014; López et al. 2016; Cornejo et al. 2016). The SNS is critical to stimulate BAT thermogenesis (Cannon and Nedergaard 2004; Silva 2006; Contreras et al. 2015; Morrison et al. 2012; Whittle et al. 2011). A higher activation of the sympathetic tone to BAT leads to norepinephrine (NE) release at the nerve terminal and activation of the β-adrenergic receptors (β-ARs, which are G protein-coupled receptors) expressed in the brown adipocytes, principally the β3 subtype (β3-AR). Upon receptor activation, the associated protein Gs stimulates adenylate cyclase (AC), increasing cAMP, which in turn activates protein kinase A (PKA), inducing thermogenesis and downstream activation of p38 mitogen-activated protein kinase (MAPK) (Cannon and Nedergaard 2004; Contreras et al. 2015; Whittle et al. 2011). PKA has both acute and chronic effects on brown adipocytes. The acute effect of PKA induces lipolysis leading to increased cytosolic free fatty acid (FFA) levels. This mechanism takes place by activation of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL; being pHSL the activated form), and monoacylglycerol lipase (MGL), the three of them sequentially hydrolyzing triglycerides to release FFAs. Carnitine palmitoyltransferase 1a (CPT1a) introduces FFAs-CoA into the mitochondria, where FA oxidation leads to the formation of NADH and FADH, which are then further oxidized in the electron transport chain (Cannon and Nedergaard 2004; Silva 2006; Contreras et al. 2015; Whittle et al. 2011).

Peripheral E2 Regulation of BAT Thermogenesis

The first study showing an association of E2 with BAT function reported the molecular binding of E2 in brown fat indicating that this tissue might be a target of E2 action (Wade and Gray 1978). But it was 5 years later when it was concluded that BAT may be involved in the process of increased energy expenditure induced by E2 (Edens and Wade 1983). In line with those findings, physiological evidence has confirmed that E2 increases brown fat lipolysis and thermogenesis, leading to increased energy expenditure in several species, such as hamsters, rats, and mice (Bartness and Wade 1984; Schneider et al. 1986; Richard 1986; Kamei et al. 2005). Of note, those effects were associated with increased NE turnover, which was reduced after OVX (Yoshida et al. 1987). This might suggest a direct effect of E2 on brown adipocytes but also a modulatory role of E2 in the central control of the

sympathetic tone to BAT. The latter mechanism would be remarkably similar to the effects of other fundamental metabolic regulators, such as THs, which, besides directly modulating UCP1 expression, increase also NE-induced lipolysis (López et al. 2013; Martinez-Sanchez et al. 2014; de Jesus et al. 2001; Ribeiro et al. 2000). However, it has been also reported that the direct effect of E2 on brown fat is probably more associated with changes in adrenergic receptors (AR) and mitochondrial biogenesis-signaling factors, such as PTEN, nuclear respiratory transcriptional factor 1 (NRF1) and possibly peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), rather than to changes in UCP1 expression (Rodriguez et al. 2002; Monjo et al. 2003; Rodriguez-Cuenca et al. 2007a) (Fig. 1).

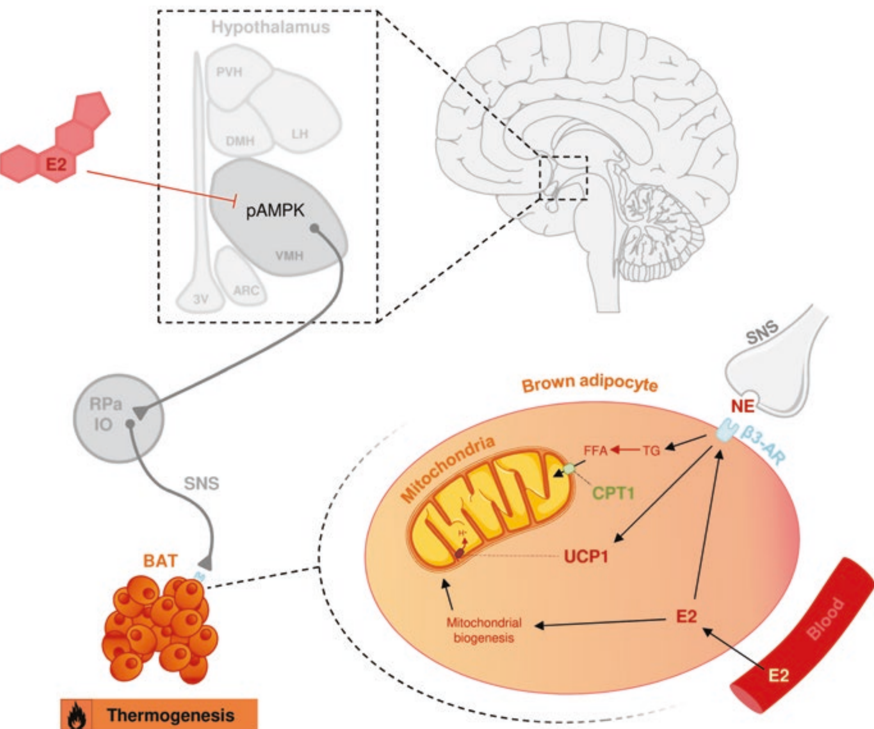

Fig. 1 Peripheral and central actions of E2 on BAT thermogenesis. Estradiol (E2) induces brown adipose tissue (BAT) thermogenesis through both mechanisms: acting directly on brown adipocytes and in hypothalamus. Peripheral actions of E2 on brown adipocytes mediate changes in ARs, where it stimulates β3-AR expression and induces mitochondrial biogenesis. At central level, E2 inhibits AMP-activated protein kinase (AMPK) in the ventromedial nucleus of the hypothalamus (VMH) activating the firing of sympathetic nervous system (SNS) to BAT. Both mechanisms will induce the thermogenic program in the brown fat producing an increase in body temperature and consequently in energy expenditure and weight loss. *3V* third ventricle, *ARC* arcuate nucleus of hypothalamus, *β3-AR* beta 3 adrenergic receptor, *CPT1* carnitine palmitoyltransferase 1, *DMH* dorsomedial nucleus of the hypothalamus, *FFA* free fatty acid, *IO* inferior olive, *LHA* lateral hypothalamic area, *NE* norepinephrine, *PVH* paraventricular nucleus of the hypothalamus, *RPa* raphe pallidus, *UCP1* uncoupling protein 1, *TG* triglyceride

Although ERs are present by the brown adipocyte, in a sexually different manner, with ERα being more expressed in male than female rats (Rodriguez-Cuenca et al. 2007b), the phenotype of ERα null mice has not produced conclusive data on the direct role of estrogens on BAT. ERα KO mice show an age-dependent enlarged adiposity (due to hyperplasia and hypertrophy) in white fat pads, but no changes are found in the mass of brown fat pads, either in males or females (Heine et al. 2000; Cooke et al. 2001). Quite opposite, Ar KO mice show enlarged BAT pads, which can be reversed by peripheral E2 administration (Hewitt et al. 2004). Regarding G protein-coupled estrogen receptor (GPER), both male and female mice with deleted GPER developed moderate weight gain with substantial reductions in energy expenditure, increased BAT lipid content (demonstrating a reduced thermogenic activity), and diminished expression of UCP1 and β3-AR (Davis et al. 2014).

The role of estrogen receptors in human BAT has not been deeply explored. It has been described that both ERα and ERβ are expressed in human fetal BAT, with ERα being more abundant (Velickovic et al. 2014). The possible role of ERs in adult human brown fat is unclear, but the discovery of functional BAT in adulthood (Nedergaard et al. 2007; Cypess et al. 2009; Marken Lichtenbelt et al. 2009; Virtanen et al. 2009) opens up the possibility that estrogens could directly regulate human brown fat activity. In this sense, it has been reported that women have a larger mass of BAT and higher ^{18}F-FDG uptake (which is an indicator of higher BAT activity) than men (Cypess et al. 2009); these evidences are suggestive of a sexual dimorphism and are compatible with a potential effect of gonadal hormones, including estrogens, in the direct control of human BAT.

Central E2 Regulation of BAT Thermogenesis

In line with the predominant role of SNS in the control of BAT thermogenesis, E2 modulation of BAT is also known to be conducted centrally, by targeting specific areas of the CNS, and more specifically the hypothalamus. This evolutionary well-conserved area is organized in discrete neuronal populations, named nuclei. While the ARC is known as the "master hypothalamic center" for appetite regulation, the VMH was the first identified hypothalamic nucleus modulating energy expenditure, in particular thermoregulation (noteworthy that VMH was postulated as "satiety center" already in the 1950s, and at that time this was mostly related with food intake (Hetherington and Ranson 1942; Anand and Brobeck 1951)). Electrical, pharmacological, and hormonal stimulation of this nucleus increases the temperature of interscapular BAT through the sympathetic tone (Martinez de Morentin et al. 2012, 2014; Perkins et al. 1981; Yoshimatsu et al. 1993; Lopez et al. 2010; Whittle et al. 2012; Seoane-Collazo et al. 2014) (for extensive review: Cannon and Nedergaard 2004; Contreras et al. 2015; Morrison et al. 2014). In addition, there are numerous brainstem areas, such as the raphe pallidus (RPa) and inferior olive (IO), two nuclei which have been functionally associated with the regulation of BAT

thermogenesis, to which VMH neurons have been proposed to relay for regulating SNS activity (Cannon and Nedergaard 2004; Contreras et al. 2015; Morrison et al. 2014).

Interestingly, neurons of VMH are target of E2 in the facilitation of the female sexual behavior, lordosis (Beyer and Gonzalez-Mariscal 1986; Moss and Dudley 1984; Pfaff et al. 2000; Pfaff and McEwen 1983). Electrophysiological data revealed that E2 affects the excitability of neurons in the VMH through a cAMP-dependent mechanism (Minami et al. 1990). Decisive evidence for a fundamental role of VMH as key hypothalamic center mediating E2's action on energy expenditure was later accumulated, mainly due to the seminal work of Clegg and coworkers. First, they demonstrated that stereotaxic administration of adeno-associated viruses silencing ERα within the VMH induced obesity and hyperglycemia and reduced energy expenditure in both mice and rats (Musatov et al. 2007). Additional work confirmed that the action of E2 on energy expenditure is mediated by steroidogenic factor-1 (SF1) neurons within the VMH. When fed on a standard diet, female (but not male) ERα/SF1 null mice presented a feeding-independent increase of body weight and visceral adiposity, which was intensified when animals were fed a high-fat diet (HFD) (Xu et al. 2011). Interestingly, such phenotype was associated with hypometabolism and reduced BAT thermogenesis, leading to reduced heat production. Actually, mice with ablated ERα in SF1 neurons showed decreased sympathetic outflow on brown fat and decreased expression of thermogenic markers, such as UCP1, peroxisome proliferator-activated receptor γ (PPARγ), PGC-1α, and β3-AR, factors known to stimulate UCP1 expression (Xu et al. 2011). In agreement with this evidence, simultaneous knockout of ERα from both SF1 and POMC neurons caused hypometabolism, hyperphagia, and severe obesity (Xu et al. 2011). Nonetheless, it must be kept in mind that part of E2 effects on BAT thermogenesis might be mediated by ERβ pathways, as administration of an ERβ-selective agonist to HFD-fed female mice induced expression of UCP1 in BAT, thereby decreasing obesity (Yepuru et al. 2010). A very recent study has also identified a new subgroup of ERα-positive cells in the ventrolateral region of the ventromedial hypothalamus (VMHVL) that modulates hormone-dependent female locomotion (Correa et al. 2015). Developmental disruption of those neurons leads to inactivity and obesity, independently of changes in fertility and more importantly in BAT thermogenesis (Correa et al. 2015), demonstrating that E2 exerts different actions on VMH neuronal populations to control energy balance.

Recent data have also established that AMPK is the key molecular mediator of E2's actions in the VMH. Actually, administration of E2 selectively within the VMH induces a catabolic response, characterized by increased neuronal activity in the RPa and IO, increased sympathetic firing, elevated BAT and core temperature , higher energy expenditure, and reduced respiratory quotient (RQ), leading to weight loss. All those effects were associated with decreased hypothalamic AMPK activity in the VMH (Fig. 1). Remarkably, virogenetic-driven reactivation

of AMPK in that nucleus prevented E2-induced stimulation of brown fat thermogenesis and weight loss (Martinez de Morentin et al. 2014). Notably, this AMPK(VMH)-SNS-BAT axis is also shared by other molecules modulating brown fat thermogenesis, such as leptin, THs, nicotine bone morphogenetic protein 8b (BMP8b), and glucagon-like peptide 1 (GLP1) (Lopez et al. 2010; Whittle et al. 2012; Tanida et al. 2013; Beiroa et al. 2014), demonstrating that this is a canonical mechanism regulating energy balance (Contreras et al. 2015). Interestingly, it is necessary to mention that some studies find a marked dichotomous action of estrogens. On one hand, peripheral administration in OVX mice increases lipid oxidation, oxygen consumption, and energy expenditure without affecting overall locomotor activity and, importantly, without increasing BAT-mediated thermogenesis. Conversely, central E2 administration increases locomotor activity, body temperature, and expression of BAT thermogenic markers (Yonezawa et al. 2012; Kim et al. 2014b). However, it is interesting to note that while the central thermogenic effects of E2 are maintained under HFD (Yonezawa et al. 2012), they are not present during pregnancy. In fact, although E2 actually inhibits hypothalamic AMPK during gestation, pregnant rats show decreased temperature and BAT function (Martinez de Morentin et al. 2015). These data suggest that pregnancy induces a state of resistance to the anorexigenic and thermogenic actions of E2, which probably preserves the physiological situation of gestational hyperphagia and adiposity, which is essential to successfully face the metabolic requests of embryonic development.

As explained above, there is another physiological mechanism closely related to BAT thermogenesis, namely, browning, whose biological effect is the transformation of the white adipocytes into a tissue with phenotypic characteristics of brown fat and as consequence greater thermogenic capacity. Therefore, body energy expenditure is enhanced and body weight reduced (Nedergaard and Cannon 2014; Contreras et al. 2015, 2016). While the effects of E2 on thermogenesis of brown fat have been extensively studied, the action of estrogens on browning of white fat remains poorly investigated. Recent data demonstrate that female mice are more responsive than males to the recruitment of brown adipocytes in gonadal WAT, and this difference is associated with greater levels of E2-dependent sympathetic innervation, thereby increasing the presence beige/brite adipocytes in female mice (Kim et al. 2016). On the other hand, ERβ influences browning of subcutaneous WAT via its actions on both sympathetic ganglia and white adipocytes. Pharmacological ERβ agonist induces browning of subcutaneous fat pad in obese female mice (Miao et al. 2016). Interestingly, human data has also emerged recently, reporting that women more potently induce browning of the perirenal WAT than men. This gender dimorphism was attributed to the sex-specific intrinsic characteristics of the mesenchymal stem cells (MSCs) in the white fat (Van Den Beukel et al. 2015).

Conclusions

In summary, estrogens are well known by its role in the control reproductive function, but its impact in energy balance and metabolism is now clearly established. As detailed in this chapter, these hormones act at central and peripheral levels, and the molecular mechanisms mediating those actions have begun to be discovered only recently. In this context, it is well established that E2 operates at central level in the hypothalamus through the AMPK(VMH)-SNS-BAT pathway in a way which is shared with other molecules such as leptin, THs, nicotine, BMP8b, and GLP1 (Lopez et al. 2010; Whittle et al. 2012; Tanida et al. 2013; Beiroa et al. 2014). Interestingly, therapeutic strategies against obesity are being developed, using estrogens and more specifically E2 as druggable target (Finan et al. 2012; Cao et al. 2014). For this, Richard DiMarchi has developed conjugated molecules with different functional moieties that aim to use the beneficial effects of E2 for the treatment of obesity, with promising results (Van Den Beukel et al. 2015). In any case, and having into account all the open questions, there is no doubt that investigation of E2 regulation of BAT thermogenesis and the central mechanism by which estrogens and AMPK modulate energy balance will be crucial in the design of new treatments for fighting against obesity and other metabolic diseases.

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Brain Estrogens and Feeding Behavior

Yong Xu

Abstract Estrogens play essential roles in suppressing food intake and preventing body weight gain. Tremendous research efforts have been focused on estrogen physiology in the context of feeding control. Estrogen receptors and the related signals have been attractive targets for development of new obesity therapies. This chapter focuses on the functional interactions between brain estrogens and other appetite-regulatory signals, the critical estrogen receptor isoforms and distinct brain regions that mediate effects of estrogens on feeding, and the intracellular signals that are involved.

Introduction

Dramatic decline in circulating 17β-estradiol (E2) in postmenopausal women has been associated with the development of obesity, type II diabetes, and the metabolic syndrome (Allende-Vigo 2008). While supplement of E2 may ameliorate these risks, the application of estrogen therapy in postmenopausal women has been very controversial. Since E2 can act upon several forms of estrogen receptors (ERs) and these ERs are coupled with complex intracellular signals, the body weight-lowering benefits provided by E2 are often associated with increased risks of heart diseases, reproductive endocrine toxicity, and breast cancer (Billeci et al. 2008). Thus, tremendous efforts have been focused on identifying the critical ER isoforms, the specific action sites of ERs, and the ER-coupled intracellular signals that are required for estrogenic actions on body weight control.

ERs have been demonstrated to be located throughout the brain and accumulating evidence pointed to a potent effect of E2, through brain ERs, to suppress food intake. This chapter will focus on functions of brain estrogens in the control of feeding behavior. We will discuss the functional interactions between E2 and other appetite-regulatory signals, the critical ER isoforms and distinct brain regions that

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mediate E2's inhibitory effects on feeding, and the intracellular signals that are involved. It should be noted that actions of ERs are also important for regulation of energy expenditure, which will not be discussed in this chapter. Audience are directed to read the other excellent chapter on this topic.

Interactions Between E2 and Other Appetite-Regulatory Signals

It has been well established that estrogens play an essential role in preventing body weight gain. For example, the withdrawal of endogenous estrogens by ovariectomy (OVX) in female animals leads to body weight gain and hyperadiposity, and this obese phenotype can be prevented by E2 treatment which suppresses food intake in OVX female animals (Blaustein 1976; Drewett 1973; Geary et al. 2001; Roesch 2006; Rogers et al. 2009; Wallen et al. 2001). Notably, oral administration of conjugated equine estrogens has been shown to reduce body weight of OVX female mice without significantly influencing food intake (Mauvais-Jarvis 2017; Kim et al. 2014), suggesting that estrogen administration with different preparations, doses, and routes may regulate energy balance via distinct mechanisms. Nevertheless, it is clear that exogenous E2 can robustly inhibit food intake in OVX female animals. Here we will first discuss the functional interactions between E2 and a number of other hormonal/neuronal signals that also regulate feeding behaviors.

Leptin

Leptin is a circulating adipokine that plays critical roles in the regulation of body mass and body composition (Leibel et al. 1997). Leptin contributes to the regulation of body weight by influencing both food intake (Alingh Prins et al. 1986; McLaughlin and Baile 1981) and energy expenditure (Dauncey 1986; Dauncey and Brown 1987; Trayhurn et al. 1977). Biological actions of leptin are thought to be primarily mediated by the long-form leptin receptor (also known as LEPR-B) (Tartaglia 1997). Accumulating evidence indicates that leptin produces anti-obesity effects by acting via LEPR-B in the brain (Halaas et al. 1997). LEPR-B is localized in several brain areas including the arcuate nucleus of the hypothalamus (ARH). It has been reported that LEPR-B expression in the ARH is co-localized with ERα (Diano et al. 1998), and E2 has been suggested to regulate the expression of LEPR-B mRNA in the ARH (Lindell et al. 2001). The co-localization of these two receptors suggests a closely coupled interaction between these peripheral signals in the regulation of energy homeostasis. Further, higher levels of E2 have been associated with increased leptin sensitivity (Clegg et al. 2006). In particular, OVX lowers sensitivity to central leptin when compared to intact females, and this can be restored by E2 treatment

(Clegg et al. 2006). In addition, exogenous E2 administration to male rats will increase sensitivity to central leptin (Clegg et al. 2006).

Cholecystokinin

Cholecystokinin (CCK) is synthesized and released from cells of the upper intestine and acts on abdominal CCK-A receptors. It plays a variety of roles in the digestive process, including slowing gastric emptying and intestinal motility (Raybould 2007). CCK exerts its satiety action primarily by activating subdiaphragmatic vagal afferent neurons (Raybould 2007). CCK-A antagonists decreased food intake to a greater extent when intact females were in estrus or in E2-treated OVX rats, and this effect was lessened in diestrus rats (Asarian and Geary 1999, 2007; Eckel and Geary 1999; Huang et al. 1993). E2 increases the potency of CCK by increasing the sensitivity of vagal CCK-A receptors, but does not increased CCK secretion or the number of CCK-A receptors (Asarian and Geary 1999; Butera et al. 1993; Geary et al. 1996). CCK influences meal size, and this has been characterized in male rats by examining the pattern of c-Fos expression after consumption (DiNardo and Travers 1997; Eckel and Geary 2001; Park and Carr 1998; Rinaman et al. 1998) or injection of CCK (Li and Rowland 1994; Rinaman et al. 1995). E2 treatment in OVX female rats increased the number of feeding- and CCK-induced c-Fos-positive cells within the nucleus of the solitary tract (NTS), the paraventricular nucleus of the hypothalamus (PVH), and the central nucleus of the amygdala (Eckel and Geary 2001; Eckel et al. 2002). These data suggest that exogenous E2 may decrease meal size by selectively increasing neuronal activity in multiple brain areas that control meal size. It is currently unknown whether a similar mechanism underlies the decrease in meal size or increase in CCK satiation, which occurs during estrus in cycling animals.

Although there is solid evidence that E2 increases the potency by which CCK exerts its direct, inhibitory control over meal size, such an interaction does not completely account for the decrease in food intake during estrus in gonadally intact animals or following E2 treatment in OVX rodents. For example, blocking the release of CCK during a meal attenuated, but did not block, the phasic inhibitory decrease in meal size, observed during estrus in gonadally intact female rats (Eckel and Geary 1999). Additionally, endogenous CCK does not appear to play a role in the tonic inhibitory action of E2 (Asarian and Geary 1999; Eckel and Geary 1999). Thus, E2 must modulate the potency of other stimuli that directly generate negative feedback during a meal.

Ghrelin

Ghrelin is produced in the stomach and acts on growth hormone secretagogue receptors (GHSRs) to increase food intake. Exogenous ghrelin is less potent in intact female rats than in male rats or OVX females (Clegg et al. 2007). Central

intra-third ventricular (i3vt) or peripheral ghrelin administration reliably increased feeding in intact male rats and OVX females (Arnold et al. 2006; Davidson et al. 2005; Nakazato et al. 2001; Tschop et al. 2000; Wren et al. 2001a, b); however, the threshold for a significant increase in feeding using either administration route was significantly reduced in intact females than in males or OVX females (Clegg et al. 2007). When OVX female rats were treated with E2, moderate intraperitoneal (i.p.) or i3vt doses of ghrelin no longer stimulated eating. Together, these data demonstrate that E2 reduces the orexigenic potency of ghrelin. Lastly, E2 treatment reduced the eating-stimulatory effect of i3vt ghrelin in male rats, indicating that the estrogenic effect exists in both sexes, which is a point of potential therapeutic relevance (Clegg et al. 2007). Careful attention to sex differences and gonadal hormone status should be included in the development of any ghrelin-based clinical control for eating behaviors.

The eating-stimulatory effect of ghrelin varies across different phases of the ovarian cycle in gonad-intact female rats (Clegg et al. 2007). Administration of i3vt ghrelin had no reliable overall effect when cycle day was not taken into account. However, when the cycle day was considered, i3vt ghrelin increased eating during diestrus 1 and diestrus 2, but not during proestrus or estrus. In addition, in OVX female rats treated with E2, ghrelin increased food intake on the days that modeled diestrus in intact rats, but not on the days that modeled proestrus or estrus. This indicates that there are cyclic variations in eating in female rats and mice, and spontaneous food intake is maximal during diestrus and minimal during estrus. Therefore, the analogous peri-ovulatory decreases in eating in women may be due to changes in estrogenic tone that affect ghrelin's eating-stimulatory action (Clegg et al. 2007). To assess the importance of ghrelin signaling in OVX-induced hyperphagia and obesity, Ghsr−/− mice lacking GHSR were ovariectomized (Clegg et al. 2007). The OVX mice, which were similar to wild-type mice in body weight and food intake presurgery, showed no increase in food intake or body weight gain after surgery. This indicates that E2 tonically inhibits endogenous ghrelin signaling in female mice and that release from this inhibition is necessary for OVX mice to increase food intake and body weight. This mechanism may account for other sex differences in eating and weight regulation previously reported in Ghsr−/− mice. For example, female Ghsr−/− mice accumulated less body weight and adiposity when given a high-fat diet (Zigman et al. 2005). Also, the magnitude of the differences in adiposity observed between Ghsr−/− and wild-type mice was greater in females than in males (Clegg et al. 2007). Ghrelin signaling appears to be a necessary component of the estrogenic control of eating and weight regulation (Clegg et al. 2007).

Central Melanocortin System

The central melanocortin system is comprised of neurons which produce endogenous melanocortins and the downstream neurons that express melanocortin receptors (Cone 2005; Elmquist et al. 1999; Williams and Schwartz 2005). The

melanocortin neurons include those expressing pro-opiomelanocortin (POMC) and those expressing neuropeptide Y (NPY) and agouti-related peptide (AgRP), which are both located in the arcuate nucleus (ARH). While POMC neurons synthesize and secret an anorexigenic peptide, α-melanocyte-stimulating hormone (α-MSH), to activate melanocortin receptors, NPY/AgRP neurons release orexigenic peptides, NPY and AgRP (Cone 2005; Elmquist et al. 1999; Williams and Schwartz 2005). Notably, AgRP is the endogenous antagonist of the melanocortin receptors (Cone 2005; Elmquist et al. 1999; Williams and Schwartz 2005). POMC and NPY/AgRP populations have been long believed to be the primary central regulators of energy homeostasis (Cone 1999; Huszar et al. 1997). In particular, ARH POMC neurons are essential to prevent obesity, as ablation of these neurons leads to hyperphagia and obesity (Xu et al. 2005; Zhan et al. 2013). Multiple hormonal and neural signals regulate POMC neuron firing to regulate body weight balance (Morton et al. 2006). For example, leptin, an adipocyte-derived hormone, stimulates POMC neuron firing to prevent obesity (Balthasar et al. 2004). Similarly, the brain neurotransmitter, serotonin, stimulates POMC neural activity through serotonin 2C receptors (5-HT_{2C}Rs) to suppress feeding and prevent obesity (Berglund et al. 2013; Sohn et al. 2011). Consistently, selective stimulation of ARH POMC neurons decreases food intake and body weight (Zhan et al. 2013). Together, these findings well establish that POMC neural activity is one key physiological regulator of energy homeostasis.

In addition to POMC neural activity, expression of POMC gene itself is physiologically important for body weight control. POMC gene products, including α-MSH, are known to act upon brain melanocortin 3 and 4 receptors to suppress food intake and body weight gain (Huszar et al. 1997; Vaisse et al. 1998; Yeo et al. 1998). Recent evidence indicates that certain stimuli (e.g., cannabinoid) can specifically increase production of β-endorphin (another POMC gene product) and result in increased feeding (Koch et al. 2015). Nevertheless, massive obesity is observed in mice with POMC gene deficiency (Yaswen et al. 1999). Consistently, humans carrying loss-of-function mutations in the POMC gene develop obesity (Challis et al. 2002; Creemers et al. 2008; Farooqi et al. 2006). Thus, POMC gene expression is another fundamental function of POMC neurons in the context of energy balance.

POMC levels are responsive to estrogenic signals. POMC mRNA levels fluctuate over the course of the estrous cycle, with the most dramatic changes on the day of proestrus (Wise et al. 1990). POMC neurons express ERα (Miller et al. 1995), and OVX decreases POMC mRNA, which is reversed by E2 treatment (Pelletier et al. 2007). Lower POMC levels are also observed in ERα knockout mice (Hirosawa et al. 2008). Using electron microscopy, the Horvath group has reported that the number of excitatory synaptic inputs to ARH POMC neurons rises as female mice enter proestrus when E2 levels are high (Gao et al. 2007). Further, central E2 administration rapidly increases the excitatory synapses on POMC neurons, an effect that is also reflected by increased miniature excitatory postsynaptic current recorded from POMC-GFP neurons (Gao et al. 2007). These synaptological rearrangements in POMC neurons are tightly paralleled with the effects of E2 on food intake, energy

expenditure, and body weight (Gao et al. 2007). E2 activates POMC neurons partly via PI3K-mediated mechanisms in female animals (Malyala et al. 2008; Qiu et al. 2003; Zhu et al. 2015). These studies demonstrated that E2 directly acts on POMC neurons and regulates their cellular activity.

NPY

Neuropeptide Y (NPY) is an endogenous neuropeptide that is co-expressed by ARH AgRP neurons. Similar to AgRP, central administration of NPY increases food intake (Paez and Myers 1991; Pierroz et al. 1996). However, neither single deletion of AgRP or NPY nor double deletion of AgRP and NPY in mice leads to abnormality in food intake and body weight (Qian et al. 2002). It has been argued that the lack of phenotypes in animals with gene mutations at the embryonic stage could results from possible genetic compensations during the early development. To circumvent this issue and establish the role of NPY/AgRP neurons in the control of body weight, several groups have used distinct genetic mouse models to achieve selective ablation of NPY/AgRP neurons during the adulthood. For example, Palmiter and colleagues used a mouse model which expresses diphtheria toxin receptor (DTR) only in AgRP-expressing cells. Injections of diphtheria toxin into these mice result in selective ablation of NPY/AgRP neurons. They found that ablation of NPY/AgRP neurons during the adulthood leads to rapid decreases in food intake and body weight (Gropp et al. 2005). Similarly, Barsh and colleagues crossed AgRP-Cre transgenic mice to a loxP-flanked mitochondrial transcription factor A (Tfam) allele to selectively delete Tfam from AgRP cells, which causes progressive loss of this population as animals grow (Xu et al. 2005). These mice with NPY/AgRP ablation display modest lean phenotypes (Xu et al. 2005). Finally, targeted expression of a neurotoxic ataxin-3 to AgRP-expressing neurons resulted in loss of the NPY/AgRP neurons and decreases in food intake and body weight (Bewick et al. 2005). Results from these three different models with genetic ablation of NPY/AgRP neuron all support a physiological role of these neurons in promoting feeding and body weight gain. This notion has been further supported by recent studies using genetic tools to selectively manipulate electrophysiological properties of NPY/AgRP neurons. For example, Aponte and colleagues generated mice expressing the light-activated cation channel, channelrhodopsin-2 (ChR2), only in NPY/AgRP neurons (Aponte et al. 2011). Light stimulation in these mice induces rapid activation of NPY/AgRP neurons, which results in increased feeding (Aponte et al. 2011). Similarly, Krashes and colleagues used the designer receptors exclusively activated by designer drugs (DREADD) (Alexander et al. 2009; Ferguson et al. 2011) to rapidly depolarize or hyperpolarize NPY/AgRP neurons in mice (Krashes et al. 2011). While depolarization of NPY/AgRP neurons promotes eating, hyperpolarization of these neurons inhibits eating (Krashes et al. 2011). Collectively, these genetic mouse models with NPY/AgRP ablated or stimulation/inhibition during the adulthood demonstrate a physiological role of NPY/AgRP neurons in the control of energy homeostasis.

In an ex vivo hypothalamic neuronal cell line, N-38, E2 affected the expression of NPY in a biphasic manner, which corresponded to changes in the ERα:ERβ ratio. When the ERα:ERβ ratio was high, NPY transcription was repressed; conversely, when the ratio was low, NPY transcription was stimulated. Additionally, a recent study has demonstrated that NPY/AgRP neurons are required to mediate the anorexigenic effects of estrogens. In this study, Xu and co-workers showed that hypothalamic expression of NPY and AgRP is tightly regulated across the estrus cycle, with the lowest levels during estrus which coincides with the plasma estrogen peak and feeding nadir in wild-type mice (Olofsson et al. 2009). They further showed that central E2 administration suppresses fasting-induced c-Fos activation in NPY/AgRP neurons and blunts the re-feeding response (Olofsson et al. 2009). Importantly, the cyclic changes in food intake and E2-induced anorexia are blunted in mice with degenerated NPY/AgRP neurons (Olofsson et al. 2009). This study indicates that neurons co-expressing NPY and AgRP are functionally required for the cyclic changes in feeding across estrous cycle and that NPY/AgRP neurons are essential mediators of E2's anorexigenic function. Surprisingly, these authors also found that ERα is completely excluded from NPY/AgRP neurons in the mouse hypothalamus (Olofsson et al. 2009), suggesting that E2 may regulate these neurons indirectly via presynaptic neurons that express ERα. However, these ERα-expressing neurons that act on NPY/AgRP neurons remain to be identified.

Serotonin

E2 decreases food intake by selectively influencing the neural controls of meal size, which likely requires the serotonergic system. The brain serotonin (5-HT) is primarily synthesized by neurons in the dorsal raphe nucleus (DRN) in the midbrain, which projects to other midbrain regions and the hypothalamus (Lechin et al. 2006). Hunger decreases 5-HT release from the DRN, while eating rapidly increases it (De Fanti et al. 2001), suggesting that dynamic 5-HT bioavailability may participate in the regulation of feeding behavior. Indeed, d-fenfluramine (d-Fen), a pharmacological agent that increases 5-HT content (Rowland and Carlton 1986), showed a potent anorexigenic activity in rodents and humans (Foltin and Moran 1989; McGuirk et al. 1991; Rogers and Blundell 1979). Conversely, treatments that suppress central 5-HT signals produce hyperphagia and weight gain (Blundell and Leshem 1974; Geyer et al. 1976; Ghosh and Parvathy 1973; Saller and Stricker 1976).

E2 was reported to increase the expression of the serotonin transporter (5-HTT) in the dorsal and median raphe nuclei in OVX female rats, allowing E2 to regulate the 5-HT system (Rivera et al. 2009). Indeed, E2 enhances the anorexia induced by increased serotonergic neurotransmission (Rivera et al. 2012). This E2/5-HT interaction may also have a clinical relevance, since dysfunctions in brain 5-HT reuptake are associated with anorexia nervosa, an eating disorder that primarily affects women (Bailer et al. 2007; Rowland et al. 2004).

Multiple Brain ERα Populations Regulate Feeding Behavior

The estrogenic effects on feeding behavior are believed to be primarily mediated by ERα, one of the "classical" estrogen receptors. Humans or mice with mutations in the ERα (Esr1) gene are obese (Heine et al. 2000; Okura et al. 2003). Further, deletion of ERα in mice blocks the anorexigenic effects of E2 treatment (Geary et al. 2001). Early studies showed that microinjections of E2 into various brain regions change animal's feeding behavior (Butera and Beikirch 1989; Palmer and Gray 1986), suggesting that ERα expressed in the brain is important for the regulation of food intake. This notion was further supported by observations from various genetic mouse models. For example, we crossed mice carrying loxP-flanked ERα alleles ($ER\alpha^{lox/lox}$) (Feng et al. 2007) to the nestin-Cre transgenic mice (Bruning et al. 2000) to produce mice lacking ERα only in the brain (Xu et al. 2011b). We demonstrated that female mutant mice develop obesity, characteristic of increased body weight and body fat. Obesity in these mice is associated with hyperphagia, decreased energy expenditure and decreased physical activity, which may all contribute to the development of obesity (Xu et al. 2011b). Notably, female mice lacking ERα in the brain display significantly elevated E2 in the circulation (Xu et al. 2011b), presumably due to the impaired negative feedback regulation by estrogens. Given that these mice develop robust obese phenotypes despite the higher E2 in the circulation, these observations further argue that compared to ERα expressed in peripheral tissues, brain ERα plays predominant roles in the regulation of energy balance.

ERα is abundantly expressed in multiple brain regions that are implicated in the regulation of feeding behavior. These include the ARH, the NTS, the DRN, and the medial preoptic area (MPOA) (Merchenthaler et al. 2004). Thus, an important question is which ERα population(s) in the brain are critical for the regulation of food intake. As discussed below, several groups have used genetic approaches to dissect out the physiological roles of ERα in various brain regions in the context of body weight control.

ERα in POMC Neurons

About 20–30% POMC neurons in the ARH co-express ERα (de Souza et al. 2011; Miller et al. 1995; Xu et al. 2011a). Using electron microscopy, Gao et al. reported that E2 can increase excitatory synaptic inputs onto ARH POMC neurons, which is associated with increased miniature excitatory postsynaptic current (Gao et al. 2007). Similarly, Malyala et al. reported that E2 stimulates POMC neurons by rapidly uncoupling $GABA_B$ receptors from the G-protein-gated inwardly rectifying K^+ channels (Malyala et al. 2008). We reported that a selective ERα agonist, propylpyrazole triol (PPT), can rapidly depolarize ERα-positive POMC neurons (Saito et al. 2015). Further, we showed that female mice lacking ERα only in POMC neurons develop hyperphagia and modest body weight gain (Xu et al. 2011b). In addition,

E2-induced anorexigenic effects are blunted in these mutant mice lacking ERα only in POMC neurons (Zhu et al. 2015). Together, these observations indicate that ERα in POMC neurons mediates E2's actions to suppress food intake (Xu et al. 2011b).

ERα in the NTS

ERα are also present in the brainstem, including the NTS (Merchenthaler et al. 2004; Osterlund et al. 1998; Schlenker and Hansen 2006). Roles of this ERα population have not yet been fully illustrated. Geary and co-workers showed that treatment of E2 in wild-type female mice suppresses food intake and potentiates CCK-induced satiation, which are accompanied by increased activity in NTS neurons (Asarian and Geary 2007; Geary et al. 2001). Interestingly, these responses are all abolished in female mice lacking ERα (Asarian and Geary 2007; Geary et al. 2001). Further, it is shown that direct administration of E2 in the NTS potentiates CCK-induced satiety signals (Thammacharoen et al. 2008). Collectively, these findings support the notion that ERα in the brainstem, such as in the NTS, may be another physiologically important site to mediate the anorexigenic effects of E2.

ERα in the DRN

ERα is abundantly expressed in the DRN (Merchenthaler et al. 2004). We further demonstrated that the majority of these ERα-positive neurons in the DRN are 5-HT neurons (Cao et al. 2014). Consistent with earlier results that E2 increases neural activities (demonstrated by c-Fos immunoreactivity) in the DRN (Dalmasso et al. 2011; Robichaud and Debonnel 2005), we showed that propylpyrazole triol (PPT, a selective ERα agonist) activates identified DRN 5-HT neurons via an ERα-dependent mechanism (Cao et al. 2014). Interestingly, Santollo et al. reported that microinjections of E2 into the DRN decrease food intake in female rats (Santollo et al. 2011). To further examine the roles of ERα in DRN 5-HT neurons, we crossed ERα$^{lox/lox}$ mice and TPH2-CreER^{T2} to generate mice lacking ERα only in 5-HT neurons (Cao et al. 2014). Interestingly, while we failed to detect any differences in basal food intake and body weight in mutant females, these mice are resistant to E2's effects to suppress binge-like eating (Cao et al. 2014), food intake that is not driven by hunger but rather than by rewards or hedonic cues. These results suggest that ERα expressed by DRN 5-HT neurons primarily functions to suppress hedonic feeding, while its roles in the hunger-driven feeding may be minor.

Certainly, the physiological functions of ERα in other brain regions have not been fully revealed. For example, Santollo et al. reported that microinjections of E2 into the MPOA decrease food intake in female rats (Santollo et al. 2011). Further, earlier studies showed that E2 implanted in the paraventricular nucleus of the hypothalamus (PVH) decreases food intake and body weight in OVX female rats (Butera

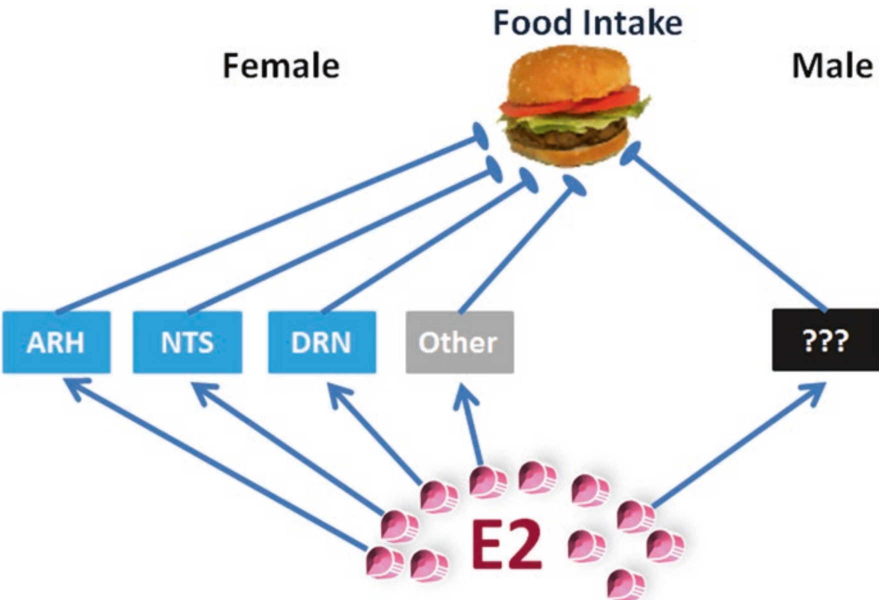

Fig. 1 Brain regions mediating E2-induced anorexia in female and male animals. In female brains, the ARH, NTS, and DRN are identified regions that mediate E2-induced anorexia; a few other regions remained to be further tested. In male brains, the exact acting sites of E2 to suppress food intake remain to be identified

and Beikirch 1989) in the absence of any signs of peripheral estrogenic stimulation. Additionally, the anorexigenic effects of subcutaneous E2 were blunted in rats with PVH lesions (Butera et al. 1992). However, subsequent studies failed to reproduce these phenotypes in rats with PVH implants (Hrupka et al. 2002). In addition, it needs to be pointed out that the PVH expresses low levels of ERα but high levels of ERβ (Merchenthaler et al. 2004). In summary, while a few structures in the female brain (e.g., ARH, NTS, and DRN) have been identified as critical mediators for E2-induced anorexia, the functions of other brain regions (e.g., the MPOA and the PVH) and the underlying mechanisms warrant further validation with genetic models (Fig. 1).

ERα in Male Brains

It is clear that actions of ERα also prevent obesity in males. For example, ERα gene deficiency results in obesity in male mice (Callewaert et al. 2009; Heine et al. 2000) and in men (Grumbach and Auchus 1999; Smith et al. 1994). In addition, administration of E2 or its analogs reduces body weight in male mice (Finan et al. 2012;

Gao et al. 2007). The major male sex hormone, testosterone, can be converted into E2 by aromatase, and both male and female aromatase knockout mice develop obesity (Jones et al. 2000). Notably, abundant aromatase is expressed in a few brain regions (Wu et al. 2009), which makes it possible that ERα in these male brain regions could be exposed to high levels of E2 despite the lack of circulating estrogens. Consistent with this notion, we showed that male mice lacking ERα in the brain develop obesity (Xu et al. 2011b), arguing that brain ERα also regulates male energy balance. However, deletion of ERα in POMC neurons or 5-HT neurons, although produces feeding and/or body weight phenotypes in females, fails to affect food intake in male mice. Thus, we speculate that different brain ERα population(s) may be responsible for E2's actions to inhibit feeding in males, which remain to be identified (Fig. 1).

ERα-Coupled Intracellular Signals

In addition to the sites of ERα actions, another major question in the field is what intracellular signals mediate ERα effects on feeding behavior and body weight balance. ERα-coupled intracellular events can be divided into several modes. First, subsets of intracellular ERα are concentrated on the cytomembrane and in the cytosol, where it regulates rapid signaling pathways, including the PI3K/Akt pathway and the AMPK pathway. It has been reported that E2 rapidly stimulates the firing activates of ARH POMC neurons (Malyala et al. 2008; Zhu et al. 2015), effects that can be blocked by a PI3K inhibitor. Importantly, genetic inhibition of PI3K in ARH POMC neurons blunts anti-obesity effects of E2 (Saito et al. 2016; Zhu et al. 2015), highlighting an important role of PI3K in mediating E2's effects on food intake and body weight balance. This notion is supported by observations from a NERKI knock-in mouse model in which E207A/G208A mutations were introduced in the DNA-binding domain of ERα which abolishes ERα's binding to the ERE motifs on the chromosome (Jakacka et al. 2002). In these mice, metabolic phenotypes affected in ERα knockout mice including body weight, glucose homeostasis, energy expenditure, and physical activity are restored to nearly normal levels (Park et al. 2011), suggesting that the ERE-dependent ERα functions are not required to maintain body weight.

However, it is worth noting that Pedram et al. generated a transgenic MOER mouse model, in which the full-length ERα protein is replaced by the E domain of the receptor, which only exists on the cytomembrane and retains capacity of initiating rapid signals (e.g., PI3K) (Pedram et al. 2008). Importantly, no ERα activity is present in the cytosol or in the nucleus in MOER mice. Interestingly, MOER mice show similar obese phenotypes as ERα knockout mice (Pedram et al. 2008). Thus, these findings suggest that rapid signals initiated by cytomembrane ERα are not sufficient to mediate anti-obesity effects of E2 (Pedram et al.

2008). Further, Handgraaf et al. recently reported that mice lacking the activation function motif-2 (AF-2) developed obesity and diabetes (Handgraaf et al. 2013). Since AF-2 domain is required for transcriptional activity of ERα, these results highlighted the importance of transcriptional activity of ERα in the regulation of energy balance.

Thus, the current findings indicate that both ERα-initiated rapid signaling pathways and ERα's transcriptional activity are required to mediate estrogenic actions to prevent body weight gain. More research efforts are needed to further pinpoint the intracellular and molecular mechanisms that mediate ERα functions to regulate feeding behavior and body weight balance.

ERβ

Compared to ERα, ERβ, another classic ER, has received less attention at least in the context of body weight balance. An earlier study by Ohlsson et al. reported that chow-fed mice (both male and female) with global deficiency in ERβ show normal body weight and fat mass compared to wild-type mice (Ohlsson et al. 2000). In addition, the authors reported that mice with compound knockout of both ERα and ERβ develop obesity with the same severity as mice only lacking ERα (Ohlsson et al. 2000). Consistent with this, both Santollo et al. (2007) and Roesch (2006) found that an ERβ agonist, diarylpropionitrile (DPN), has no effects on food intake and body weight in chow-fed OVX rats, while PPT (the ERα agonist) at similar doses can significantly reduce food intake and body weight. While these earlier studies suggest a minor role of ERβ in body weight control in chow-fed animals, Foryst-Ludwig et al. demonstrated that ERβ knockout mice, when fed on a high-fat diet (HFD), developed obesity compared to HFD-fed wild-type mice (Foryst-Ludwig et al. 2008). This increased sensitivity to diet-induced obesity is associated with normal food intake but increased energy expenditure and decreased fat oxidation (Foryst-Ludwig et al. 2008). Consistently, Yepuru et al. developed new selective ERβ agonists (β-LGNDs) and found these agonists attenuate HFD-induced body weight gain associated with increased energy expenditure (Yepuru et al. 2010). Thus, the current data suggest that ERβ may play an important role in preventing obesity when animals are challenged by obesogenic diets, while ERβ's functions in animals fed on regular chow diets appear to be minimal. Certainly, the ERβ-mediated control of energy homeostasis warrants further investigation. For example, the action sites of ERβ on energy balance remain to be confirmed, although both Foryst-Ludwig et al. and Yepuru et al. suggested a contribution from ERβ in the peripheral tissues (Foryst-Ludwig et al. 2008; Yepuru et al. 2010).

GPR30

GPR30 (also known as GPER) is a G-protein-coupled estrogen receptor, bound to the cell membrane. In vitro studies confirmed that E2 binds to GPR30. Body weight phenotypes among several independent GPR30 knockout mouse lines are controversial. For example, both Haas et al. (2009) and Sharma et al. (2013) observed obese phenotypes in male and female GPR30 knockout mice, which were generated by Wang et al. (2008); however, Liu et al. reported no difference in body weight in the same GPR30 knockout stain (Liu et al. 2009). Otto et al. constructed an independent GPR30 knockout line and found no obese phenotypes in female mutants (2009). Interestingly, another GPR30 knockout line generated by Martensson et al. showed reduced body weight only in females, but not in males (Martensson et al. 2009). More recently, Davis et al. carefully characterized Wang's GPR30 knockout mice and reported that both male and female mutants are significantly heavier than wild-type littermates, which appears to depend on reduced energy expenditure independent of physical activity, but not on food intake (Davis et al. 2014). Importantly, body weight-lowering effects of E2 are attenuated in OVX GPR30 knockout mice compared to OVX wild-type mice (Davis et al. 2014). The discrepancy from these studies may be attributed to different strategies to construct the GPR30 knockout alleles, different genetic backgrounds that mice were maintained on, different facility environments, etc. Nevertheless, observations from Wang's GPR30 knockout line are largely consistent and suggest a potential role of GPR30 in estrogenic regulation on body weight homeostasis. Obviously, effects of GPR30 on energy balance need further validation.

Conclusions

In this chapter, we have discussed the important roles of E2 to suppress food intake and the functional interactions between E2 and other endogenous appetite-regulatory signals. We summarized current evidence regarding the functions of ERα in different brain regions in the regulation of feeding behavior. With regard to ERα-coupled intracellular signals, both rapid signals (e.g., PI3K) and "classic" transcriptional activities of ERα appear to contribute to E2's regulation on food intake and body weight, although the overall picture of this complex signal network is still not clear. Effects of other ERs (ERβ and GRP30) on body weight balance may have been underappreciated in the past; fortunately, revisits of various knockout models started to reveal previously unrecognized roles of these receptors, and these warrant further investigations.

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Sex Differences and Role of Estradiol in Hypoglycemia-Associated Counter-Regulation

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Abstract Vital nerve cell functions, including maintenance of transmembrane voltage and information transfer, occur at high energy expense. Inadequate provision of the obligate metabolic fuel glucose exposes neurons to risk of dysfunction or injury. Clinical hypoglycemia rarely occurs in nondiabetic individuals but is an unfortunate regular occurrence in patients with type 1 or advanced insulin-treated type 2 diabetes mellitus. Requisite strict glycemic control, involving treatment with insulin, sulfonylureas, or glinides, can cause frequent episodes of iatrogenic hypoglycemia due to defective counter-regulation, including reduced glycemic thresholds and diminished magnitude of motor responses. Multiple components of the body's far-reaching energy balance regulatory network, including the hindbrain dorsal vagal complex, provide dynamic readout of cellular energetic disequilibrium, signals that are utilized by the hypothalamus to shape counterregulatory autonomic, neuroendocrine, and behavioral outflow toward restoration of glucostasis. The ovarian steroid hormone 17β-estradiol acts on central substrates to preserve nerve cell energy stability brain-wide, thereby providing neuroprotection against bio-energetic insults such as neurodegenerative diseases and acute brain ischemia. The current review highlights recent evidence implicating estrogen in gluco-regulation in females by control of hindbrain metabolic sensor screening and signaling of hypoglycemia-associated neuro-energetic instability. It is anticipated that new understanding of the mechanistic basis of how estradiol influences metabolic sensory input from this critical brain locus to discrete downstream regulatory network substrates will likely reveal viable new molecular targets for therapeutic simulation of hormone actions that promote positive neuronal metabolic state during acute and recurring hypoglycemia.

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Abbreviations

4CIN	Alpha-cyano-4-hydroxycinnamate
6-OHDA	6-Hydroxydopamine
AICAR	5-Aminoimidazole-4-carboxamide-riboside
AMPK	Adenosine 5′-monophosphate-activated protein kinase
ANLSH	Astrocyte-neuron lactate shuttle hypothesis
ARH	Arcuate hypothalamic nucleus
CA	Catecholamine
CaMMKβ	Ca^{++}/calmodulin-dependent protein kinase-beta
CRH	Corticotropin-releasing hormone
CV4	Caudal fourth ventricle
DAB	1,4-Dideoxy-1,4-imino-d-arabinitol
DBH	Dopamine-beta-hydroxylase
DMH	Dorsomedial hypothalamic nucleus
DVC	Dorsal vagal complex
ERα	Estrogen receptor-alpha
ERβ	Estrogen receptor-beta
FD	Food deprivation
GABA	γ-Aminobutyric acid
$GAD_{65/67}$	Glutamate $decarboxylase_{65/67}$
GCK	Glucokinase
GE	Glucose-excited
GI	Glucose-inhibited
GnRH	Gonadotropin-releasing hormone
GP	Glycogen phosphorylase
GS	Glycogen synthase
HAAF	Hypoglycemia-associated autonomic failure
icv	Intracerebroventricular
ir	Immunoreactivity
K_{ATP}	ATP-dependent potassium channel
LH	Luteinizing hormone
LHA	Lateral hypothalamic area
NE	Norepinephrine
nNOS	Neuronal nitric oxide synthase
NPY	Neuropeptide Y
OGDH	Alpha ketoglutarate dehydrogenase
ORDX	Orchidectomy
OVX	Ovariectomy
pAMPK	PhosphoAMPK
PFKL	Phosphofructokinase
PHTPP	4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl] phenol
PVH	Paraventricular hypothalamic nucleus

RIIH	Recurring insulin-induced hypoglycemia
rPO	Rostral preoptic area
NTS	Nucleus of the solitary tract
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
VMH	Ventromedial hypothalamic nucleus

Introduction

Energy homeostasis is the ideal physiological circumstance wherein cellular energy yield is adequate to perform essential life-sustaining functions. Cell health and survival depend upon myriad energy-reliant activities and are thus endangered by energy insufficiency. The body as a whole is protected against harmful effects of metabolic deficiency by a complex neural regulatory circuitry (composed of widespread neuroanatomically characterized integrative, premotor, and motor components) that coordinates endocrine, autonomic, and behavioral motor outflow to align systemic energy supply with demand. Cellular energy stability is dynamically assessed in distinct sites in both brain and gastrointestinal tract; alongside hormone signals that relate peripheral fuel storage abundance, this continuous metabolic readout supplies vital sensory data to this control circuitry. The central nervous system expends a prodigious fraction of acquired energy due to the high energy cost of vital neuron functions, including transmembrane voltage and information transfer, which renders it uniquely vulnerability to energy deficit-associated dysfunction or injury. It is thus unremarkable for the brain to utilize, in part, self-derived sensory information on nerve cell energy imbalance to guide corrective motor responses to metabolic disequilibrium. This internal input derives from a small select set of neural structures, including the hypothalamus, e.g., arcuate and ventromedial nuclei and lateral hypothalamic area and hindbrain dorsal vagal complex (DVC), where specialized neurons adjust synaptic firing in response to diminished substrate fuel supply.

Sex Differences in Clinical Insulin-Induced Hypoglycemia

Iatrogenic hypoglycemia is a primary complication of therapeutic management of endogenous insulin deficiency, namely, type I (T1DM) and advanced type 2 (T2DM) diabetes mellitus, owing to factors such as therapeutic insulin release and absorption rates that contribute to de-correlation of circulating insulin versus glucose (de Galan et al. 2006). In nondiabetic individuals, progressive decrements in circulating glucose initiate a redundant and hierarchical series of counterregulatory

responses (Cryer 1993). As glucose levels begin to fall, leading edge physiological reactions include diminished insulin secretion (when glucose falls below the lower boundary or physiological euglycemia, e.g., 4.5 mM/L), followed by increased release of pancreatic glucagon and adrenomedullary epinephrine (glucose levels below 3.8 mM), which enhance hepatic glucose production via glycogenolysis and gluconeogenesis. Heightened hypoglycemia (3.5 mM/L glucose) recruits additional, albeit less effective gluco-stimulatory hormones, e.g., adrenocorticoid glucocorticoid and pituitary growth hormone, followed ultimately (3.5–3.0 mM/L glucose) by sympathetic neural activation-driven neurogenic (confusion, blurred vision, difficulty thinking or speaking, experience of faintness, drowsiness, and/or dizziness) and autonomic (sweating, tingling, trembling, palpitations, anxiety) symptoms that trigger behavioral responses such as carbohydrate ingestion. Neuroglucopenia, e.g., brain glucose deficiency, occurs at 2.6 mM/L glucose and correlates with cognitive impairment; glucose levels less than 1.5 mM/L cause severe neuroglucopenia and coma and seizures (de Galan et al. 2006). The magnitude of responses to hypoglycemia is an inverse function of the glucose nadir, not the rate of fall in glucose (Santiago et al. 1980; Amiel et al. 1987; Mitrakou et al. 1993). Suboptimal counterregulatory outflow is liable to occur in diabetic patients when these thresholds are up- or downregulated by persistent hyper- or hypoglycemia, respectively. T1DM-associated impairments of glucose counter-regulation include dysregulation of paracrine control of pancreatic glucagon secretion that occurs within a short time after disease onset; with this development, epinephrine release and neurogenic/autonomic symptoms become primary defenses against hypoglycemia.

Despite similarity of glycemic thresholds between the sexes, clinical and experimental studies consistently document sex-dimorphic, e.g., blunted counter-regulation, yet reduced risk of development of severe hypoglycemia in women versus men. Clinical research shows that insulin elicits dissimilar temporal glucose profiles, magnitude of counterregulatory glucagon and epinephrine secretion despite similar glycemic thresholds, and adjustments in peripheral and hepatic insulin sensitivity in adult nondiabetic women compared to men (Amiel et al. 1993; Fanelli et al. 1994; Davis et al. 2000). There is limited understanding of sex-specific gonadal steroid regulation of counterregulatory hormone profiles; yet, available information from animal studies reveals that estradiol stimulates hypoglycemia-associated hyperglucagonemia and hypercorticosteronemia in female rats (Briski and Nedungadi 2009). The latter findings are consistent with reports that ovariectomized animals exhibit significantly lower basal and stress-associated plasma corticosterone levels (Seale et al. 2004). Those workers also found that orchidectomy (ORDX) stimulates the release of this hormone under nonstress and stress conditions. Ongoing studies in our laboratory show that ORDX decreases or increases the magnitude of glucagon and corticosterone secretion, respectively, in insulin-injected male rats, responses that are reversed by exogenous testosterone [Briski, personal communication]. Collectively, these data point to the likelihood of differential androgen- versus estrogenic regulation of glucagon secretory responses to hypoglycemia.

Cellular/Molecular Mechanisms of CNS Detection of Glucopenia: Impact of Estradiol

Metabolism of glucose, the primary energy source to the brain, is compartmentalized by cell type and involves exchange of metabolites between astrocytes and neurons (Laming et al. 2000). The astrocyte-neuron lactate shuttle hypothesis (ANLSH) postulates that glucose is acquired from the circulation by astrocytes and either stored as glycogen, a complex branched polymer, or catabolized to the oxidizable fuel L-lactate for trafficking to neurons (Pellerin and Magistretti 1994); Pellerin 2003). This cell-to-cell transfer is accomplished by the glia- and nerve cell-specific monocarboxylate transporters MCT1 and MCT2, respectively (Bröer et al. 1997; Pierre et al. 2000; Debernardi et al. 2003). Lactate is released into the extracellular space as a vital energy substrate for nerve cell aerobic respiration. Despite high-energy needs, neuron cells are ironically devoid of energy stores and exhibit a truncated glycolytic pathway that favors pentose phosphate metabolism and antioxidative protection over energy production (Barros 2013). Nerve cell reliance upon astrocyte-derived lactate is implied by its preferred use over glucose as an in vivo energy substrate when both substrates are available (Wyss et al. 2011). This energy reserve is dynamic during normal brain activity and metabolic stasis and is an important reserve of lactate equivalents during states of heightened activity or glucose deficiency (Stobart and Anderson 2013). Unlike neurons, astrocytes maintain a high rate of glycolysis; internal glycogen stores thus favor glial and neuronal energetic stability as stored glucose can be rapidly converted to energy and exportable fuel for respective needs of each cell type (Obel et al. 2012).

The notion that the caudal dorsomedial hindbrain is a critical site for screening of glucose-derived energy is bolstered by proof that caudal fourth ventricular (CV4) administration of the monocarboxylate transporter inhibitor alpha-cyano-4-hydroxycinnamate (4CIN) elicits dose-dependent increases in circulating glucose, whereas magnitude and duration of hypoglycemia are exacerbated by exogenous lactate infusion to that location (Patil and Briski 2005). The hypothalamus functions as the final common conduit for efferent control of brain stem/spinal cord autonomic cell groups and the anterior pituitary gland. Lactoprivic signals of caudal dorsomedial hindbrain impact downstream hypothalamic elements of the brain gluco-regulatory circuitry as Fos immunolabeling of hypothalamic structures relevant to glucostasis, including the ventromedial (VMH), dorsomedial (DMH), paraventricular (PVH), and arcuate (ARH) nuclei and lateral hypothalamic area (LHA), is increased in proportion to CV4 4CIN dosage (Briski and Patil 2005). The ultrasensitive, evolutionarily conserved energy gauge adenosine 5′-monophosphate-activated protein kinase (AMPK) is activated by phosphorylation in response to metabolic stressors, e.g., exercise, starvation, hypoglycemia, hypoxia, etc., that increase the intracellular AMP/ATP ratio (Hardie 2003; Kahn et al. 2005) and provides crucial input on neuron ATP availability to brain regulatory circuits (Ronnett et al. 2009). Hypothalamic AMPK activity is critical for optimal counterregulatory hormone secretion (Han et al. 2005). Caudal dorsomedial hindbrain lactate repletion

normalizes hypothalamic AMPK activity and metabolic neuropeptide profiles and attenuates hyperglucagon/corticosteronemia, results that emphasize the relevance of hindbrain energy state to hypothalamic metabolic monitoring and effector transmitter functions that govern counterregulatory rectification of hypoglycemia (Gujar et al. 2014; Shrestha et al. 2014). Hindbrain lactate repletion intensifies hypoglycemia in both sexes, but the magnitude of this decline is amplified by estradiol in females (Vavaiya and Briski 2007, 2008).

Electrophysiological mapping studies of the DVC reveal that among its sensory (nucleus of the solitary tract; NTS), motor (dorsal motor nucleus vagus nerve), and circumventricular organ (area postrema) elements, neurons exhibiting electroreactivity to substrate fuel abundance in vitro and in vivo reside primarily in the caudal NTS (Mizuno and Oomura 1984; Adachi et al. 1995). These cells differ from neighboring electrically unresponsive neurons by expression of the biomarker glucokinase (GCK), a low-affinity, high-Km hexokinase whose activity varies according to glucose concentration (Balfour et al. 2006). The NTS is typical of brain nuclei in its heterogeneous neurotransmitter cell composition. This cellular diversity complicates efforts to determine molecular mechanisms of metabolosensory function in that site, necessitating the selective analysis of homogeneous cell samples. Laser-catapult microdissection is a powerful technology that permits acquisition of single CNS neurons of interest, based upon morphological or neurochemical criteria, for downstream molecular (DNA, RNA, protein) analyses. Caudal DVC A2 noradrenergic neurons regulate a wide array of physiological, behavioral, and cognitive functions that maintain homeostasis, including control of emotional, endocrine, and autonomic responses to stress (Rinaman 2011). Using a combinatory analytical approach involving in situ immunocytochemistry, laser-catapult microdissection, and single-cell quantitative real-time PCR methods, we found that these cells express mRNAs that encode GCK and the inwardly-rectifying, ATP-dependent potassium channel, K_{ATP} (thereby demonstrating a molecular basis for intracellular glucose monitoring and transduction of energy status into membrane voltage, respectively), and that both profiles are upregulated by insulin-induced hypoglycemia (Briski et al. 2009). A2 neurons exhibit augmented Fos protein immunostaining (Patil and Briski 2005) and dopamine-β-hydroxylase (DβH) mRNA expression (Li et al. 2006) following glucose antimetabolite administration, indicating functional reactivity to glucopenia. DVC AMPK regulates feeding and glucostasis (Ibrahim et al. 2013; Alenazi et al. 2016). A2 neurons remain the sole caudal DVC cell group known to express AMPK (Briski et al. 2014) and are unique among medullary catecholamine populations by virtue of AMPK reactivity to hypoglycemia-associated lactoprivation (Shrestha et al. 2014). A2 cells mediate brain stem-to-hypothalamus norepinephrine (NE) signaling during hypoglycemia as NE accumulation in distinct hypothalamic gluco-regulatory loci is prevented by caudal dorsomedial hindbrain lactate repletion (Shrestha et al. 2014). Lastly, A2 neuron function is necessary for hindbrain lactoprivic regulation of blood glucose levels, feeding, counterregulatory hormone secretion, and hypothalamic AMPK activity as such responses are effectively blunted by caudal dorsomedial hindbrain catecholamine neurotoxic lesioning (Gujar et al. 2014). A2 neurons express mRNAs

that encode glucose (GLUT3, GLUT4) as well as monocarboxylate (MCT2) transporter proteins (Briski et al. 2009), indicating the putative ability to screen availability of both glucose and lactate. Based upon observations that these gene profiles diverge during acute hypoglycemia (GLUT3/GLUT4 and MCT2 mRNAs were, respectively, elevated or decreased) and that caudal hindbrain lactate repletion normalizes MCT2 gene expression, it is presumed that glucose may be a primary energy source to these cells during hypoglycemia, whereas decreased lactate uptake may be a critical monitored manifestation of systemic glucose deficiency at the cellular level.

A2 neurons express estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ) proteins (Ibrahim et al. 2013), indicating a likely function as substrate for estrogen regulation of metabolic deficit signaling to the gluco-regulatory network. Estradiol is reported to enhance glycolytic, tricarboxylic acid (TCA) cycle, and respiratory chain enzyme expression and activity (Kostanyan and Nazaryan 1992; Nilsen et al. 2007; Chen et al. 2009) and oxidative respiration (Irwin et al. 2008) in whole brain or select brain region, e.g., cerebral cortex, cerebellum, etc. Our work on pure A2 cell samples shows that that estradiol increases basal expression of rate-limiting glycolytic (phosphofructokinase; PFKL) and TCA (isocitrate dehydrogenase) enzymes and pyruvate dehydrogenase, Complex II, and ATP synthase subunits (Tamrakar et al. 2015a). During hypoglycemia, these cells exhibit further augmentation of PFKL and upregulation of a second key TCA enzyme alpha ketoglutarate dehydrogenase (OGDH) in the presence of estradiol. Estrogen-dependent resistance of A2-activated profiles to hypoglycemia implies hormonal promotion of cellular metabolic stability during this energetic stress. AMPK refractoriness to hypoglycemia, despite likely increases in energy production and lack of change in upstream kinase Ca^{++}/calmodulin-dependent protein kinase-beta (CaMMKβ) expression, may reflect estrogenic stabilization of generated versus expended energy ratio, despite systemic glucose deficit. Alternatively, hypoglycemic upregulation of A2 pAMPK expression in non-estrogen-treated ovariectomized (OVX) rats may indicate, in part, augmented CaMMKβ expression and/or imbalance of energy yield relative to use. Results also showed that A2 neurons express the key lipogenic enzymes acetyl coA-carboxylase and fatty acid synthase in an estrogen-dependent manner, suggesting that estradiol can inhibit A2 de novo fatty acid synthesis during eu- and hypoglycemia. Estradiol governs AMPK activation in both the A2 cell group and the hypothalamus, alongside hypothalamic metabolic neurotransmitter and counter-regulatory profiles in response to administration of the AMP mimic 5-aminoimidazole-4-carboxamide-riboside (AICAR) to the OVX female rat caudal hindbrain (Ibrahim et al. 2013; Alenazi et al. 2014; Ibrahim and Briski 2014). Importantly, that work emphasizes dependency of hindbrain-hypothalamic AMPK functional interaction on estrogen, as AMPK activity is differentially altered, e.g., augmented (ARH and PVH) or diminished (VMH) in estradiol- versus oil-implanted OVX rats after hindbrain AMPK activation.

Hypothalamic Targets of Estrogen-Controlled Hindbrain Hypoglycemia-Associated Lactoprivic Signaling

In ovary-intact female rat, estradiol secretion fluctuates over the 4-/5-day estrous cycle with circulating hormone levels gradually increasing four to fivefold from baseline (metestrus) to peak concentrations on proestrus afternoon (Butcher et al. 1974; Goodman 1978). Circadian-driven food intake during the daily dark phase varies over the estrous cycle, declining during the proestrus evening after the pituitary luteinizing hormone surge (the night of ovulation and behavioral estrus) (ter Haar 1972; Asarian and Geary 2002), whereas light-phase consumption is relatively constant over the cycle (Asarian and Geary 2006). Studies analyzing energy balance over the rat estrous cycle indicate that fluctuations in whole-body energy state correlate with E secretion (Giles et al. 2010). Net energy equilibrium shifts from positive imbalance (diestrus) to a balanced/slightly negative state (estrus) during escalation of estradiol release from baseline to maximal. This amplified hormone signal paradoxically inhibits food intake on proestrus evening despite a net decline in caloric excess over that day; yet, the extent of this reduction in consumption is likely mitigated by signals of decreased positivity of energy state.

Our studies show that in female rats, estradiol establishes unique hypothalamic neuropeptide transmitter targets of hindbrain AMPK activation, namely, ARH neuropeptide Y (NPY) and PVH corticotropin-releasing hormone (CRH), whereas pro-opiomelancortin protein profiles were responsive to this signal in both estradiol- and vehicle-implanted OVX rats (Alenazi et al. 2016). It was thus of interest to us to examine the premise that stage-specific patterns of estradiol output over the rat estrous cycle determine neuroanatomical and molecular foci of hindbrain substrate deficit signaling during hypoglycemia. We observed that insulin-injected rats exhibited lactate-reversible augmentation of NE accumulation in all preoptic/hypothalamic structures examined, excluding the DMH where hindbrain lactate infusion either suppressed or enhanced NE content in OVX rats given estradiol replacement designed to replicate nadir versus peak circulating hormone levels, respectively. These results diverge from the male where preoptic NE activity was refractory to hypoglycemia (Shrestha et al. 2014). As shown in Table 1, expression profiles of hypoglycemia-reactive metabolic neuropeptides were without exception normalized (albeit with greater efficacy in the presence of peak estradiol concentrations) by fourth ventricular L-lactate infusion of insulin-injected rats. Results imply that differential patterns of estradiol release typical of the adult female rat estrous cycle thus evidently determine common, as well as unique hypothalamic metabolic neurotransmitter targets of hindbrain lactate deficit signaling as DMH RFamide-related peptide-1 and DMH RFamide-related peptide-3, ARH NPY and prepro-kisspeptin, and VMH neuronal nitric oxide synthase (nNOS) protein responses to hypoglycemia varied according to estradiol dose. Further effort is warranted to ascertain whether differential downstream forebrain target activation by hindbrain signals of hypoglycemic lactoprivation involves estradiol concentration-dependent cell population-level regulation of receptivity of individual neurotransmitter groups to NE input and/or magnitude of NE stimulation.

Table 1 Effects of caudal fourth ventricular infusion of L-lactate on hypoglycemic patterns of hypothalamic metabolic neuropeptide transmitter protein expression in the presence of estrous cycle peak- versus nadir-like estradiol concentrations

	EN (30 μg/mL)[a]			EP (300 μg/mL)[b]		
Neuropeptide transmitter	V/V	I/V	I/L	V/V	I/V	I/L
Arcuate hypothalamic nucleus						
Neuropeptide Y (NPY)		↕̸[c]	↕̸	>[d]	↑[e]	↓[f] (N)[g]
Proopiomelanocortin (POMC)		↕̸	↕̸	>	↕̸	↕̸
Pre-/pro-kisspeptin		↑	↓ (N)		↕̸	↓
Ventromedial hypothalamic nucleus						
Neuronal nitric oxide synthase (nNOS)		↕̸	↓		↑	↓ (N)
Glutamate decarboxylase $_{65/67}$ ($GAD_{65/67}$)		↓	↑		↓	↑ (N)
Dorsomedial hypothalamic nucleus						
RFamide-related peptide 1 (RFRP-1)		↑	N	>	↕̸	↓
RFamide-related peptide 3 (RFRP-3)		↕̸	↓		↑	↓ (N/↓)[h]
Lateral hypothalamic area						
Orexin-A (ORX-A)		↑	↓ (N)		↑	↓ (N/↓)
Melanin-concentration hormone (MCH)		↓	↑	>	↓	↑ (N)
Paraventricular hypothalamic nucleus						
Corticotropin-releasing hormone		↑	↓ (N)	>	↑	↓ (N/↓)
Medial preoptic nucleus						
$GAD_{65/67}$		↓	↑ (N)		↓	↑ (N/↑)
Anteroventral periventricular nucleus						
Pre-pro-kisspeptin		↓	↑ (N)		↓	↑ (N)
Rostral preoptic area						
Gonadotropin-releasing hormone (GnRH)		↓	↑		↓	↑ (N)

[a]Ovariectomized (OVX) rats were implanted with *sc* silastic capsules filled 30 μg estradiol/mL safflower oil
[b]Ovariectomized (OVX) rats were implanted with *sc* silastic capsules filled 30 μg estradiol/mL safflower oil
[c]Not different from V/V controls treated with same estradiol dosage
[d]Elevated relative to V/V group implanted with 30 μg E/mL
[e]Increased versus V/V controls
[f]Decreased versus V/V controls
[g]Normalized to V/V controls
[h]Normalization significantly exceeded V/V group mean

Estradiol Regulation of Hypothalamic Glycogen Metabolism During Hypoglycemia

Brain astrocytes engage in numerous activities that benefit nerve cell function and survival, including release of regulatory glio-transmitters, control of local blood supply, and provision of oxidative phosphorylation substrates (Stobart and Anderson 2013). Glucose, the primary energy source to the brain, is taken up from the circulation for use by neurons and astrocytes and stored as glycogen within the latter cell

compartment (Nehlig et al. 2004). In the brain and elsewhere, glycogen metabolism is controlled by antagonistic actions of glycogen synthase (GS) and glycogen phosphorylase (GP). CNS glycogenolysis is increased when energy provision falls short of demand circumstances, e.g., during seizure, sleep deprivation, and hypoglycemia (Gruetter 2003; Brown 2004). There is keen interest in clarifying the capability of the brain glycogen reserve to protect neurological function during energy imbalance (Gruetter 2003; Suh et al. 2007; Herzog et al. 2008; Oz et al. 2009). Neural and endocrine signals regulate astrocyte glycogen metabolism. NE stimulates glycogen breakdown in cerebral cortex in vivo (Harik et al. 1982) and cultured cortical astrocytes in vitro (Pellerin et al. 1997), but its role in astrocyte glycogen metabolism during hypoglycemia is unclear. Astrocytes express ERα and ERβ (Azcoitia et al. 1999; Hösli et al. 2000) and are implicated in estrogenic neuroprotective and anti-inflammatory actions on the female brain (Spence et al. 2013). Estradiol controls noradrenergic input to the preoptic area-hypothalamus through regulation of NE neurotransmission volume (Wise et al. 1981; Adler et al. 1983) and direction of NE effects on substrates (Herbison and Dyer 1991). We investigated the premise that estradiol regulates hypothalamic astrocyte glycogen metabolism during normo- and hypoglycemia in vivo through dorsomedial hindbrain catecholamine (CA)-dependent mechanisms (Tamrakar et al. 2015b). Individual astrocytes identified in situ by glial fibrillary acidic protein immunolabeling were laser-microdissected from several metabolic loci for Western blot analyses of glycogen metabolic enzyme protein expression. Our results showed that estradiol-mediated stimulation (VMH; LHA) or suppression (PVH; ARH) of GS expression was averted in the former three sites by caudal fourth ventricular pretreatment with the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) (Fig. 1). 6-OHDA also reversed (VMH; PVH) or did not modify (LHA) estrogenic inhibition of GP protein profiles. Hypoglycemic augmentation of ventromedial, arcuate, and lateral hypothalamic GP expression in the presence of estradiol was abolished by 6-OHDA. These findings reveal site-specific effects of estrogenic regulation of normo- and hypoglycemic patterns of astrocyte glycogen metabolic enzyme expression in the female rat hypothalamus and identify locations where dorsomedial hindbrain catecholamine input is required for such action. Our observations that estradiol augments GS, while at the same time reducing GP protein levels in the VMH and LHA, but stimulates the latter protein profile during hypoglycemia suggest that estradiol may act in a neuroprotective manner concerning glycogen content and turnover in those sites during glucose sufficiency and shortage. Interestingly, GS and GP reactivity to hypoglycemia and involvement of hindbrain NE signaling in regulation of basal and hypoglycemic patterns of expression are apparently sex-dependent as these protein profiles are for the most part refractory to hypoglycemia in male rats and are augmented relative to baseline by 6-OHDA pretreatment (Fig. 2). Steroid-controlled hindbrain regulation of hypothalamic astrocyte glycogen turnover may have relevance for metabolic sensory signaling in that location. For example, the VMH contains electro-reactive neurons and provides signals on substrate fuel availability that shape counter-regulation. VMH γ-aminobutyric acid (GABA)- (Chan et al. 2006, 2007; Zhu et al. 2010) and nNOS- (Routh et al. 2014) expressing neurons are presumed to function as or

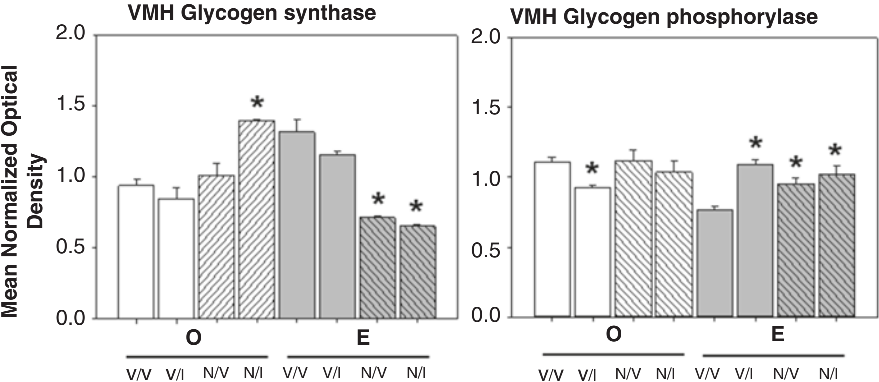


Fig. 1 Effects of caudal fourth ventricular (CV4) administration of the catecholamine neurotoxin, 6-hydroxydopamine (6-OHDA) on ventromedial hypothalamic nucleus (VMH) astrocyte glycogen metabolic enzyme protein expression during insulin-induced hypoglycemia in estradiol (E)- and oil (O)-implanted ovariectomized (OVX) female rats. Groups of 6-OHDA [N; *cross-hatched bars*]- and vehicle [V; *solid bars*]-pretreated O [*white bars*] and E [*gray bars*] rats were injected neutral protamine Hagedorn insulin [I; 12.5 U/*kg bw*] or V *sc* at time zero (t_o); animals were and sacrificed 2 h later. Lysates of n = 50 laser-microdissected glial fibrillary acidic protein-immunopositive VMH astrocytes per treatment group (n = 12/13 cells per rat) were analyzed in triplicate by Western blot for GS (*left-hand side*) or GP (*right-hand side*); target protein optical densities were normalized to alpha-tubulin. *Bars* depict normalized protein band optical densities (O.D.) +/– S.E.M. for groups of V/V (n = 5 O; n = 5 E), (2) V/I (n = 5 O; n = 5 E), (3) N/V (n = 5 O; n = 5 E), and (4) N/I (n = 5 O; n = 5 E) animals. *$p < 0.05$ compared to V/V

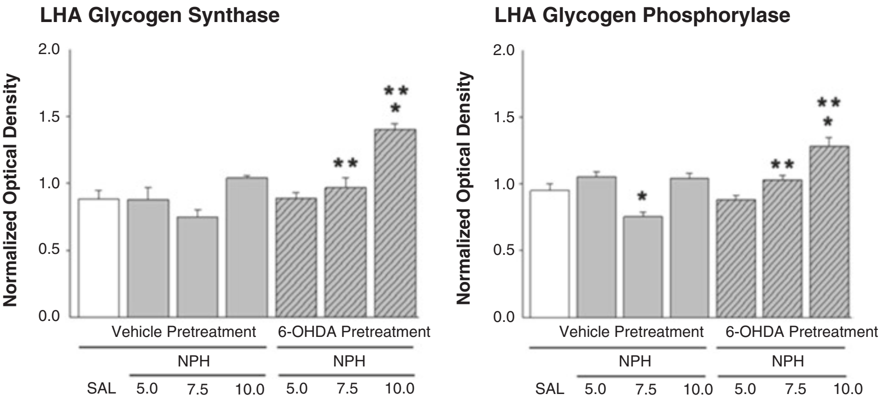
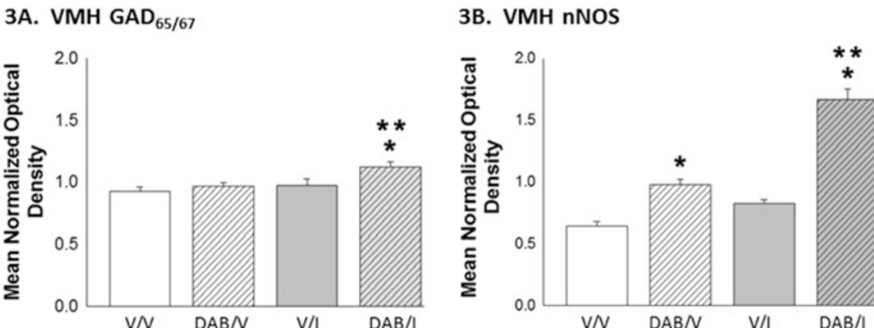


Fig. 2 Effects of 6-OHDA pretreatment on hypoglycemic patterns of GS and GP protein expression in the male rat lateral hypothalamic area (LHA). *Bars* depict mean normalized LHA GS (*right-hand side*) or GP (*left-hand side*) protein O.D. measures +/– S.E.M. for vehicle (V; *solid bars*)- or 6-OHDA (*horizontal-striped bars*)-pretreated rats injected at t_o with I [5.0 U/kg (*light gray* fill), 7.5 U/kg (*medium gray* fill), or 10.0 U/kg (*dark gray* fill)] or V/V controls (*white* fill) (n = 4/group). *$p < 0.05$ versus V/V; *$p < 0.05$ versus V/I; #$p < 0.05$, versus N/I $_{10\ \mathrm{U/kg}}$



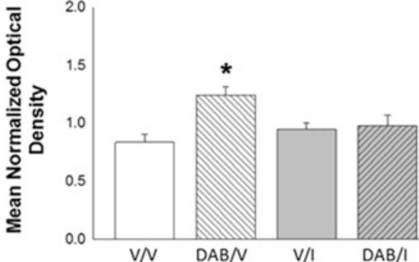


Fig. 3 Effects of the GP inhibitor 1,4-dideoxy-1,4-imino-D-arabinitol (DAB) on VMH glutamate decarboxylase$_{65/67}$ (GAD$_{65/67}$), neuronal nitric oxide synthase (nNOS), and steroidogenic factor-1 (SF-1) protein expression in eu- and hypoglycemic male rats. Micropunch-dissected VMH tissue from groups of male rats pretreated by intra-VMN administration of DAB (150 pM) or V to *sc* injection with I or V was analyzed by Western blot for GAD$_{65/67}$ (**a**), nNOS (**b**), and SF-1 (**c**) protein content. *Bars* depict illustrate mean normalized protein O.D. measures +/– S.E.M. for the following treatment groups (n = 4 rats/group): V_{VMN} plus V_{sc} (*solid white bars*), DAB_{VMN} plus V_{sc} (*diagonal-striped white bars*), V_{VMN} plus I_{sc} (*solid gray bars*), and DAB_{VMN} plus I_{sc} (*diagonal-striped gray bars*). *$p < 0.05$

downstream of "glucose-excited" (GE) or "glucose-inhibited" (GI) sensors, respectively, as manipulation of these respective marker proteins alters counterregulatory function. We evaluated effects of GP inhibitor 1,4-dideoxy-1,4-imino-d-arabinitol (DAB) on VMH glutamate decarboxylase$_{65/67}$ (GAD$_{65/67}$) and nNOS profiles in each sex. Our data show that DAB elevated nNOS expression in both sexes but only increased AMPK activity in males or decreased GAD$_{65/67}$ protein in females (Figs. 3 and 4). These outcomes provide novel evidence that glycogen-derived substrate fuel provision represses VMN AMPK activity and neurotransmitter signals of metabolic deficiency in a sex-specific manner.

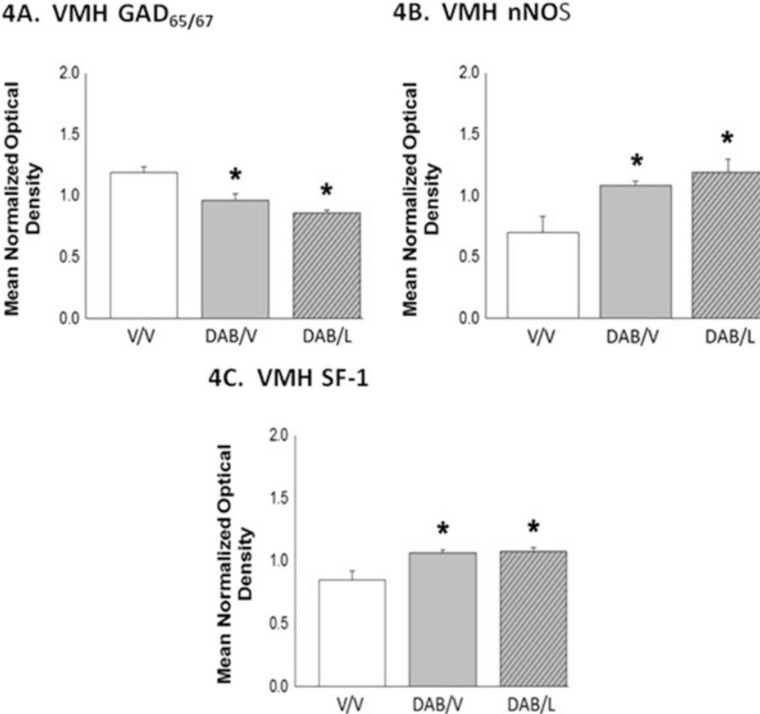


Fig. 4 Effects of DAB on VMH glutamate decarboxylase$_{65/67}$ (GAD$_{65/67}$), neuronal nitric oxide synthase (nNOS), and steroidogenic factor-1 (SF-1) protein expression in E-implanted OVX female rats: Impact of L-lactate infusion. Groups of OVX+E animals were injected into the VMH with DAB (150 pM) or V prior to initiation of continuous intra-VMH infusion of L-lactate (Patil and Briski 2005) or V. VMH tissue was analyzed by Western blot for GAD$_{65/67}$ (**a**), nNOS (**b**), and SF-1 (**c**) protein content. *Bars* depict illustrate mean normalized protein O.D. measures +/– S.E.M. for the following treatment groups ($n = 4$ rats/group): V/V (*solid white bars*), DAB/V (*solid gray bars*), and DAB/L (*diagonal-striped gray bars*). *$p < 0.05$ versus V/V

Hindbrain-Mediated Coordinate Control of Reproductive Neuroendocrine and Counterregulatory Responses to Hypoglycemia

Negative energy balance impairs fecundity in women, food animals, and laboratory species. Female reproduction (encompassing ovulation, conception, pregnancy, and lactation) is a high energy cost activity; thus, its impedance or suspension by metabolic deficit cues is logical as successful outcome of this complex process is likely to be jeopardized and energy investment thereby wasted (Wade and Jones 2004). Clinical and experimental research substantiates the key regulatory impact of metabolic status on reproduction in female mammals. Substrate fuel shortage

inhibits gonadotropin-releasing hormone (GnRH) output from the brain to anterior pituitary gonadotropes (Clarke et al. 1990; Chen et al. 1992). Steroid positive-feedback activation of the GnRH-pituitary luteinizing hormone (LH) neuroendocrine axis triggers a critical mid-cycle signal to the ovary that controls oogenesis, ovulation, and corpus luteum function. GnRH regulation by extra-preoptic metabolic sensors is affirmed by hindbrain glucoprivation-induced reversal of steroid positive-feedback activation of GnRH neurons and induction of the LH surge (Briski and Sylvester 1998). Indeed, the hindbrain, not the hypothalamus, functions as the primary source of metabolic deficit signals that restrain LH (Ohkura et al. 2000). As discussed above, our studies show that hypoglycemic patterns of preoptic NE activity (rostral preoptic area, anteroventral periventricular nucleus, medial preoptic nucleus) and expression of neurotransmitter proteins and biosynthetic enzymes implicated in reproductive neuroendocrine function (GnRH, prepro-kisspeptin, and $GAD_{65/67}$) are normalized by hindbrain lactate repletion, results that suggest that the hindbrain mediates energy partitioning away from the hypothalamic-pituitary-gonadal axis during metabolic shortfall. These outcomes emphasize a need for greater understanding of systems-level organization, interaction, and involvement of hindbrain lactoprivic-sensitive neuropeptide transmitters in regulatory integration of diverse corrective responses to energy imbalance.

Caudal DVC AMPK activity and ERβ protein expression strengthen in parallel during short-term food deprivation or simulation of local energy shortage by AICAR (Ibrahim et al. 2015). Hypoglycemia elevates A2 pAMPK and ERβ (but not ERα) protein profiles in steroid-primed OVX female rats (Shrestha and Briski 2015), suggesting that ERβ protein responses to AMPK activity state may underlie A2 noradrenergic inhibitory metabolic signaling to the GnRH-pituitary LH axis. Recent studies (Briski and Shrestha 2016) assessed effects of intra-caudal fourth ventricular administration of the selective ERβ antagonist, 4-[2-phenyl-5,7-*bis*(trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) to determine if caudal hindbrain ERβ is involved in hypoglycemic repression of steroid positive-feedback activation of the LH surge. Since intra-hindbrain glucose antimetabolite delivery (Andrew et al. 2007) or AICAR activation of caudal hindbrain AMPK in gonadal steroid- (but not non-steroid)-replaced OVX female rats (Ibrahim et al. 2013) elevates glucagon and corticosterone secretion, we also assessed whether hypoglycemic patterns of glucagon and corticosterone secretion are also subject to control by ERβ-contingent hindbrain mechanisms. Western blot analysis of laser-microdissected A2 neurons revealed hypoglycemic augmentation of AMPK activity and PHTPP-reversible stimulation of dopamine-β-hydroxylase protein expression. PHTPP normalized LH, glucagon, and corticosterone secretory patterns but did not prevent inhibition of rostral preoptic area (rPO) GnRH-I protein in hypoglycemia rats. Hindbrain ERβ antagonism prevented hypoglycemic augmentation of NE activity in the ARH, but not other preoptic/hypothalamic structures of relevance for reproduction while reversing at the same time adjustments in arcuate pre-/pro-kisspeptin protein expression. These novel outcomes suggest that caudal hindbrain substrates for ERβ may function to integrate reproductive and counterregulatory hormone responses to hypoglycemia. Discrepant GnRH-I precursor protein and LH secretory responses to

PHTPP pretreatment imply that ERβ-controlled hindbrain signaling may inhibit hypothalamic GnRH output to the pituitary by action on neurotransmitter exocytosis and/or degradation within median eminence axon terminals. Intriguing outcomes indicating site-specific control by caudal hindbrain ERβ of hypoglycemic patterns of NE activity in forebrain projection sites, specifically the ARH, support the possibility that pre-/pro-kisspeptin may be a local target of that signaling.

Sex Differences in Recurring Hypoglycemia and Hypoglycemia-Associated Autonomic Failure (HAAF)

Scrupulous therapeutic management of T1DM with the objective of tight glycemic control is highly correlated with iatrogenic hypoglycemia; this complication has immediate and far-ranging deleterious effects as it reduces hypoglycemic awareness and counterregulatory outflow during subsequent bouts of hypoglycemia (Cryer 2001; Raju et al. 2003). Antecedent hypoglycemia is a primary factor in the development of HAAF, a pathophysiological syndrome in which a vicious cycle of severe, exacerbated hypoglycemia, propelled by counterregulatory collapse and symptomatic unawareness (inability to perceive hypoglycemia), poses a substantial risk to patient safety (Cryer 2005). T1DM-associated derangements in insulin decrements and glucagon increments shift reliance to epinephrine as the principal counterregulatory defense against hypoglycemia in those patients. HAAF is characterized by repressed epinephrine outflow; resultant attenuation of the three leading edge counterregulatory responses puts T1DM patients at significantly high risk of severe hypoglycemia (White et al. 1983; Bolli et al. 1984). HAAF also limits awareness of hypoglycemia until the point of onset of neuroglucopenia, which typically precludes patient intervention. Iatrogenic hypoglycemia remains the primary barrier obstacle to optimal glycemic stabilization due to patient fear of negative cumulative cognitive and behavioral consequences of repetitive insulin overdosage.

Clinical studies provide clear evidence of sex-dimorphic effects of antecedent hypoglycemia on counter-regulation, as outcomes show that women exhibit greater resistance over a range of antecedent glucose decrements as well as significantly lesser blunting of these responses during reexposure to hypoglycemia (Davis et al. 2000). Indeed, that study showed that epinephrine responses were only diminished by exposure to antecedent glucose levels of 2.9 mM/L but not lesser reductions in women but blunted in men previously experiencing glucose decline to 3.9 or 3.3 mM/L. Glucagon release was impaired in men, but not women 1 day after glucose reductions to the lowest evaluated decrement, 2.9 mM/L. Counterregulatory adaptation to recurring insulin-induced hypoglycemia (RIIH) is also sex-dependent in adult rats. Our studies show that antecedent hypoglycemia diminishes glucagon but not corticosterone output in insulin-injected male rats (Paranpape and Briski 2005), whereas hypoglycemic hyperphagia is unabated in these animals (Sanders

et al. 2006). In females, RIIH increased food intake in both estradiol- and vehicle-implanted OVX rats, relative to acute hypoglycemic hyperphagia (Briski and Nedungadi 2009). Net food consumption did not differ between estradiol- and oil-treated animals after either single or serial insulin dosing, but estradiol maintained temporal patterns of intake between day 1 and day 4 of insulin treatment. Area-under-the-curve analyses showed that total glucagon and corticosterone secretion were both greater in estradiol- versus vehicle-implanted OVX rats after single and serial insulin dosing. These results point to likely gender disparities concerning adaptation of glucagon and food intake to RIIH.

The Brain as Substrate for Estradiol Regulation of RIIH

There is an urgent need to clarify mechanisms underlying maladaptive counter-regulation that can be leveraged toward development of therapies designed to prevent or minimize HAAF and its deleterious effects on patient quality of life. An obvious focus of interest is sex-specific habituation of the brain gluco-regulatory network to RIIH. Studies involving Fos immunocytochemical mapping of rat fore- and hindbrain after single versus serial injection of insulin disclose sex differences in RIIH-associated acclimation of genomic activity in the DVC and hypothalamus. Male rats exposed to RIIH exhibit diminished Fos immunoreactivity in the PVH, DMH, and LHA (Paranpape and Briski 2005), yet OVX rats implanted with estradiol, but not oil, exhibit consistent patterns of Fos labeling in those sites after acute versus repeated hypoglycemia (Nedungadi et al. 2006). These observations imply that diverse sensory, integrative, and premotor components of the male, but not female gluco-regulatory circuitry are likely capable of acclimation to RIIH. An obvious question that emerges from this work concerns where estradiol may act in the brain to maintain glycemic profiles during RIIH. In situ hybridization and immunocytochemical mapping studies show that ER are expressed throughout the gluco-regulatory network in the female rat brain (Shughrue et al. 1997; Osterlund et al. 1998; Mufson et al. 1999). We observed that OVX rats treated by continuous intracerebroventricular (*icv*) estradiol infusion exhibited uniform patterns of hypoglycemia after one or four insulin doses, on as many days, and that recovery from both single and multiple bouts of hypoglycemia was more rapid in rats infused with higher hormone doses (Nedungadi and Briski 2012). Mapping of nuclear ERα-immunoreactivity (−ir) in *icv* estradiol-infused animals revealed site-specific staining patterns in ER-expressing metabolic loci characterized by nonadaptive of Fos labeling during RIIH. Evidence for relatively high levels of ERα-ir in the ARH and VMH and lesser staining of the DVC prompted us to investigate whether central actions of estradiol on glycemic responses to acute and chronic insulin dosing involve substrates in one or more of these structures. An equivalent estradiol dose applied to the ventromedial and arcuate nuclei either delayed recovery from both acute and chronic hypoglycemia or did not alter hypoglycemia but attenuated RIIH, respectively. Moreover, a lower steroid dose delivered to the arcuate nucleus

impeded recovery from RIIH. Estradiol similarly exerted dose-dependent effects on RIIH after administration to the caudal dorsomedial hindbrain, as glycemic profiles were either, respectively, unchanged or reduced compared to acute hypoglycemia in high- versus low estradiol dose-treated animals. Outcomes of these efforts imply that whole-brain exposure to relatively high estradiol levels may promote counterregulatory outflow that truncates hypoglycemia and sustains glycemic glucose profiles during repetitive exposure to hypoglycemia. In contrast, actions of comparatively lower levels of this steroid on CNS substrates may prompt adaptive adjustments that result in lower circulating blood during chronic versus acute hypoglycemia. Importantly, these results reveal that estradiol exerts site-specific, concentration-dependent effects on RIIH. Further studies are needed to determine if and how CNS-specific effects of estradiol influence glucostatic defenses, e.g., detection of cellular metabolic deficiency, counterregulatory behavioral and endocrine responses, hepatic glucose metabolism, and peripheral insulin sensitivity. Evidence for concentration-dependent effects of *icv* estradiol infusion on RIIH raises the issue of how estrous and menstrual cycle-associated variations in endogenous hormone secretion, as well as pregnancy and senescence-related patterns of hormone output may influence acute and chronic hypoglycemia. Since the continuous *icv* infusion paradigm employed above does not replicate dynamic fluctuations in brain hormone levels that predictably occur in adult ovary-intact females, it is acknowledged that brain responses to static estradiol levels, low or high, may not mimic that elicited by endogenous estrogens.

Sex Differences in DVC A2 Noradrenergic Nerve Cell Metabolic-Sensory Function During RIIH

Prompted by strong evidence supporting A2 noradrenergic nerve cell function as a critical source of sensory signaling on cellular energetic sequelae of hypoglycemia, we addressed the premise that RIIH causes sex-specific maladaptations in A2 metabolic stability that correlate with altered substrate fuel transporter expression using tandem mRNA and protein analytical techniques in combination with laser-catapult microdissection (Cherian and Briski 2011, 2012). In male rats, precedent hypoglycemia reduced basal A2 MCT2, GLUT3, and GLUT4 profiles and inhibited MCT2, GLUT4, and glucokinase responses to recurring hypoglycemia. While acute hypoglycemia increased A2 AMPK activity, baseline phosphoAMPK (pAMPK) levels were elevated after recovery from antecedent hypoglycemia and were further augmented to a minimal extent upon reexposure to hypoglycemia. This evidence for diminished basal A2 glucose and lactate uptake and attenuated pAMPK-mediated detection of hypoglycemia-associated energy deficits suggests that in this sex, A2 cells adjust to RIIH by adopting a new metabolic steady state characterized by energy paucity and reduced sensitivity to hypoglycemia. Interestingly, OVX female rats implanted with estradiol exhibit opposite A2 protein responses to acute and

chronic hypoglycemia. These animals exhibited increased A2 MCT2 protein expression during acute hypoglycemia as well as elevated baseline MCT2 profiles following antecedent hypoglycemia, which were further increased by RIIH, results that correlate with incrementally greater reductions in cellular DBH RNA content. Interestingly, A2 nerve cell pAMPK levels were progressively suppressed or elevated by acute and chronic hypoglycemia in OVX animals in the presence versus absence of estradiol, respectively. These results are consistent with the concept that estradiol likely enhances A2 lactate utilization during acute hypoglycemia, thereby lessening AMPK activation relative to euglycemic controls. In females, it can be concluded that A2 cell acclimation to RIIH may involve estradiol-dependent augmentation of lactate and GLUT3-mediated glucose uptake and hormone-independent increases in GLUT4 expression, coincident with diminished pAMPK-mediated noradrenergic signaling of energy deficiency.

Estradiol regulation of RIIH effects on A2 neurotransmitter synthesis correlates with neuroprotective effects on energy metabolic pathway function during and between bouts of hypoglycemia (Tamrakar and Briski 2017). Precedent hypoglycemia caused opposite changes in basal A2 DβH protein expression in estradiol (downregulated)- versus oil (upregulated)-implanted OVX female rats, plus exacerbation of these divergent adjustments by reexposure to hypoglycemia. Opposite direction of DβH habituation to recurring hypoglycemia in estradiol- versus non-steroid-replaced OVX female rats implies that potential acclimation of A2 signaling to this stress is hormone-dependent. This estradiol effect is consistent with the view that it promotes a positive energy state during this recurring metabolic stress. Estradiol also regulates effects of recurrent hypoglycemia on enzyme proteins involved in energy metabolism and fatty acid synthesis. A2 cells exhibit elevated baseline expression of the rate-limiting glycolytic enzyme PFKL in the aftermath of hypoglycemia, coincident with diminished C-V-alpha and ATP synthase-α protein profiles, suggesting that estradiol may stimulate glycolysis, yet suppress mitochondrial aerobic respiration/energy production. Thus, between hypoglycemic bouts, these neurons may acquire, when estradiol is present, heightened needs for glycolytic metabolites or derived energy, coincident with decreased reliance upon oxidative phosphorylation. During RIIH, estradiol permitted OGDH upregulation and prevented downregulation of ATP synthase-α, implying that it may beneficially alleviate energy state negativity in A2 cells during reexposure to this stress. This view is bolstered by evidence that A2 AMPK activation is greater in O versus E rats in response to recurring hypoglycemia. Taken together, our findings suggest that estradiol-mediated reductions in A2 noradrenergic signaling may reflect, in part, energetic resilience of these cells to recurring hypoglycemia. A2 neurons are implicated in neural regulation of a wide array of physiological, behavioral, and cognitive functions that maintain homeostasis, including control of emotional, endocrine, and autonomic responses to stress. Further research is needed to determine effects of adaptive adjustments in between hypoglycemia A2 neurotransmission on glucostatic and, importantly, non-glucostatic functions governed by this cell group.

The Hindbrain Is a Substrate for Estrogen Regulation of Discriminative Metabolic Responses to Distinctive Energy-Shortfall States

In addition to iatrogenic causes, hypoglycemia in diabetic patients can also result from missed or delayed meals (Hopkins 2004; King and Clark 2015). The ideal circumstance of unfettered ability to eat at will, in response to a complex interplay of physiological, psychological, social, and genetic factors that influence meal timing, quantity of food intake, and food preference, can be elusive in reality. Indeed, short-term suspension of food intake, planned or unplanned, is often unavoidable and unpredictable in modern life. In a model of short-span cessation of feeding, wherein food was removed for 12 h commencing 2 h after lights off, we observed that estradiol reduced refeeding and attenuated activation of DVC AMPK relative to oil-implanted OVX rats (Ibrahim and Briski 2015). Sensor activity was similarly divergent in full-fed estradiol- versus oil-implanted animals after caudal fourth ventricular administration of AICAR, implying estrogen-mediated improvement in local energy balance subsequent to each treatment. This evidence for relative AMPK deactivation during concurrent suspension of fuel acquisition in estradiol-treated food-deprived (FD) animals is noteworthy, suggesting that estradiol may mitigate detrimental effects of mild/moderate bio-energetic insults on DVC energy stability. On the other hand, simultaneous FD plus AICAR treatment increased DVC AMPK activity in both estradiol- and oil-implanted OVX groups, albeit to a lesser extent in the former. These findings imply that estrogenic energy-stabilizing mechanisms initiated by short-term feeding cessation are apparently overridden by imposition of a second energetic challenge, e.g., simulated AMP elevation, but that estradiol still affords neuroprotection against heightened compound metabolic stress. Further studies are needed to investigate whether estrogen mitigates threats to energy stability, as signaled by A2 neurons, consequent to combinatory meal omission and ill-matched insulin therapy in diabetic patients, and to determine if this hormone may regulate sensor cross adaptation to different modes of metabolic stress.

In summary, emerging awareness of the physiological relevance of DVC lactoprivic-sensitive A2 noradrenergic neurons AMPK to neuro-gluco-regulatory function, including participation in a functional, interactive hindbrain-hypothalamic AMPK sensor axis, is expected to spur efforts to chart neuroanatomical and functional connections of this critical sensor element with downstream integrative, premotor, and motor cell groups that cooperatively govern counteractive autonomic, neuroendocrine, and behavioral outflow. A corollary necessity involves characterization of the broad array of nutrient, hormonal, and neurotransmitter signals that converge to shape dynamic sensory reactivity of this critical cell group to hypoglycemia imposed upon distinctive systemic energy balance states determined by feeding patterns, energy expenditure (exercise), and, in the female, reproductive state-specific patterns of estradiol secretion. New perspectives will likely challenge the prevailing notion that metabolic sensors functioning in multiple locations within

and external to the brain provide independent input to downstream integrative and premotor elements of the brain's energy regulatory circuitry. Our body of work supports the unorthodox concept that hindbrain metabolo-sensory signals supersede those of hypothalamic origin. An anticipated departure from the norm of exclusive focus on hypothalamic, hindbrain, or portal vein sensors is expected to be replaced by a unitary approach that addresses the novel prospect of sensor interaction and cooperation. This "inclusionist" perspective represents a paradigm shift from the customary view of the hypothalamus as a self-contained, singular source of sensory indicators of cellular metabolic imbalance. There is particular need for insight on how afferent neural input from peripheral sensors may shape brain metabolo-sensory functionality, specifically hindbrain A2 neurons. We predict research outcomes emphasizing that metabolic sensors assimilate a unique repertoire of metabolic stimuli, e.g., they are not interchangeable, and that neural communication between sensors is a mechanism for disseminating distinctive signals and ultimately, collective assessment of the body's metabolic status. Moreover, A2 nerve cells likely mediate estrogenic defense of cellular and systemic energy homeostasis in the face of recurring hypoglycemic in diabetic patients, superimposed against normal or lifestyle-associated feeding abstinence or excessive exercise. Ongoing efforts to elucidate the molecular mechanisms of this beneficial estrogenic action will undoubtedly identify potential therapeutic targets for maintenance of positive energy balance during RIIH in diabetes patients of both sexes.

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The Role of Estrogens in Pancreatic Islet Physiopathology

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Abstract In rodent models of insulin-deficient diabetes, 17β-estradiol (E2) protects pancreatic insulin-producing β-cells against oxidative stress, amyloid polypeptide toxicity, gluco-lipotoxicity, and apoptosis. Three estrogen receptors (ERs)—ERα, ERβ, and the G protein-coupled ER (GPER)—have been identified in rodent and human β-cells. This chapter describes recent advances in our understanding of the role of ERs in islet β-cell function, nutrient homeostasis, survival from pro-apoptotic stimuli, and proliferation. We discuss why and how ERs represent potential therapeutic targets for the maintenance of functional β-cell mass.

Introduction

The gonadal hormone 17β-estradiol (E2) is involved in reproductive, bone, cardiovascular, and neuronal physiology via estrogen receptors (ERs) (Deroo and Korach 2006). Over the past decade, ERs have also emerged as important regulators of glucose homeostasis and energy balance (Liu and Mauvais-Jarvis 2010; Mauvais-Jarvis 2011; Tiano and Mauvais-Jarvis 2012a; Mauvais-Jarvis et al. 2013).

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This chapter integrates recent advances in our understanding of the role of estrogens and pancreatic islet β-cell ERs in β-cell survival and function and the protection of functional β-cell mass in diabetic conditions. We will also discuss novel therapeutic avenues to enhance estrogens' action in β-cells without the adverse effects of general estrogen therapy.

Estrogens' Protection of β-Cells in Rodent Models

Rodent models of diabetes with β-cell failure exhibit a male bias and are thus critical to exploring the role of E2 in islet protection. These models have been described in detail elsewhere (Liu and Mauvais-Jarvis 2010; Mauvais-Jarvis 2016; Tiano and Mauvais-Jarvis 2012a). Here, we will focus on the most important.

While the nonobese diabetic (NOD) mouse comes closest to modeling human T1D, surprisingly, it shows a female predominance (Liu et al. 2010). The sexual dimorphism seen in NOD mice results from an organ-specific gonadal hormone effect on the immune system and a specific gene that segregates with diabetes in female NOD mice (Fox 1992; Pearce et al. 1995; Rosmalen et al. 2001; Bao et al. 2002). Still, in a recent study, a systemic E2 treatment at a high physiological dose prevented insulitis and T1D in NOD mice by restoring the immunomodulatory functions of iNKT cells (Gourdy et al. 2016). This protection was abolished in iNKT cell-deficient NOD mice, suggesting that E2 acts as an immune modulator to prevent T1D at least partially via iNKT cells.

Mice with diabetes induced by streptozotocin (STZ) or alloxan are classical models of β-cell apoptosis in vivo with insulin deficiency and exhibit a female protection. In these models, female mice are protected from STZ- or alloxan-induced insulin deficiency, while E2-deficient female mice, either from ovariectomy or aromatase knockout, are susceptible to STZ or alloxan (Puah and Bailey 1985; Le May et al. 2006; Kilic et al. 2014). In these models, E2 treatment protects β-cells from oxidative stress-induced apoptosis in vivo (Paik et al. 1982; Le May et al. 2006; Liu et al. 2009). Importantly, the female protection against STZ- or alloxan-induced islet destruction is observed in the animal from different genetic backgrounds, suggesting that modifier genes play a minor role (Maclaren et al. 1980; Paik et al. 1982; Puah and Bailey 1985; Le May et al. 2006; Liu et al. 2009).

The transgenic mouse overexpressing the human islet amyloid polypeptide (hIAPP) in β-cells is another classical sexually biased model of T2D. The IAPP produces pathogenic islet aggregates and is associated with T2D in humans but not rodents (Betsholtz et al. 1989a, b; Ohagi et al. 1991). Thus, to study the pathogenicity of IAPP in rodents, researchers have overexpressed the human IAPP (hIAPP) in mouse islets. Adult transgenic males overexpressing hIAPP develop β-cell failure and reduced β-cell mass; females do not (Janson et al. 1996; Geisler et al. 2002). This male predominance suggests that E2 may protect against hIAPP-induced β-cell damage. Indeed, ovariectomy promotes amyloid aggregates in female mouse islets. However, this was not associated with β-cell failure (Kahn et al. 2000). Still, E2

treatment of male hIAPP transgenic mice decreased islet amyloid aggregates and prevented the progression to β-cell failure (Geisler et al. 2002).

The Zucker diabetic fatty (ZDF) rat is also a classical model of T2D with a sex dimorphism. Male ZDF rats show progressive β-cell failure and develop overt diabetes following islet lipid accumulation (Lee et al. 1994). Female ZDF rats, however, are protected from islet lipid accumulation, retain normal β-cell function, and do not develop diabetes (Lee et al. 1994). Tiano et al. showed that E2 treatment of male ZDF rats suppresses islet lipogenesis and prevents β-cell failure (Tiano et al. 2011). Islets from control nondiabetic Zucker fatty (ZF) rats—which do not develop β-cell failure—exhibit islet lipolysis and fatty acid oxidation that compensates for the increased lipogenesis, thus favoring islet lipid detoxification (Nolan et al. 2006). In contrast, islets from ZDF rats exhibit increased lipogenesis without compensation via lipolysis/β-oxidation, provoking islet lipid accumulation. E2 treatment decreases de novo fatty acid synthesis and esterification in ZDF islets, thus preventing the accumulation of toxic lipid intermediates leading to gluco-lipotoxicity (Tiano et al. 2011). Similarly, Garris et al., using the *db/db* mouse (the mouse equivalent of ZDF rats), reported that E2 treatment reduced islet lipid accumulation. This was associated with a prevention of islet destruction and function resulting in diabetes improvement (Garris and Garris 2005).

The Akita mouse provides another interesting T2D model with a sex dimorphism. These mice have a missense mutation in the *Ins2* gene leading to proinsulin misfolding (Yoshioka et al. 1997; Wang et al. 1999). The misfolded proinsulin is retained in the endoplasmic reticulum, causing chronic endoplasmic reticulum stress leading to β-cell failure (Oyadomari et al. 2002). Male Akita mice quickly progress to severe diabetes; conversely, female mice exhibit milder hyperglycemia (Yoshioka et al. 1997; Oyadomari et al. 2002). Preliminary studies in the male Akita mouse suggest that estrogens act as pharmacological endoplasmic reticulum stress mitigators. In the islets of male Akita mice, the accumulation of unfolded proinsulin promotes endoplasmic reticulum stress, which induces the expression of the pro-apoptotic transcription factor CCAAT-enhancer-binding protein homologous protein (CHOP). In these mice, treatment with estrogens reduced islet CHOP expression and prevented islet destruction and the development of insulin-deficient diabetes (Xu et al. 2015).

Estrogen Receptors Expression in β-Cells

In most cells, estrogens signal via ERα, ERβ, and the G protein-coupled estrogen receptor (GPER). Using single mouse islets cells, Angel Nadal initially suggested the presence of ERs, and reported a rapid insulinotropic effect of E2 on K^{+}_{ATP} channel activity and calcium influx through a membrane receptor (Nadal et al. 1998) unrelated to ERα or ERβ (Nadal et al. 2000). These nonclassical, rapid actions of E2 in β-cells were not blocked by the ER antagonist ICI182,780 (Ropero et al. 2002; Quesada et al. 2002). Subsequently, John Geisler confirmed the presence of

classical ERs in β-cells by showing protein expression of ERα in mouse and human islets (Geisler et al. 2002), and Juan Contreras showed that ER antagonists could reverse E2 actions in islets (Contreras et al. 2002). The functional significance of ERs in β-cells in vivo was first reported by Mauvais-Jarvis and coworkers (Le May et al. 2006). Rodent and human β-cells express both the long 66 kDa isoform and a shorter 58 kDa isoform of ERα (Le May et al. 2006) as well as ERβ (Liu et al. 2009). INS-1 β-cells express ERα36 (Tiano et al. 2011), a short ERα isoform of 36 kDa lacking both transcriptional activation domains and a functional DNA-binding domain (Kang et al. 2010). Although these ERs can be found in the nucleus of β-cells (Le May et al. 2006), where they can activate estrogen response elements (ERE) on the promoter of target genes (Liu et al. 2009), they exhibit a predominant extranuclear localization (Liu et al. 2009).

Estrogens and Islet Survival

A powerful survival hormone, E2, protects from oxidative stress and pro-inflammatory cytokine-induced apoptosis (Contreras et al. 2002; Eckhoff et al. 2003; Le May et al. 2006; Liu et al. 2009; Liu and Mauvais-Jarvis 2009; Balhuizen et al. 2010; Kumar et al. 2011), as discussed below. E2 can promote islet survival via ERα in mice of both sexes via a pathway independent from the classical ERE (Liu et al. 2009). This protection involves the activation of extranuclear ERs and a predominant ERα effect (Liu et al. 2009). It appears that ERα and ERβ prevent apoptosis via redundant pathways leading to an inhibition of caspase 3/7. The precise signaling pathways underlying this protection are still under investigation. In cultured β-cells, E2 antiapoptotic action can be induced independently of gene transcription or de novo protein synthesis, suggesting that this cytoprotection can happen independently of nuclear events (Liu and Mauvais-Jarvis 2009, 2010). This could involve rapid events such as an alteration in the phosphorylation of proteins or the function of ion channels. Interestingly, in MIN6 β-cells, non-feminizing stereoisomer 17α-estradiol shows weak transcriptional activity compared to E2 on a reporter construct containing an ERE (Liu and Mauvais-Jarvis 2009). However, exposure of human islets to E2 or 17α-estradiol produced a similar and robust protection against H_2O_2-induced apoptosis (Liu and Mauvais-Jarvis 2009). The possibility that 17α-estradiol signals through the recently discovered ERα36 is intriguing and warrants further exploration. Importantly, 17α-estradiol may be a candidate for gender-neutral antiapoptotic therapy in diabetes because it has few of the biological effects associated with female hormone activity. Also of interest, a high dose of genistein, a phytoestrogen found in soy that binds ERs, prevents oxidative stress-induced islet destruction by alloxan in culture and in vivo (Yang et al. 2011). In addition to its rapid actions, E2 can also promote survival via classical genomic mechanisms involving ERα. The liver receptor homolog (LRH-1), an orphan nuclear receptor (NR5A2) that regulates cholesterol homeostasis and cell plasticity, is upregulated at the mRNA level by E2 via ERα. Silencing LRH-1 by siRNA abrogates the protective effect conveyed by E2 on rat islets against cytokines. This ERα

pathway could induce glucocorticoid biosynthesis in islets, thereby blunting inflammation-induced apoptosis in islets (Baquie et al. 2011).

The G protein-coupled estrogen receptor (GPER) is a membrane receptor for E2 that mediates rapid non-genomic signals (Revankar et al. 2005; Maggiolini et al. 2004). In vivo female mice deficient in GPER are susceptible to STZ-induced islet β-cell death (Liu et al. 2009). In addition, in cultured mouse and human islets, pharmacological activation of GPER by the agonist G1 protects from oxidative stress- and pro-inflammatory cytokine-induced apoptosis (Liu et al. 2009; Balhuizen et al. 2010; Kumar et al. 2011).

Directing E2 to islets using GLP1 as a vector has been proposed as another means to protect functional β-cell mass in diabetes, without the adverse effects of general estrogen therapy. Indeed, islet cells express both ERs and GLP1 receptors (GLP1R). Conjugates combining GLP1 stably linked to E2 in a single molecule have been synthesized. In male diet-induced obese mice, these GLP1-E2 compounds produced a synergistic anti-obesity effect above that observed with a single agonist, without inducing E2 reproductive effects. The effect was due to a synergistic effect between E2 and GLP1 in the hypothalamus that resulted in suppression of food intake (Finan et al. 2012). The effect of the GLP1-E2 conjugates was also efficient in preventing type 2 diabetes (T2D) in the male New Zealand obese mouse (Schwenk et al. 2014). The diabetes prevention in these mice was a consequence of hypothalamic targeting by GLP1-E2, resulting in an improvement in obesity via anorectic action, indirectly improving islet function. Further, in male mouse and cultured human islets, the GLP1-E2 conjugates did not enhance the insulinotropic effect of GLP1 over GLP1 alone (Tiano et al. 2015). Studies are underway to determine the therapeutic effects of GLP1-E2 conjugates in β-cell survival (Tiano et al. 2012).

Another approach to ER targeting in islet protection consists of pairing estrogens with a selective estrogen receptor modulator (SERM) that has antagonistic action in the breast and uterus. For example, pairing conjugated equine estrogens (CE) with the SERM bazedoxifene (Komm 2008) is a new menopausal hormone therapy providing the benefits of CE on menopausal symptoms while at the same time antagonizing CE action in the uterus and breast with bazedoxifene (Kim et al. 2014). The combination of CE/bazedoxifene and bazedoxifene alone reduced the severity of islet destruction and STZ-induced insulin-deficient diabetes in female mice (Kim and Mauvais-Jarvis 2016). The prevention of STZ-induced β-cell apoptosis in mice is a marker of estrogen agonist activity (Le May et al. 2006). Thus, the prevention of STZ-induced diabetes by bazedoxifene demonstrates that, in female mice, bazedoxifene acts as an ER agonist in β-cells.

Estrogens and Islet Insulin Secretion and Biosynthesis

The insulinotropic action of E2 has been well described. E2 effects in rodents in vivo range from islet hypertrophy to increase in pancreas insulin concentration and insulin secretion (Costrini and Kalkhoff 1971; Geisler et al. 2002; Choi et al. 2005; Alonso-Magdalena et al. 2006; Wong et al. 2010). E2 also increases insulin

secretion in the perfused rat pancreas (SutterDub 1976; Senzen 1978) and in cultured rodent islets (Faure and Sutter-Dub 1979; Alonso-Magdalena et al. 2008). The direct effect of E2 on insulin secretion and ion channel activity has been shown in INS-1 insulin-secreting cells. In this model, E2 did not influence insulin content, basal and stimulated insulin output, or Ca^{2+} fluxes (Horn et al. 2000). However, these cells overexpressed ERs and thus exhibit increased ER output in basal condition, thereby blunting the stimulatory effect. In rat insulinoma (RIN) cells, E2 induced an increase in glucokinase activity, thus increasing glucose-stimulated insulin release (Magnaterra et al. 1997). In MIN6 cells and human primary β-cells, E2 produced depolarization and Ca^{2+} influx, thereby triggering insulin release (Al-Majed et al. 2005). Thus, with regard to secretion, E2 may influence β-cell responsiveness to other stimuli such as glucose. The predominant estrogen receptor, ERα, is also important in stimulating insulin synthesis. Activation of ERα by E2 stimulates β-cell proinsulin gene transcription and insulin synthesis via an extranuclear ERα that signals to Src and ERK kinases (Alonso-Magdalena et al. 2008; Wong et al. 2010). This potentiates the glucose-induced nuclear translocation of the transcription factor NeuroD1 and its binding to the insulin promoter (Wong et al. 2010). E2 also rapidly reduces K_{ATP} channel activity to enhance glucose-induced Ca^{2+} signals and insulin release in cultured mouse islets via ERβ. This insulinotropic action of ERβ requires the membrane atrial natriuretic peptide receptor (Soriano et al. 2009). Thus, during pregnancy, high E2 concentration may promote islet adaptation to the increased metabolic demand by enhancing insulin biosynthesis (via ERα) and insulin secretion (via ERβ) (Alonso-Magdalena et al. 2008; Soriano et al. 2009; Wong et al. 2010).

The insulinotropic effects of GPER have also been described. In one study, GPER-deficient mice and cultured islets displayed altered GSIS (Martensson et al. 2009). Sharma et al. reported that GPER was involved in E2 stimulation of basal and glucose-dependent insulin secretion from β-cells because E2 effects were lost in $GPERKO^{-/-}$ islets and MIN6 cells with GPER silenced by siRNA. The insulinotropic action of GPER seems to signal via the epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK) (Sharma and Prossnitz 2011). However, GPER does not appear to be involved in E2-induced insulin biosynthesis (Wong et al. 2010).

Estrogens and Islet Lipid Homeostasis

In rodents, in vivo E2 treatment suppresses key lipogenic genes in the liver, white adipose tissue, and muscle, which is associated with a reduction in adiposity and liver lipid content and improvement in glucose metabolism (D'Eon et al. 2005; Gao et al. 2006). Tiano et al. further demonstrated that estrogens suppress islet de novo fatty acid synthesis and esterification into triglycerides in rodent and human islets via ERα, ERβ, and GPER (Tiano et al. 2011). This antilipogenic action of activated

ERs is associated with a protection against lipotoxic β-cell failure in the ZDF rat model of T2D. E2 acts on islet ERα in vivo to suppress fatty acid synthase expression and activity, which reduces islet toxic lipid accumulation and is associated with a protection against β-cell failure (Tiano et al. 2011). These effects depend on extranuclear ERs activating STAT3 and AMP-activated protein kinase and promoting the suppression of the master regulator of lipogenesis, liver X receptor (LXR), and its lipogenic targets, sterol regulatory element-binding protein 1c (SREBP1c), and carbohydrate response element-binding protein (ChREBP) (Tiano and Mauvais-Jarvis 2012b). These studies demonstrate the importance of estrogen signaling in promoting islet lipid homeostasis.

Estrogens and Islet Transplantation

Human pancreatic islet transplantation (PIT) offers a physiological therapeutic approach to T1D. However, procuring sufficient islet yield requires several deceased human donors; novel strategies are needed to improve islet graft function to achieve insulin independence with fewer islets. The female sex is a predictor of insulin independence after total pancreatectomy and islet cell autotransplantation, suggesting that E2 improves PIT in women (Johnston et al. 2015). Brain death (BD) is characterized by activation of pro-inflammatory cytokines (PICs) with reduced islet yields and reduced functionality of islets from cadaver donors (Takada et al. 1998; Contreras et al. 2003). In vivo E2 treatment to BD donor rats improved in vitro islet recovery as well as viability and functionality (Eckhoff et al. 2004). E2 also promotes cultured human islet survival and improves islet functionality after PIC exposure (Contreras et al. 2002). The mechanisms could involve an E2-induced decrease in pro-inflammatory NF-κB and c-Jun N-terminal kinase (JNK) activation and a decrease in mitochondrial cytochrome c release and activation of caspase-9 (Contreras et al. 2002; Eckhoff et al. 2003). Using nude mice rendered diabetic by STZ injection and transplanted with human islets, Liu el. showed that a transient treatment with E2 at the time of PIT improves human islet engraftment in diabetic nude mice of both sexes (Liu et al. 2013). The protective effect of E2 was secondary to an acute protection of islet graft from hypoxia, oxidative stress and apoptosis, and chronic induction of islet revascularization. These effects were predominantly mediated via ERα.

Silencing inducible nitric oxide synthase (iNOS) is another strategy to promote the survival of transplanted islets (Hwang et al. 2015). Hwang et al. used peptide micelles that co-deliver small interfering RNA (siRNA)-iNOS along with E2, loaded together in the hydrophobic core. The delivery of these peptide micelles improved islet engraftment following transplantation in male diabetic syngeneic mice. Further studies are necessary to assess the efficacy of local E2 delivery to improve islet engraftment during PIT.

ERs and β-Cell Proliferation

The effect of estrogen on islet regeneration was suggested by Houssay and Rodriguez after subtotal pancreatectomy followed by implantation of an estrogen pellet in the remaining pancreas, leading to local islet regeneration (Houssay et al. 1954). Islet regeneration was also observed in rats with alloxan-induced diabetes (Goodman and Hazelwood 1974). More recently, E2, used at pharmacological doses, promoted β-cell regeneration in rat pancreatic islets damaged by STZ. This effect was observed only in mildly hyperglycemic but not in severely hyperglycemic rats (Yamabe et al. 2010). In addition, E2 and exercise have been reported to exhibit a synergistic action in promoting rodent β-cell proliferation following ovariectomy. This effect was associated with an increase in IRS-2 and Pdx-1 expression (Choi et al. 2005). Finally, E2 was reported to promote the proliferation and inhibit the differentiation of adult human islet-derived precursor cells without an effect on fetal islet-derived precursor cells (Ren et al. 2010). These intriguing observations merit further investigation.

GPER has been implicated in the expansion of functional β-cell mass during pregnancy. In rodent pregnancy, GPER is markedly upregulated, which is associated with decreased expression of the islet microRNA miR-338-3p (Jacovetti et al. 2012). Downregulation of miR-338-3p promoted rodent β-cell proliferation, while increased miR-338-3p expression decreased rodent β-cell mass. In cultured rat islets, treatment with E2 or the GPER agonist G1 also decreased miR-338-3p, which was associated with increased β-cell proliferation. These E2 effects depend on cAMP and protein kinase A. Although E2 exposure also reduced the level of miR-338-3p in cultured human islet cells (Jacovetti et al. 2012), neither E2 nor silencing of miR-338-3p stimulated replication of cultured human β-cells. Likewise, neither E2 nor G1 treatments increased proliferation of human β-cells transplanted in male mice (Liu et al. 2013). Thus, the effect of GPER activation and the resulting suppression of miR-338-3p observed in rodent β-cell proliferation are not observed in human β-cells.

Partial duct ligation (PDL) is a rodent model of pancreatic injury leading to β-cell expansion. Following PDL, cells expressing Neurogenin3 (NGN3+) are generated near the duct and can differentiate into β-cells (Van de Casteele et al. 2014). In PDL, β-cells are mostly generated via replication, but they can also derive from neogenesis via a NGN3+ stage (Van de Casteele et al. 2014). Treatment with the ERα antagonist tamoxifen or knockout of ERα in male mice similarly decreased NGN3 expression and β-cell proliferation in the PDL model, suggesting that ERα is involved in this process (Yuchi et al. 2015). Interestingly, PDL increased local E2 concentration in the ligated portion of the pancreas and stimulated ERα nuclear localization in β-cells (ERα is cytosolic in β-cells under normal conditions). Tamoxifen inhibition of ERα in the embryonic pancreas, or its deletion as in the ERα-knockout mouse, also decreased NGN3 expression and NGN3+ progenitors at the end of gestation (Yuchi et al. 2015). Thus, in the mouse, it appears that the generation of NGN3+ cells and the resulting β-cell mass expansion in developing or injured pancreas are both stimulated by ERα (Yuchi et al. 2015).

The transgenic (Tg) mouse model with β-cell-directed expression of the inducible cAMP early repressor-Iγ exhibits decreased β-cell replication and severe loss of β-cell mass, leading to diabetes only in males. In this model, treatment of orchidectomized male Tg mice with E2 enhanced β-cell proliferation by stimulating the neogenesis of new β-cells in ducts and the replication of existing β-cells in islets (Inada et al. 2014). E2 stimulation of β-cell proliferation was associated with an increased expression of pancreatic duodenal homeobox-1 (PDX-1).

Thus, in these two rodent models of β-cell regeneration, E2 can induce β-cell mass expansion via ERα signaling by inducing the replication of existing β-cells or the neogenesis of new β-cells via activation of NGN3 or PDX-1. The validity of these findings for human β-cells remains to be established (Bernal-Mizrachi et al. 2014).

The ERβ could also be involved in β-cell regeneration. A selective ERβ agonist was shown to increase β-cell proliferation in STZ-induced diabetic male C57BL6 mice and in the diabetic db/db mice (Alonso-Magdalena et al. 2013).

In summary, ER activation in β-cells promotes survival and mass expansion in rodent models. Selective activation of ERs in islets may be a strategy to protect functional β-cell mass if we can harness their beneficial properties.

Conclusions and Perspectives

E2 promotes islet adaptation to metabolic stresses via islet ERs. ERs favor islet survival, lipid homeostasis, and glucose-stimulated insulin biosynthesis and secretion as well as mass expansion under specific conditions. Estrogens are FDA approved for postmenopausal therapy, but the fear of hormone-dependent cancers stands as a major obstacle to the use of ER ligands to protect β-cells in diabetes. Further studies are thus needed to identify and develop new ligands that protect β-cells without risks.

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Nuclear and Membrane Actions of Estrogen Receptor Alpha: Contribution to the Regulation of Energy and Glucose Homeostasis

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Abstract Estrogen receptor alpha (ERα) has been demonstrated to play a key role in reproduction but also to exert numerous functions in nonreproductive tissues. Accordingly, ERα is now recognized as a key regulator of energy homeostasis and glucose metabolism and mediates the protective effects of estrogens against obesity and type 2 diabetes. This chapter attempts to summarize our current understanding of the mechanisms of ERα activation and their involvement in the modulation of energy balance and glucose metabolism. We first focus on the experimental studies that constitute the basis of the understanding of ERα as a nuclear receptor and more specifically on the key roles played by its two activation functions (AFs). We depict the consequences of the selective inactivation of these AFs in mouse models, which further underline the prominent role of nuclear ERα in the prevention of obesity and diabetes, as on the reproductive tract and the vascular system. Besides these nuclear actions, a fraction of ERα is associated with the plasma membrane and activates nonnuclear signaling from this site. Such rapid effects, called membrane-initiated steroid signals (MISS), have been characterized in a variety of cell lines and in particular in endothelial cells. The development of selective pharmacological tools that specifically activate MISS as well as the generation of mice expressing an ERα protein impeded for membrane localization has just begun to unravel the physiological role of MISS in vivo and their contribution to ERα-mediated metabolic protection. Finally, we discuss novel perspectives for the design of tissue-selective ER modulators.

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Introduction

Although estrogens are classically viewed as reproductive hormones in mammalian species, it is now well recognized that they induce cellular changes in almost all tissues and influence numerous physiological or pathophysiological situations. For instance, complex interactions have been described with the development or the progression of some cancers or endometriosis, but these hormones have beneficial effects on the skin, bone, and cardiovascular system, as well as on energy balance and glucose metabolism. The characterization of these latter properties progressively led to consider the estrogen pathway as an effective target to prevent the development of visceral adiposity and type 2 diabetes.

Supporting the beneficial effect of estrogens on insulin action and glucose homeostasis, it is well recognized that menopause favors visceral fat deposition and insulin resistance, leading to a significant increase in type 2 diabetes risk (Wedisinghe and Perera 2009; Mauvais-Jarvis et al. 2017). Noteworthy, hormonal replacement therapy reduces the incidence of type 2 diabetes in postmenopausal women. Indeed, in randomized placebo-controlled studies, a 21–35% decrease in diabetes occurrence was observed in menopausal women receiving the association of conjugate equine estrogens and medroxyprogesterone acetate (Kanaya et al. 2003; Margolis et al. 2004; Bonds et al. 2006). Accordingly, in animal models such as monkeys and rodents, bilateral ovariectomy was shown to impair insulin sensitivity and glucose metabolism, a deleterious metabolic effect that can be reversed by the chronic administration of estrogens (Louet et al. 2004). In addition, subjects bearing inactivating genetic mutations of aromatase, leading to the abolition of estrogen synthesis, develop visceral adiposity, insulin resistance, and impaired glucose tolerance (Morishima et al. 1995; Grumbach and Auchus 1999). Finally, these clinical observations have been confirmed in genetically engineered mice models, since aromatase gene invalidation similarly favors several features of the metabolic syndrome in both males and females (Jones et al. 2000). Thus, both clinical and experimental data concur to demonstrate that estrogens elicit strong beneficial actions on energy and glucose homeostasis.

The actions of estrogens are essentially mediated by two molecular targets, the estrogen receptors alpha (ERα) and beta (ERβ), which are expressed by most cell types in mammals (Ascenzi et al. 2006). The crucial role of ERα in fat mass distribution and glucose homeostasis was first suggested by the unique clinical observation of a man bearing a mutation in the ERα gene who developed a premature and severe metabolic syndrome associated to vascular dysfunctions (Smith et al. 1994). A few years later, ERα gene invalidation in mice led to a similar phenotype characterized by weight gain, visceral adiposity, insulin resistance, and glucose intolerance in both males and females (Heine et al. 2000; Cooke et al. 2001). Accordingly, we established that the ERα pathway could represent an effective therapeutic target to fight metabolic disturbances induced by a nutritional stress in mice. Indeed, E2 administration exerts a protective effect against high-fat diet (HFD)-induced glucose intolerance and insulin resistance, and this beneficial action is totally abolished

in ERα-deficient (ERα$^{-/-}$) mice (Riant et al. 2009). Interestingly, treatment with estrogens enhances insulin synthesis and secretion (Nadal et al. 2009) and also preserves murine pancreatic beta cells from apoptosis through mechanisms involving ERα (Le May et al. 2006; Liu and Mauvais-Jarvis 2010). These beneficial actions of estrogens on endocrine pancreas are out of the scope of the present chapter but have been reviewed elsewhere (Mauvais-Jarvis 2016; Tiano and Mauvais-Jarvis 2012a). In a classical model of estrogen action, estrogen binds to the ER in the cytoplasm, leading to ER dimerization and translocation to the nucleus, where this complex interacts with specific DNA sequences (estrogen-responsive element, ERE) in target genes, providing the basis of the initial "two-step mechanism" of hormone action (Jensen and Desombre 1973). Through its two activation functions (AF1 and AF2), the estrogen-ER complex then recruits transcriptional co-regulators and components of the RNA polymerase II complex that altogether subsequently regulate the transcription of target genes (Metzger et al. 1995). However, it is now clear that, in numerous cells, ER is mainly located in the nucleus, even in the absence of ligand (Palierne et al. 2016). Importantly, these nuclear, transcriptional actions could not account for all of the biological functions of ERα. Over the last two decades, it has become apparent that a fraction of ERα is associated with plasma membrane, in the caveolae (or lipid rafts), where it can activate membrane-initiated steroid signaling (MISS), also termed rapid or non-genomic or even nonnuclear effects, in a variety of cell types (Wu et al. 2011; Mendelsohn and Karas 2010; Levin and Pietras 2008). In the present chapter, we will summarize our current understanding of these nuclear and MISS effects of ERα with a focus on available data regarding their respective contributions to the metabolic and vascular actions of estrogens. As in most other fields of physiology, major progress has been recently facilitated by the generation of transgenic mouse models (affecting specific AFs and membrane-addressing elements) but also by the design of specific compounds that electively activate ERα MISS effects. Finally, we will consider how the experimental in vivo molecular dissection of ERα activation could shed light on the possible mechanisms of action of actual and future selective estrogen receptor modulators (SERMs), in particular to optimize their protective actions against obesity and diabetes.

ERα Structure and Function as a Nuclear Transcription Factor

Functional Domains of ERα

The alignment of the amino acid sequences of nuclear receptors allowed to propose that the members of this superfamily of transcription factors share a similar modular organization subdivided into six domains (A to F) (Green et al. 1986; Krust et al. 1986), as illustrated for ERα (Figs. 1 and 2a). ERα and ERβ share a high degree of homology in their C and E domains with, respectively, 97% and 60% homology, while the other domains are more divergent (Mosselman et al. 1996).

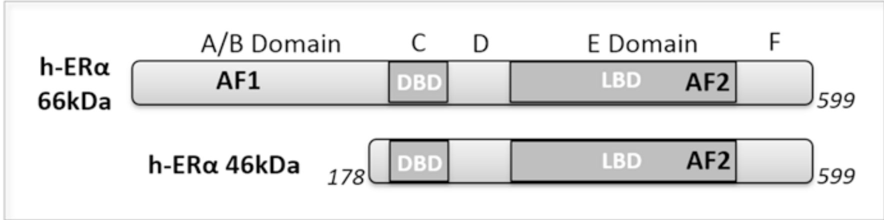

Fig. 1 Modular structure of estrogen receptor alpha (ERα) isoforms. Schematic representation of the full-length 66 kDa ERα (595 amino acids) and the short 46 kDa ERα isoform (421 amino acids). Molecular structure of the 66 kDa ERα includes a DNA-binding domain (DBD), a ligand-binding domain (LBD), and two transcriptional activation functions, respectively, named AF-1 and AF-2. The N-ter A/B domain contains AF-1, and the C-ter domain binds to DNA motifs called EREs. The D domain is called the hinge region and contributes to DNA-binding specificity and nuclear localization of the ERs. The LBD is located in the E domain and interacts with estrogens or SERMs. The 46 kDa ERα isoform lacks the NH2-terminal region harboring AF-1

In most nuclear receptors, N-terminal domains contain an activation function and/or a silencing function (Kumar and Thompson 1999). Sequence alignment allowed delineation of two domains in this region, called A and B (Krust et al. 1986). The A domain in human ERα corresponds to the first 38 N-terminal amino acids. It was shown to interact with the C-terminal region of the receptor in the absence of ligand, competing with helix 12 (H12) and corepressors for an identical binding site (Metivier et al. 2002). Thus, through this interaction, the domain A hinders ligand-independent transcription and trans-repression.

The B domain bears the transactivation function 1 (ERαAF-1). This function is described as hormone-independent because of its constitutive transcriptional activity in the absence of estradiol when isolated from the rest of the protein (Lees et al. 1989). However, it remains a ligand-dependent function in the entire protein, in which its activity is repressed by the C-terminal region in the absence of ligand as mentioned above. Serine residues of the B domain are phosphorylation target sites of several intracellular kinases involved in signaling pathways of growth factors (Kato et al. 1995). These include serine104/serine106 or 118, whose phosphorylation controls the recruitment of various coactivators, thereby increasing ligand-dependent or ligand-independent activity of the receptor (Ali et al. 1993; Bunone et al. 1996).

Fig. 2 (continued) (**c**) The first complete knockout (referred to as *ERα−/−*mice) was based on the introduction of LoxP sites to allow excision of the second coding exon of *Esr1* gene, coding for parts of the DBD (Dupont et al. 2000). (**d**) *ERαAF-1°* mice were generated by targeting the first exon of *Esr1* gene coding for the A and most of the B domains and thereby AF-1 (deletion corresponding to amino acids 2–148) (Billon-gales et al. 2009b). (**e**) The *ERαAF-2°* mice model was generated through the deletion of seven amino acids (543–549) in the helix 12 that constitutes the core of AF-2 (Billon-Gales et al. 2011). Another model of AF-2 (not shown) targeting through two point mutations, L543A and L544A (*ERαAF2-KI* mice), was also generated (Arao et al. 2011). (**f**) The first model of membrane ERα inactivation was obtained with a point mutation of the palmitoylation site of ERα (*C451A-ERα*, the mouse counterpart of the C447A residue of human ERα) (Adlanmerini et al. 2014; Pedram et al. 2014)

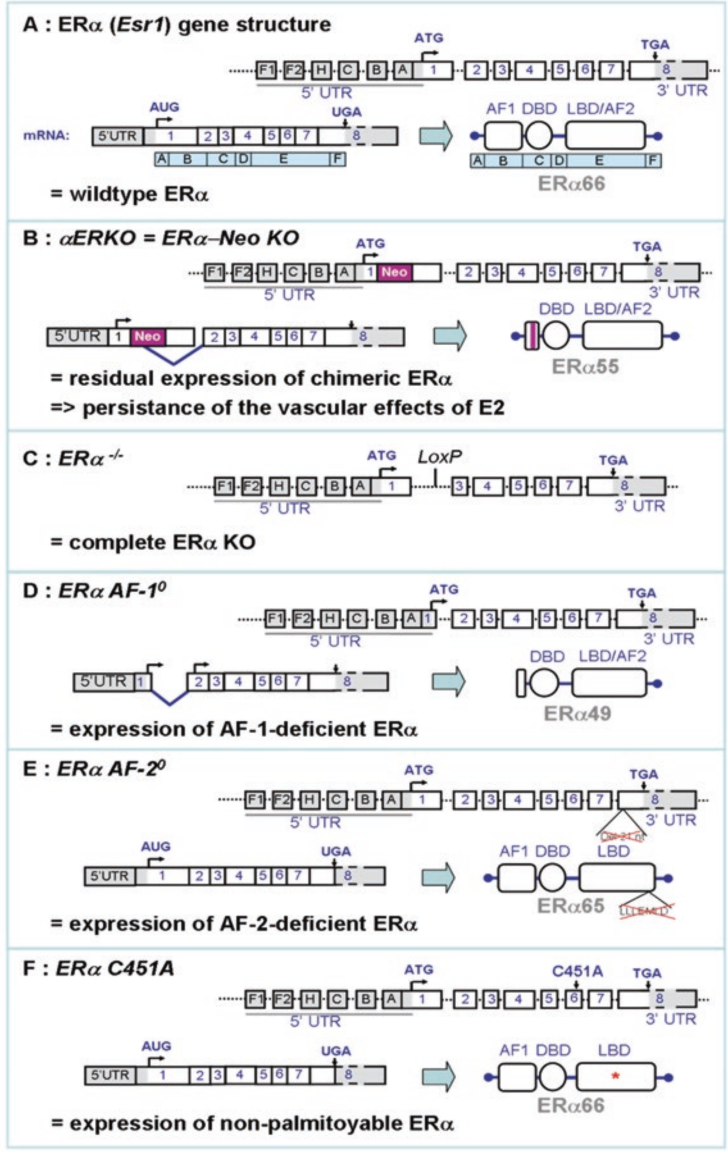

Fig. 2 Transgenic strategies for the generation of knockout or knock-in ERα mouse models. (**a**) Schematic representation of the mouse ERα (*Esr1*) gene, which encompasses eight coding exons. As described in Fig. 1, the ERα protein is composed of six domains (A to F) comprising a DNA-binding domain (DBD), a ligand-binding domain (LBD), and two activation functions (AF-1 and AF-2). (**b**) The first strategy used to invalidate ERα expression involved the insertion of a neomycin cassette in the first exon of the *Esr1* gene (*αERα-KO*, rather referred to as *ERα-Neo-KO*) (Lubahn et al. 1993). However, as a consequence of a nonnatural splicing involving the neomycin cassette, the *ERα-Neo-KO* expresses a chimeric 55 kDa isoform probably devoid of AF-1 function.

The central region of nuclear receptors comprises the DNA-binding domain (DBD), within the C region, which is considered to be the phylogenetic signature of the NR superfamily of transcription factors. The crystal structure of the C domain revealed a compact globular structure created by two zinc fingers, each formed by the tetrahedral coordination of two zinc ions with four cysteine residues. It is the α-helix of the first zinc finger module that controls/regulates DNA binding by lodging directly into the major groove of DNA. This α-helix is the only sequence of the nuclear receptor in direct contact with nucleotides that allows their specific recognition through five amino acids, constituting the "P-box" (Green and Chambon 1987). ERs recognize DNA sequences termed estrogen-responsive elements (EREs), which have a 13-base-pair consensus sequence (GGTCAnnnTGACC). The binding of an ER dimer to such an inverted palindrome implies that the two monomers are arranged in symmetrical way, face-to-face. The D domain is a hinge region between the C and E domains (Krust et al. 1986). It is involved in ERα conformational changes upon DNA and E2 binding and in protein-protein interactions (Carson-Jurica et al. 1990). This domain is thus required for the receptor interaction with other transcription factors such as Fos/Jun (AP1 sites) or SP1, leading to indirect interactions with DNA. It contains nuclear localization signals (NLS), which are important for receptor translocation from the cytoplasm to the nucleus (Ylikomi et al. 1992). Finally, the D domain is subjected to various posttranslational modifications that modulate the activity of the receptor, including phosphorylation, acetylation, methylation, ubiquitination, or sumoylation events.

The E domain identified as the ligand-binding domain (LBD) is a relatively large and complex region, both structurally and functionally. The structure of the E domain is organized around 3 layers of 12 α-helices and 2 β sheets that overall form a hydrophobic pocket for the ligand (Moras and Gronemeyer 1998). Upon ligand binding, several helices including H12 are repositioned to generate a more compact structure. Hence, in the absence of any ligand, the LBD adopts an "open conformation" in which H12 can be located away from the body of the receptor. In contrast, the H12 holds a position on the hormone-mediated three-dimensional reorganization at the presumed entry/exit route for ligands, a process termed a "mouse trap" mechanism (Moras and Gronemeyer 1998). The E domain contains a ligand-dependent activation function AF-2 included in H12 (small region covering amino acids 538 and 552 in ERα) (Danielian et al. 1992).

The E domain is the principal dimerization interface of the receptor, although the DNA-binding domain (C) of each monomer also contributes in the dimer stability (Brzozowski et al. 1997; Schwabe et al. 1990). However, unliganded LBD or E domains can form stable dimers, which are further stabilized by ligands. The F domain of ERs is the least conserved within the nuclear receptor superfamily. Since the F domain is directly adjacent to H12, it has been proposed that this region is influenced by the position of H12 and, conversely, may affect H12 (Green and Chambon 1987; Krust et al. 1986; Carson-Jurica et al. 1990; Ylikomi et al. 1992). Finally, in addition to the "classic" most abundant full-length 66 kDa ERα (ERα66), another ERα isoform has been identified in mammals. Indeed, ERα46 is a 46 kDa

isoform which lacks the first 173 N-terminal amino acids, thus devoid of the A/B domain and consequently of ERα AF-1 (Fig. 1). This isoform has been reported to be expressed in various cell types such as human osteoblasts, macrophages, and vascular endothelial cells (Wu et al. 2011). ERα46 is even expressed in most breast tumors, highlighting the importance of the choice of antibodies used for the diagnosis of breast cancer (Chantalat et al. 2016). It can be generated by either alternative splicing, proteolysis, or translation through an internal ribosome entry site (IRES) (Chantalat et al. 2016). However, the extent to which ERα46 is expressed in normal tissues and the physiological role it might be playing are essentially unknown.

Recruitment of Cofactors Determines the ERα Transcriptional Machinery

Nuclear receptors regulate the transcription of their target genes by recruiting transcriptional co-regulators through their transactivation domains AF-1 and AF-2. Co-regulators, which include coactivators and corepressors, generally exhibit intrinsic enzymatic activities involved in chromatin remodeling, histone posttranslational modifications, initiation of transcription, RNA elongation, RNA splicing, or transcriptional termination. Some of these co-regulators are also docking platforms facilitating the recruitment of other cofactors or components of the transcriptional machinery. Nearly 300 co-regulators of NR have been identified (Lonard and O'malley 2007), and co-recruitment of 17 to more than a hundred of these co-regulators by ERα has been demonstrated (Foulds et al. 2013; Liu et al. 2014).

Structurally, the interaction of ERs with coactivators occurs mainly through the direct contact with a hydrophobic surface created within the ER C-terminal domain upon structural modifications induced by ligand binding. This surface accommodates the binding of LxxLL motifs termed "NR boxes" (Mckenna and O'malley 2002) which are, for instance, found in the p160 family of coactivators that includes three analogous factors: SRC-1/NCoA-1, SRC-2/NCoA-2/TIF-2/GRIP-1, and SRC-3/NCoA-3/pCIP/AIB-1/ACTR/RAC3/TRAM-1. Other NR box-containing cofactors are the CBP and p300 proteins.

Nuclear receptors are also able to recruit corepressors involved in transcriptional repression, such as NCoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptor) via their CoRNR box motifs (Mangelsdorf and Evans 1995; Horwitz et al. 1996). These proteins generally act through the mobilization of HDACs (histone deacetylases). In contrast to some members of the nuclear receptor family (such as TR or RAR-subtype receptors), ERα does not recruit NCoR/SMRT complexes in the absence of ligand. The NCoR/SRMT repressive complexes are only present on the pS2 promoter in MDA-ERα46 transfected cells since the surface of interaction of the NCoR with the E domain is masked by the A domain of the unliganded ERα (Metivier et al. 2004). Recruitment

of such corepressors is also observed when ERα is bound to certain compounds such as tamoxifen (Lavinsky et al. 1998). The binding of this SERM to ERα allows its AF-1 to directly interact with HDAC4, which in turn can contribute to the trans-repression of some estrogen-regulated genes (Leong et al. 2005).

In Vivo Approaches to Delineate the Specific Roles of Nuclear ERα

The development of experimental genetic models has greatly increased our understanding and knowledge of the physiological roles of ERs. These models initially presented global inactivation of each gene, leading to mice lacking functional ERα (*ER*α*-KO*) or ERβ (*ERβ-KO*). Subsequently, selective inactivation of ERs in specific cell types or tissues provided tissue-selective KO mice, and the selective inactivation of a given function (limited deletions or point mutations) allowed an in vivo dissection of ER function, in particular of ERα. The strategies used to generate knockouts (KOs) or knock-ins of ERα are shown in Fig. 2, and the respective phenotypes of these mouse models are summarized in Table 1, as recently described elsewhere (Arnal et al. 2017). We will mainly detail here the vascular and metabolic phenotypes resulting from these genetic modifications.

ERα Knockout Mouse Models

P. Chambon's team (Strasbourg, France) generated a mouse model of ERα gene disruption, consisting of floxing the second exon, which allowed deletion of the C domain (Fig. 2c) (Dupont et al. 2000). Using this model of complete deletion of ERα (*ER*α$^{-/-}$), our group demonstrated for the first time that ERα, but not ERβ, is absolutely necessary for the accelerating effect of E2 on reendothelialization (Brouchet et al. 2001) and on endothelial NO production (Darblade et al. 2002). Then, Pare et al. showed the same obligatory role of ERα for the preventive effect of E2 on medial hyperplasia after vascular injury (Pare et al. 2002) (Table 1).

Importantly, Korach et al. had previously developed the first model of ERα deletion, inserting a neomycin resistance cassette into the exon 2 to create the frameshift mutation (Fig. 2b). However, these *ER*α*-Neo-KO* mice were shown to still express an N-terminal truncated 55 kDa mutant form of ERα, generated by nonnatural alternative splicing (Pendaries et al. 2002). We investigated the order of magnitude of the functional vascular actions of this chimeric 55 kDa isoform produced as a consequence of this transcriptional leakage (Pendaries et al. 2002). Such an ERα isoform, lacking a major part of the B domain and thus probably the functional AF-1, was sufficient to mediate the E2 effects on endothelial NO production (Pendaries et al. 2002), on post-injury medial hyperplasia (Iafrati et al. 1997), as well as on

Table 1 Phenotypic characteristics (including response to 17β-estradiol, E2) of the main knockout and knock-in ERα mouse models (*ERα−/−*, *ERαAF-1°*, *ERαAF-2°*, and *C451A-ERα*)

	WT	ERα−/−	AF1° ERα	AF2° ERα	C451A-ERα
Female fertility	*Normal*	*Infertile*	*Infertile*	*Infertile*	*Infertile*
Ovarian cycle	Normal	Met/diestrus	Met/diestrus	Met/diestrus	Estrus
Ovarian function and morphology	Ovulatory	Anovulatory polycystic +++	Anovulatory polycystic ++	Anovulatory polycystic +++	Anovulatory polycystic +
Reproductive organs	*Normal*	*Atrophic*	*Atrophic*	*Atrophic*	*Normal*
Uterus response to E2	Growth and proliferation	Atrophy unresponsive	Atrophy unresponsive	Atrophy unresponsive	Growth and proliferation
Mammary gland: response to E2	Growth and proliferation	Atrophy unresponsive	ND	ND	ND
Vascular actions of E2	*Protective*	*Abrogated*	*Protective*	ND	ND
Effect of E2 on endothelial no production/ vasodilation	Stimulation	No effect	Stimulation	Stimulation	No effect
Effect of E2 on endothelial healing (reendothelialization)	Acceleration	No effect	Acceleration	Acceleration	No effect
Atheroprotective effect of E2	Protective	Abrogated	Protective	Abrogated	ND
Metabolic phenotype (normal chow diet)	*Normal*	*Obesity IGT*	*Normal*	*Obesity IGT*	*ND*
HFD-induced metabolic disorders	*Normal*	*Exacerbated*	*Normal*	*Exacerbated*	*ND*
Prevention of HFD-induced metabolic disorders by E2	*Protective*	*Abrogated*	*Protective*	*Abrogated*	*ND*
Prevention of bone demineralization by E2	*Protective*	*Abrogated*	*Protective for cortical bone*	*Abrogated*	*Abrogated*

ND Not Determined

reendothelialization (Chambliss et al. 2010). As mentioned above, all these E2 actions are fully abrogated in the second mouse model (*ER*α$^{-/-}$) which is unambiguously and fully deficient in ERα (Dupont et al. 2000; Pendaries et al. 2002; Pare et al. 2002; Brouchet et al. 2001). Along with pharmacological approaches using selective ERα or ERβ agonists (Bolego et al. 2005), it is now clear that ERα activation is absolutely required for most of the beneficial vascular actions of E2 (Arnal et al. 2010).

The same conclusion applies to the influence of estrogens on energy balance and glucose homeostasis. Indeed, as summarized in the introductive part of this chapter, both *ER*α*-Neo-KO* and *ER*α$^{-/-}$ mice, either males or females, spontaneously developed an obese and dysmetabolic phenotype characterized by decreased energy expenditure, decreased locomotion, and increased adiposity, insulin resistance, and glucose intolerance (Heine et al. 2000; Cooke et al. 2001; Handgraaf et al. 2013). Furthermore, we demonstrated that ERα deletion (in *ER*α$^{-/-}$ mice) exacerbates high-fat diet-induced weight gain and hyperglycemia and totally abrogates the metabolic protection conferred by exogenous estrogen administration (Riant et al. 2009; Handgraaf et al. 2013).

Consequences of Altered ERα DNA Binding

Because the tissue-specific effects of some ERα ligands may be caused by tissue-specific mechanisms of action of ERα, two groups aimed to identify whether the mode of DNA recognition by ERα in vivo may be responsible for these differences (Jakacka et al. 2002; O'brien et al. 2006; Ahlbory-Dieker et al. 2009). To distinguish between direct interactions of ERα with ERE and indirect interactions at AP1 sites through tethering with FOS/JUN dimers on AP1 sites (Mcdevitt et al. 2008), mutations were introduced within the DBD, to modify its DNA sequence selectivity (Mader et al. 1989).

A first mouse model was generated thanks to a knock-in strategy resulting in specific mutations at amino acids 207 and 208 of the mouse ERα, two residues that have been demonstrated to govern DNA sequence selectivity. This mutant allele (E207A/G208A, named AA) was then introduced onto the *ER*α$^{-/-}$ mutant background to obtain *ER*α$^{-/AA}$ transgenic mice (although referred as KIKO mouse model (Jakacka et al. 2002). Suggesting a substantial role for the ERE-independent noncanonical pathway, Park et al. reported that the AA mutant allele largely rescues the obese and dysmetabolic phenotype observed in *ER*α$^{-/-}$ mice through the normalization of energy expenditure, including locomotor activity (Park et al. 2011). However, further investigations performed in K. Korach's group revealed that the KIKO ERα retains DNA-binding activity. Indeed, whole uterine chromatin immunoprecipitation sequencing demonstrated enrichment of KIKO ERα binding to hormone response elements (HREs) motifs, and this binding was verified using reporter gene and DNA-binding assays (Hewitt et al. 2014).

Ahlbory-Dieker et al. (2009) generated another mutated ERα mouse with four amino acid exchanges in the DNA recognition helix (Y201E, K210A, K214A, R215E), hence named EAAE (Ahlbory-Dieker et al. 2009). Importantly, in contrast with the KIKO model, the EAAE/EAAE has no ability to induce the expression of ERE reporter gene induction nor to bind to DNA HRE motifs (Hewitt et al. 2014). The phenotype of the EAAE/EAAE mice is similar to the general loss-of-function phenotype of ERα$^{-/-}$ mice, with impaired uterine growth and transcriptional activity, hemorrhagic ovaries, and absence of mammary gland development. The EAAE-ERα DBD mutant mouse demonstrates that ERα DNA binding is crucial for biological and transcriptional processes in reproductive tissues and that ERα tethering may not contribute to estrogen responsiveness in vivo. Furthermore, examination of the transcriptome of livers from EAAE/EAAE mice provided evidence of a near-complete loss of E2-sensitive gene regulations in comparison with wild-type mice. This demonstrates that gene responses to E2 in the liver require an intact ERα DBD in vivo (Ahlbory-Dieker et al. 2009).

Respective Roles of ERα Activation Functions (ERαAF-1 and ERαAF-2)

A mouse deficient in ERαAF-1 was also generated and resulted in the expression of a short 49 kDa *ER*α isoform, deficient in most of the AB domain, in place of the full-length 66 kDa ERα (Fig. 2d, named *ER*α*AF-1^{0}*). We first reported that ERαAF-1 was necessary for the reproductive actions of E2 while it is dispensable for three major vascular protective actions of E2, namely, the reendothelialization process, NO production, and prevention of atheroma (Billon-gales et al. 2009b), contrasting with the full abrogation of these E2 actions in *ER*α$^{-/-}$mice (Billon-Gales et al. 2009a) (Table 1). The role of ERαAF-1 was also studied in cortical bone demineralization (Borjesson et al. 2011), and, interestingly, the protective effect of E2 mice was also preserved in *ER*α*AF-1^{0}* (Borjesson et al. 2011).

In contrast to *ER*α$^{-/-}$mice that spontaneously develop an obese and dysmetabolic phenotype, body weight gain, body composition, and glucose tolerance of male and female *ER*α*AF-1^{0}* mice maintained on a chow diet were similar to those observed in their wild-type littermates (Handgraaf et al. 2013). In addition, no differences were found in terms of body weight, adiposity, insulin sensitivity, and glucose metabolism between *ER*α*AF-1^{0}* and wild-type mice submitted to a high-fat diet (HFD), at least until 7 months of age (Handgraaf et al. 2013). Altogether, these studies demonstrate that, whereas ERαAF-1 is required for the proliferative actions of E2 on the uterus and mammary gland, its role seems to be dispensable for the vascular (including atheroma), metabolic (HFD-induced obesity and type 2 diabetes), and cortical bone protection conferred by estrogens (Table 1).

To assess the role of ERαAF-2, a targeted deletion of seven amino acids (543–549) (*ERαAF-2^{0}* mice, Fig. 2e) (Billon-Gales et al. 2011), or two point mutations

(L543A and L544A, *ERαAF2-KI* mice) (Arao et al. 2011), has been introduced in the H12 of ERα using knock-in strategies in mice. These animal models first allowed to demonstrate that ERαAF-2 is absolutely required for the effects of estrogens on the reproductive tract (Billon-Gales et al. 2011). In particular, gene expression response to acute E2 administration is completely abrogated in the uterus of mutant mice (Arao et al. 2011; Adlanmerini et al. 2014). At variance to $ER\alpha AF\text{-}1^0$ mice, the protective effects of E2 against bone demineralization (Borjesson et al. 2011) and atheroma constitution (Billon-Gales et al. 2011) were abolished in $ER\alpha AF\text{-}2^0$ mice. The role of ERαAF-2 in the regulation of energy balance and glucose metabolism was then studied, and we found that, such as $ER\alpha^{-/-}$ mice, both male and female $ER\alpha AF\text{-}2^0$ mice developed severe obesity and metabolic disturbances, even when maintained on a chow diet. HFD feeding exacerbated this dysmetabolic phenotype with a significant increase in adiposity and hyperglycemia levels as compared to wild-type and $ER\alpha AF\text{-}1^0$ mice (Handgraaf et al. 2013). Accordingly, the insulin-resistant status resulting from ERαAF-2 inactivation was assessed by hyperinsulinemic-euglycemic clamp (Handgraaf et al. 2013). Furthermore, the protective effects of E2 against weight gain and hyperglycemia, as well as the regulation by E2 of the expression of key genes involved in adipose tissue and liver metabolism, were totally abrogated in $ER\alpha AF\text{-}2^0$ mice, in contrast to data obtained from wild-type and $ER\alpha AF\text{-}1^0$ mice (Handgraaf et al. 2013).

Whereas most of the actions of E2 were abrogated in $ER\alpha AF\text{-}2^0$ mice, a phenotype that was very reminiscent to that of $ER\alpha^{-/-}$ mice, the acceleration of endothelial repair in response to E2 was found to be similar to that observed in wild-type mice (Billon-Gales et al. 2011). This result showed that the $ER\alpha AF\text{-}2^0$ mutant is sufficient to mediate this vascular effect and thus probably to elicit MISS effects in response to E2. Accordingly, estrogen-dendrimer conjugate (EDC), a synthesized estrogen-macromolecule conjugates reported to selectively activate membrane ERα as described below (section "Selective Activation of ERα MISS with Pharmacological Compounds"), was reported to stimulate in vitro endothelial cell proliferation and migration, as well as nitric oxide synthase (eNOS) activation, through mechanisms independent of ERα nuclear activation (Chambliss et al. 2010). In addition, EDC was recently tested in $ER\alpha AF\text{-}2^0$ mice and found to accelerate endothelial repair as does E2, demonstrating the ability of $ER\alpha AF\text{-}2^0$ mutant to mediate MISS effects (Adlanmerini et al. 2014) (Table 1).

Finally, it is important to specify that the interplay between the two ERαAFs is still poorly understood in vivo. Thus, the metabolic influence induced by a specific activation of ERαAF1 remained to be explored. To this end, we recently considered the effects of tamoxifen, a selective ER modulator that acts as an ERαAF1 agonist/ERαAF2 antagonist, in ovariectomized female mice concomitantly fed a HFD (Guillaume et al. 2017). In wild-type mice, tamoxifen significantly reduced food intake and totally prevented adiposity, insulin resistance, hyperglycemia, and liver steatosis. These effects were abolished in $ER\alpha^{-/-}$ and $ER\alpha AF\text{-}1^0$ mice, revealing the specific role of this latter activation function in response to tamoxifen. Accordingly, hepatic gene expression changes elicited by tamoxifen in wild-type mice were abrogated in $ER\alpha AF\text{-}1^0$ mice. This combination of pharmacological and transgenic

experimental approaches thus indicates that selective ERαAF1 activation by tamoxifen is sufficient to elicit metabolic protection, contrasting with the specific requirement of ERαAF2 in the metabolic actions of E2 (Guillaume et al. 2017). The redundancy in the ability of the two ERαAFs to separately mediate metabolic prevention strikingly contrasts with the contribution of both of them in pathophysiological situations such as breast cancer proliferation.

Identification of Membrane-Initiated Steroid Signal (MISS) Effects of ERα

In Vitro Studies First Revealed ERα MISS Effects

From immunohistochemistry or immunocytochemistry data, it has been accepted for many years that ERα is predominantly characterized as a nuclear protein regardless of whether it is complexed with a ligand. This recurring observation of the predominant location of ERα in the nucleus, along with the definition of ERα-positive breast cancer based on the percentage of cancer cells with ERα nuclear staining, has certainly contributed to the dogma that ERα is nothing more than a nuclear receptor, exclusively regulating transcription of target genes.

The first observation that suggested the hypothesis of membrane actions was the rapid occurrence of some ERα-mediated events (in the order of seconds to minutes) as opposed to the time required for transcriptional effects. The terms “extranuclear,” “nonnuclear,” or “non-genomic” have been used by several authors, but “membrane-initiated steroid signaling (MISS)” is a widely accepted designation that encompasses all steroid signaling events initiated within the membrane compartment, which may, in fact, result in activation of different pathways of cytoplasmic or nuclear signaling that may extend to long-term effects. MISS was viewed as rapid activation of many signaling pathways acting through changes in the levels of classic second messengers such as cyclic AMP and calcium mobilization or of various kinase pathways such as MAPKs and PI3K/Akt. These various MISS actions have been mainly reported in numerous cancer cell lines (see reviews Hammes and Levin 2007; Lange et al. 2007; Levin and Hammes 2016) and in the endothelium (Wu et al. 2011).

We will now focus on the endothelium since ERα MISS appears to play a prominent role in this cell type in vivo, i.e., in differentiated cells, as detailed below. Endothelial cells express endothelial nitric oxide synthase (eNOS), an enzyme that converts arginine into citrulline and NO. Once synthesized, NO rapidly diffuses and then relaxes underlying smooth muscle cells and prevents platelet activation/adhesion as well as leukocyte-endothelial cell adhesion. Besides the basal NO production by endothelial cells, eNOS activity can be acutely stimulated, within seconds, by several agonists such as acetylcholine, but the most important stimulus, at least in vivo, is the blood flow through the shear stress (Zhou et al. 2014). Estrogens

rapidly stimulate eNOS enzymatic activity in an ERα-dependent manner (Lantin-Hermoso et al. 1997; Caulin-Glaser et al. 1997), and, noteworthy, short-term vasodilative properties of estrogens were also demonstrated in vivo in humans (Reis et al. 1994).

Most of the studies on the endothelium have identified full-length ERα (66 kDa) as the predominant plasma membrane ERα isoform associated with MISS signaling (Chen et al. 1999; Arnal et al. 2010; Dan et al. 2003), but the hypothesis that other receptors mediate extranuclear estrogen signaling has been proposed. As mentioned above, the N-terminus-deleted isoform, ERα46, has been found in some human endothelial cell lines (Li et al. 2003). ERα46 colocalizes with caveolin-1 in caveolae and effectively transduces MISS responses to E2 (Russell et al. 2000; Li et al. 2003). When expressed in cell lines, ERβ is also able to elicit MISS actions in a fashion rather similar to that of ERα (Chambliss et al. 2002). However, although ERβ plays a key role in reproduction (Heldring et al. 2007; Thomas and Gustafsson 2011), it appears to play a minor role in the vascular and metabolic actions of E2. Finally, the G-protein-coupled receptor 30 (GPR30), localized in the plasma membrane or in the endoplasmic reticulum, was proposed as a nonclassical ER (Revankar et al. 2005; Filardo et al. 2000; Meyer et al. 2011). However, the four transgenic mouse KO for GPR30 display varying phenotypes (Langer et al. 2010), although none of them show cycling or fertility abnormalities in female mice, questioning GPR30 as a biologically relevant protein for estrogen response in the reproductive function (Levin 2009). Finally, although Clegg et al. recently provide new information concerning a sexual dimorphism in GPER function in the development of postpubertal energy balance (Davis et al. 2014), the role for GPR30 as a modulator of energy homeostasis also remains to be clarified and is discussed in another chapter of this book.

ERα Posttranslational Modification and Membrane Localization

Studies of subcellular localization and signaling using CHO cell line transfected with ERα or ERβ (Razandi et al. 1999) revealed that only 2–3% of ERα/ERβ was localized to the plasma membrane. Caveolae, a subset of lipid rafts, are specialized cholesterol-rich plasma membrane organelles that compartmentalize signal transduction molecules at the cell surface. Noteworthy, eNOS is targeted to caveolae via myristoylation and palmitoylation posttranslational modifications (Garcia-Cardena et al. 1996). In isolated caveolar membranes, both E2 and acetylcholine activate eNOS with very similar profiles (Chambliss et al. 2002). A direct interaction between ERα and G proteins appears to be crucial for the activation of kinase cascades and the resulting enhanced phosphorylation of eNOS Ser-1177 in the endothelium (Kumar et al. 2007; Chen et al. 1999; Simoncini et al. 2000).

The Cys-447 located in the ERα LBD is a palmitoylation site that appears crucial for membrane localization of ERα (Acconcia et al. 2005; Marino and Ascenzi 2008). This S-palmitoylation is a reversible lipid modification which dynamically

regulates intracellular traffic, palmitoylated ERα becoming localized at the plasma membrane in association with caveolin-1. Other posttranslational modifications have been reported to be involved in membrane ERα signaling (Le Romancer et al. 2011). For instance, in MCF-7 breast cancer cells, the methylation of Arg-260 in the ERα DNA-binding domain by the arginine methyltransferase PRMT1 has been demonstrated to trigger the interaction of the receptor with the PI3K p85 subunit and with c-Src (Le Romancer et al. 2008).

In Vivo Studies to Approach the Role of ERα MISS Actions

Selective Activation of ERα MISS with Pharmacological Compounds

Membrane impermeable steroids, typically conjugated with bovine serum albumin (BSA) (Hafezi-Moghadam et al. 2002), have been initially useful to characterize the MISS actions and to exclude the involvement of nuclear ERs (Hafezi-Moghadam et al. 2002). For instance, E2-BSA caused a rapid increase in intracellular calcium or eNOS activation in arterial endothelial cells, as observed with E2 (Stefano et al. 2000; Chen et al. 2004). More recently, J. Katzenellenbogen et al. synthesized estrogen-macromolecule conjugates (estrogen-dendrimer conjugate, EDC) in which ethinylestradiol is attached to a large and positively charged poly(amido)amine (PAMAM) dendrimer (Harrington et al. 2006). EDC allows optimal ligand access to membrane ER, but does not enter the nucleus. EDC also provides a unique tool to interrogate MISS estrogen actions in vivo (Chambliss et al. 2010; Kukowska-Latallo et al. 2005).

We have already pointed that EDC administration to ovariectomized mice is sufficient to induce some of the ERα-dependent vascular protective actions such as endothelial NO production and endothelial repair following intravascular injury (Chambliss et al. 2010). In contrast to E2, EDC failed to prevent atherosclerosis in hypercholesterolemic apoE-deficient mice and also to protect female mice fed with a western diet from increased adiposity and glucose intolerance (Chambliss et al. 2016). However, E2 and EDC have similar beneficial effects to limit diet-induced hepatic steatosis, through the downregulation of genes involved in fatty acid and triglyceride synthesis in the liver. Finally, in contrast to E2 that also mediates nuclear activation of ERα, EDC has been shown to be devoid of proliferative actions on the uterus or breast tumor cells (Chambliss et al. 2010).

In 2016, J. Katzenellenbogen's group published a new strategy for the development of structurally novel compounds that preferentially activate ER extranuclear pathways and result in favorable target tissue-selective activity. Through a process of structural alteration of estrogenic ligands with reduced binding affinity for ERs, they designed "pathway preferential estrogens" (PaPEs), which interacted with ERs to activate the extranuclear-initiated signaling pathway preferentially over the

nuclear-initiated pathway (Madak-Erdogan et al. 2016). PaPE-1 was shown to regulate metabolism-related genes through activation of mTOR and MAPK signaling pathways without ERα DNA binding. In ovariectomized mice, PaPE-1 triggered beneficial responses in metabolic tissues (adipose tissue and liver) leading to the prevention of body weight gain and fat accumulation and to decreased levels of plasma triglycerides. In the vasculature, PAPE-1 accelerated the repair of endothelial damage, similarly to E2. Very interestingly, as observed with EDC, PaPEs did not stimulate reproductive and mammary tissues or breast cancer cells (Madak-Erdogan et al. 2016).

The design of these new pharmacological compounds that electively activate ERα MISS actions undoubtedly constitutes an important progress for the understanding of the respective contributions of the ERα nuclear and extranuclear pathways to the effects of estrogens. They have already led to the conclusion that selective activation of membrane ERα is sufficient to induce some endothelial and metabolic beneficial actions. However, these pharmacological tools do not permit a clear exploration of the complex interactions between MISS and nuclear effects, to finally explore the physiological roles of ERα MISS. To this important aim, new genetic mouse models have been recently generated as described below.

Phenotype of the Membrane-Only ERα (MOER) Mouse Model

As previously mentioned (see "Respective Roles of ERα Activation Functions (ERαAF-1 and ERαAF-2)" section), *ERαAF-2*^{0} mice appear to express an ERα protein devoid of nuclear actions, due to the inactivation of AF2, but retaining the ability to transduce MISS effects in response to EDC (Adlanmerini et al. 2014). However, its activation by E2 is not sufficient to elicit a detectable metabolic protection, suggesting a minor, if any, role of MISS effects in this beneficial action of estrogens (Handgraaf et al. 2013). More exactly, this observation allows us to conclude that, in the absence of functional nuclear ERα and thus of direct transcriptional regulation, activation of MISS effects is insufficient to confer a significant metabolic protection.

Another strategy was followed by Levin's group to dissociate MISS from nuclear effects, using a construct expressing only the E domain of ERα adorned with multiple palmitoylation sites from a neuromodulin-derived peptide to enforce its association with the plasma membrane. This transgene was used to generate the membrane-only ERα (MOER) mouse (Pedram et al. 2009), after breeding with *ERα*$^{-/+}$ mice to express the ERα E domain transgene in an *ERα*$^{-/-}$ background. Results obtained with the MOER mouse suggested that the E domain was not sufficient to mediate most of the physiological functions of ERα, since they had a reproductive phenotype similar to the one of *ERα*$^{-/-}$ mice (Pedram et al. 2009). Furthermore, MOER females have been demonstrated to gain more weight and to accumulate more abdominal fat than their wild-type control mice (Pedram et al. 2016). However, contents in cholesterol, triglycerides, and fatty acids were

decreased in the livers from both WT and MOER mice exposed to the ERα agonist propyl pyrazole triol (PPT), but not in the livers from *ERα*$^{-/-}$ mice (Pedram et al. 2013). The transcriptional profiles of liver tissue samples following PPT administration revealed that the expressions of many genes involved in lipid synthesis were similarly decreased in livers from MOER and wild-type mice, but not suppressed in the liver from *ERα*$^{-/-}$ mice, suggesting that membrane-localized ERα E domain is able to exert metabolic actions that did not require nuclear ERα (Pedram et al. 2013). Similarly, an extranuclear ERα suppresses lipogenic genes in pancreatic islets (Tiano et al. 2011; Tiano and Mauvais-Jarvis 2012b). However, these mice have not been challenged with a HFD, and their vascular phenotype has not been reported to date.

Mouse Models with Abrogated ERα Membrane Localization

To further investigate the physiological roles of MISS in vivo, our group (Adlanmerini et al. 2014) and Levin et al. (Pedram et al. 2014) generated mouse models expressing ERα with a point mutation at the palmitoylation site (*C451A-ERα*, the mouse counterpart of the human C447A residue of ERα, Fig. 2f). This mutation was predicted to produce a membrane-specific loss of function of ERα (Acconcia et al. 2005; Marino and Ascenzi 2008; Pedram et al. 2007). Indeed, transfection of HeLa cancer cells with wild-type or nonpalmitoylable *C447A ERα* mutant led to the demonstration that palmitoylation of ERα is needed for its interaction with the membrane protein caveolin-1 and association with the plasma membrane, as well as for the induction of non-genomic activities, including the activation of signaling pathways and cell proliferation (Acconcia et al. 2005).

The abrogation of membrane localization of ERα in *C451A-ERα* mice was confirmed in primary hepatocytes, and this point mutation resulted in female infertility with ovarian abnormalities (lack of corpora lutea and presence of hemorrhagic cystic follicles) and increased plasma levels of luteinizing hormone (Adlanmerini et al. 2014). Some of the vascular actions in response to E2 such as rapid vasodilation, acceleration of endothelial repair, and eNOS phosphorylation were also abrogated in *C451A-ERα* mice (Table 1). In contrast, E2 effects on the uterus were preserved in *C451A-ERα* mice, including the early gene response and endometrial epithelial proliferation induced by an acute physiological dose of E2 as well as the long-term response to chronic E2 that were quite similar to those observed in wild-type mice (Adlanmerini et al. 2014). However, Levin et al. (Pedram et al. 2014) reported that their strain of *C451A-ERα* mice had an abnormal uterine response to chronic exposure at a pharmacological dose of E2. A careful and systematic comparison of both *C451A-ERα* models must then be performed in the future. However, the uterus of *C451A-ERα* mice is clearly and definitively responsive to E2. *C451A-ERα* mice allowed for the first time a genetic segregation of membrane versus nuclear actions of a steroid hormone receptor, demonstrating their respective tissue-specific roles in vivo. This mouse model also enables a cross-validation of

results obtained by pharmacological approaches engaging MISS effects such as the use of EDC or PaPEs.

Noteworthy, the palmitoylation site of ERα is not only required for endothelial actions of estrogens and fertility but also plays a role in the bone protection by E2, as recently reported (Vinel et al. 2016). It is thus interesting to analyze the contribution of ERα palmitoylation and MISS actions on the regulation of energy homeostasis and glucose metabolism by estrogens using the *C451A-ERα* mouse model.

Recently, Pedram et al. have indeed reported that the *C451A-ERα* female mice generated by E. Levin's group, also called NOER (nuclear-only estrogen receptor) mice, exhibited extensive abdominal visceral fat deposition and weight gain as compared with wild-type mice (Pedram et al. 2016). Since similar phenotypes were observed in both MOER and NOER mice, they concluded that extranuclear and nuclear ERα pools collaborate to suppress adipocyte development, although in vitro experiments suggest that inhibition of lipid synthesis in mature adipocytes depends on extranuclear ERα (Pedram et al. 2016). Further data support a role for ERα MISS in the metabolic protection exerted by endogenous estrogens. Indeed, our preliminary results indicate that *C451A-ERα* female mice are prone to obesity and insulin resistance, although in a lesser extent than $ER\alpha^{-/-}$ and $ER\alpha AF\text{-}2^{0}$ mice (unpublished data), highlighting the physiological importance of signaling interactions induced by membrane and nuclear ERα.

Conclusion and Perspectives

It is now well accepted that ERα is a key regulator of energy homeostasis and glucose metabolism and should thus be considered as a pertinent target for the prevention or the treatment of dysmetabolic conditions linked to obesity such as insulin resistance, type 2 diabetes mediates, or nonalcoholic fatty liver diseases. As summarized in this chapter, the experimental strategies developed in the last decade provide important new insights into the mechanisms of ERα activation that contribute to these beneficial metabolic actions.

First, ERα-mediated nuclear effects play a prominent role, and the ERαAF2 function is absolutely required for the prevention of obesity and hyperglycemia conferred by endogenous or exogenous estrogens. However, a selective ERαAF1 activation with tamoxifen was shown to exert a similar protective effect against HFD-induced metabolic disorders, demonstrating a clear redundancy in the ability of the two ERαAFs to separately mediate metabolic prevention. This latter consideration strikingly contrasts with the absolute need of both to worsen pathophysiological proliferative processes leading to uterine or breast cancer progression.

Second, recent observations indicate that ERα MISS effects could mediate, or at least potentiate, the beneficial actions of estrogens on energy balance, insulin sensitivity, and glucose metabolism. Indeed, selective activation of the extranuclear ERα pool is not only sufficient to induce some endothelial actions but also to limit adiposity and fatty liver accumulation. Moreover, preliminary data obtained from the

C451A-ERα mouse models support a significant role of membrane ERα pool and MISS effects in the metabolic protective effects of estrogens.

Beyond the new insights into the understanding of estrogen actions on energy and metabolic homeostasis, these findings open immediate perspectives to characterize the possible mechanism of action of available SERMs such as bazedoxifene or raloxifene. Finally, pursuing such a strategy of systematic dissection of the mechanisms of ERα activation will be very helpful to design new SERMs mediating metabolic and vascular protection but devoid of side effects on reproductive targets.

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G-Protein-Coupled Estrogen Receptor (GPER) and Sex-Specific Metabolic Homeostasis

Geetanjali Sharma and Eric R. Prossnitz

Abstract Obesity and metabolic syndrome display disparate prevalence and regulation between males and females. Human, as well as rodent, females with regular menstrual/estrous cycles exhibit protection from weight gain and associated chronic diseases. These beneficial effects are predominantly attributed to the female hormone estrogen, specifically 17β-estradiol (E2). E2 exerts its actions via multiple receptors, nuclear and extranuclear estrogen receptor (ER) α and ERβ, and the G-protein-coupled estrogen receptor (GPER, previously termed GPR30). The roles of GPER in metabolic homeostasis are beginning to emerge but are complex and remain unclear. The discovery of GPER-selective pharmacological agents (agonists and antagonists) and the availability of GPER knockout mice have significantly enhanced our understanding of the functions of GPER in normal physiology and disease. GPER action manifests pleiotropic effects in metabolically active tissues such as the pancreas, adipose, liver, and skeletal muscle. Cellular and animal studies have established that GPER is involved in the regulation of body weight, feeding behavior, inflammation, as well as glucose and lipid homeostasis. GPER deficiency leads to increased adiposity, insulin resistance, and metabolic dysfunction in mice. In contrast, pharmacologic stimulation of GPER in vivo limits weight gain and improves metabolic output, revealing a promising novel therapeutic potential for the treatment of obesity and diabetes.

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Introduction: Obesity, Metabolism, and Sex Differences

Obesity represents a grave public health concern in the modern world. It is now recognized as a global epidemic with the number of obese individuals increasing drastically over the last two to three decades in both developed and more recently developing nations (Flegal et al. 2010; Guh et al. 2009). According to the latest demographic data from the Centre for Disease Control, it is estimated that in the United States alone, more than 65% of population is either overweight or obese (Center for Disease Control and Prevention: Adult Obesity Facts 2017). Obesity is not merely the presence of excessive body weight but represents a major risk factor for metabolic syndrome, a collection of conditions that includes high blood sugar, increased waist circumference, high blood pressure, and abnormal cholesterol (i.e., low high-density lipoprotein levels) or triglyceride levels (Han and Lean 2016; Keller and Lemberg 2003; Alberti et al. 2006; Mittendorfer 2011). Together, these conditions raise the risk of diabetes, heart disease, stroke, as well as certain forms of cancer (Eckel and Krauss 1998; Poirier et al. 2006; Kernan and Dearborn 2015; Basen-Engquist and Chang 2011). The socioeconomic burden of obesity is enormous, with current annual cost estimates in the United States alone ranging from \$147 to 210 billion, and simultaneous reductions in the quality of life and life expectancy (Guh et al. 2009; Hammond and Levine 2010). Obesity can result from a multitude of factors ranging from genetic, behavioral to environmental (McAllister et al. 2009). However, the principal reasons for the recent obesity epidemic include increased consumption of calorie-dense foods, high in saturated fats and refined sugars, coupled with a sedentary lifestyle, resulting in a chronic energy imbalance. The precise mechanisms responsible for the development of obesity and metabolic dysfunction are complex and not yet fully understood. As a result, there is an urgent need to identify novel molecular targets and therapeutic agents capable of preventing or limiting the development of obesity and its resulting metabolic abnormalities.

Obesity, diabetes, and cardiovascular diseases exhibit a significant sexual dichotomy, with the incidence being lower in females in their premenopausal years compared to age-matched men or postmenopausal women (Kotani et al. 1994; Garaulet et al. 2002; Nakhjavani et al. 2014; Tandon et al. 2010; Regitz-Zagrosek et al. 2006). Furthermore, the quantity and site of fat distribution also varies between men and women, leading to differential health outcomes (Regitz-Zagrosek et al. 2006; Blaak 2001). Overall, men have less body fat than women; however, in men this fat is distributed in the upper body or abdominal area, reflecting an "android" pattern, whereas women display a "gynoid" pattern, with lower body or subcutaneous fat distribution. Properties of android and gynoid fat differ, with the former being more prone to lipolysis and secreting increased levels of pro-inflammatory cytokines and therefore associated with an increased risk of metabolic syndrome and cardiovascular disease (Kotani et al. 1994; Blaak 2001; Jensen 2008; Ritchie and Connell 2007; Monteiro et al. 2014; Shulman 2014). Typical sex-specific fat distributions in males and females suggest distinct regulatory mechanisms that control energy balance and adiposity. Obesity leads to altered secretory profiles of adipose-specific hormones

and cytokines, increased levels of circulating glucose and lipids, and systemic inflammation, resulting in ectopic lipid deposition in peripheral metabolic tissues, such as the pancreas, liver, and skeletal muscle (Shulman 2014; Klop et al. 2013; Consitt et al. 2009; Bays et al. 2013; Shoelson et al. 2007; Tchernof and Despres 2013). The ensuing glucotoxicity, lipotoxicity, and inflammation result in dysfunction of these tissues, including defects in insulin production and secretion by the pancreas, inhibition of insulin-stimulated glucose uptake in skeletal muscle, and increases in glucose production by the liver (Consitt et al. 2009; Muoio and Neufer 2012; Mota et al. 2016; Brons and Vaag 2009; Poitout and Robertson 2008).

Differences also exist in insulin sensitivity between the sexes with premenopausal women being more insulin sensitive compared to age-matched men or postmenopausal women (Walton et al. 1993; Geer and Shen 2009; Lindheim et al. 1994; Mauvais-Jarvis 2011). Postmenopausal women experience a decline in metabolic health due to increased android fat deposition, reduced energy expenditure, insulin resistance, impaired glucose/lipid metabolism, and inflammation (Mauvais-Jarvis 2011; Poehlman et al. 1995; Lobo 2008; Abu-Taha et al. 2009; Godsland 2005; Bruns and Kemnitz 2004; Zhang et al. 2002; Andersson et al. 1997; Mauvais-Jarvis et al. 2013). The systemic loss of estrogen after menopause is linked to an elevated risk of age-related metabolic disease and cardiovascular disease in women (Tandon et al. 2010). Conversely, hormone replacement therapy in postmenopausal women improves multiple aspects of metabolism (Bonds et al. 2006; Gurney et al. 2014; Kanaya et al. 2003; Margolis et al. 2004; Pereira et al. 2015). With increases in overall life expectancy, it is critical to develop therapeutic agents that will ameliorate weight gain and the effects of metabolic dysfunction in women and improve the quality of life after menopause. As an experimental model, mice also display similar sex differences in weight gain and metabolism (Hong et al. 2009; Della Vedova et al. 2016). Male mice fed a high-fat diet exhibit more pronounced effects on adiposity, hormonal imbalance, and impaired glucose metabolism compared to females (Yang et al. 2014; Pettersson et al. 2012; El Akoum et al. 2011). In both human and rodent females, these protective metabolic effects are largely attributed to the female hormone estrogen. Although recognized for its effects on reproduction and development, the complex mechanisms of estrogen action on metabolic tissues remain incompletely understood.

Estrogen Action in Metabolic Tissues

Estrogen (most importantly17β-estradiol, E2) is a steroid hormone critical for the development and function of reproductive organs as well as the development of secondary sex characteristics. In addition, E2 action is involved in the nervous, immune, vascular, muscular, skeletal, and endocrine systems, all of which contribute to multiple aspects of metabolism (Deroo and Korach 2006; Prossnitz et al. 2008a). The regulation of metabolic functions by E2 and its receptors has been a topic of great interest owing to the sexual dimorphisms that exist in body weight,

food intake, glucose/lipid homeostasis, and insulin sensitivity (Mauvais-Jarvis 2011, 2015; Barros and Gustafsson 2011; Barros et al. 2006; Meyer et al. 2011). The decline of circulating E2 due to either natural or surgical menopause induces rapid changes in whole body metabolism, fat distribution, inflammation, and insulin action both in animals and humans (Abu-Taha et al. 2009; Mauvais-Jarvis et al. 2013; Lee et al. 2009; Straub 2007). Loss of E2 or its function increases the risk of central weight gain, abnormal lipid profiles, diabetes, and cardiovascular disease (Hewitt et al. 2005; Louet et al. 2004). Similarly, E2 insufficiency in male and female mice lacking aromatase, a key enzyme in the biosynthesis of E2 from testosterone, results in increased adiposity, higher circulating lipid and insulin levels, as well as reduction in lean mass (Jones et al. 2000). In rodent models of obesity, such as high-fat (aka Western) diet, leptin deficiency, or ovariectomy, supplementation with E2 or its mimetics attenuates weight gain and alleviates metabolic abnormalities (Stubbins et al. 2012; Lundholm et al. 2008; Shen et al. 2014). In postmenopausal women, although hormone replacement therapy is a viable treatment option to alleviate the symptoms of menopause (Kaunitz and Manson 2015), it is associated with oncogenic and cardiovascular risks (*Hormone Replacement Therapy and Cancer* 2001; Nabulsi et al. 1993).

One of the most widely used rodent models to study E2 action in vivo is ovariectomy, wherein surgical removal of the ovaries drastically reduces the levels of circulating endogenous E2 (Diaz Brinton 2012). The systemic loss of E2 in ovariectomized mice reveals that E2 is critical to several aspects of metabolism. E2 mediates certain metabolic effects via the central nervous system, as revealed by increased food intake, reduced energy expenditure, and weight gain in ovariectomized mice compared to ovary-intact littermates (Mauvais-Jarvis et al. 2013; Brown and Clegg 2010). In ovariectomized mice, the pancreas exhibits a loss of β-cell function and death (Louet et al. 2004; Le May et al. 2006). In contrast, supplementation with E2 promotes pancreatic β-cell survival in a proapoptotic environment (e.g., exposure to oxidative stress and/or pro-inflammatory cytokines), induces glucose-stimulated insulin secretion (GSIS), and suppresses lipogenesis, the latter by downregulating the expression of key transcription factors involved in lipid synthesis (Liu and Mauvais-Jarvis 2009; Liu et al. 2009; Balhuizen et al. 2010; Tiano et al. 2011; Tiano and Mauvais-Jarvis 2012). Furthermore, ovariectomy leads to aberrant glucose and lipid homeostasis resulting in increased fat mass, insulin resistance, glucose intolerance, dyslipidemia, and ectopic fat deposition (Vieira Potter et al. 2012; Yonezawa et al. 2012; Lin et al. 2013). Following ovariectomy, mice exhibit increases in visceral fat with larger adipocytes and increased expression of lipogenic and pro-inflammatory genes (Hong et al. 2009; Vieira Potter et al. 2012; Kamei et al. 2005; D'Eon et al. 2005). In addition, loss of E2 action increases susceptibility to oxidative stress and lowers fatty acid oxidation in multiple metabolic tissues (Muthusami et al. 2005; Paquette et al. 2009; Bokov et al. 2009). Rodent models of obesity and patients with type 2 diabetes also exhibit increased oxidative stress and inflammation (Furukawa et al. 2004; Fernandez-Sanchez et al. 2011; Wright et al. 2006; Folli et al. 2011; Domingueti et al. 2016). E2 supplementation in mice increases the expression of antioxidant enzymes and reduces inflammation (Monteiro et al. 2014;

Strehlow et al. 2003; Borras et al. 2010). Finally, ovariectomized mice display increased susceptibility to the deleterious effects of HFD that can be reversed by supplementation with E2 at physiological concentrations (Stubbins et al. 2012; Litwak et al. 2014). Surprisingly, male mice fed a HFD also exhibit reduced body weight and improved glucose tolerance upon treatment with E2 or its mimetics (Huang et al. 2017; Vinayagam and Xu 2015; Kim et al. 2005).

Many natural or synthetic compounds with estrogenic activity can regulate endocrine signaling pathways, exerting either beneficial or adverse effects. Animal studies have revealed that prenatal exposure to endocrine disruptors, such as bisphenol A and diethylstilbestrol, causes abnormal programming of differentiating E2 target tissues that leads to the development of obesity later in life (Garcia-Arevalo et al. 2014; Liu et al. 2013; Newbold et al. 2007, 2009). On the other hand, dietary intake of genistein, an isoflavone that mimics certain actions of E2, exerts antidiabetic effects by improving hyperglycemia, glucose tolerance, and insulin levels in multiple mouse models of metabolic dysfunction (Lei et al. 2011; Liu et al. 2006). Taken together, these observations suggest that E2 exerts pleiotropic effects on multiple tissues involved in metabolism. Thus, a more complete understanding of the mechanisms underlying E2 action on metabolic tissues requires a thorough investigation of the roles of individual estrogen receptors.

Estrogen Receptors, Signaling, and GPER Selectivity

The actions of E2, as an important physiological modulator of the complex events that regulate body weight and metabolism in multiple tissues, are mediated by its multiple receptors. The classical genomic actions through nuclear estrogen receptors (ERα and β) involve dimerization upon ligand activation and eventual binding to ER response elements in the promoters of target genes to facilitate regulation of gene expression (Barkhem et al. 2004; Dahlman-Wright et al. 2006; Jia et al. 2015). In addition to genomic responses, E2 also elicits rapid non-genomic signaling through extranuclear ERs and the G-protein-coupled estrogen receptor (GPER, previously known as GPR30) (Prossnitz et al. 2008a, b, c; Levin 2015). Long-term effects of GPER activity, however, also involve transcriptional regulation of target genes (Pandey et al. 2009; Prossnitz and Barton 2014; Vivacqua et al. 2015). GPER was initially discovered as an orphan receptor (Owman et al. 1996) but has since been demonstrated to bind E2 and activate multiple non-genomic, as well as genomic, pathways (Prossnitz et al. 2008a; Barton et al. 2017; Prossnitz and Arterburn 2015; Revankar et al. 2005, 2007; Filardo et al. 2000, 2002). GPER is expressed in diverse cell types and tissues, including the reproductive tissues, pancreatic islets, adipose, liver, skeletal muscle, CNS, heart, intestine, and inflammatory cells (Prossnitz and Barton 2011). It has been functionally implicated in metabolic regulation (Prossnitz and Barton 2014; Sharma et al. 2013; Sharma and Prossnitz 2016; Martensson et al. 2009; Barton and Prossnitz 2015), immune regulation (Blasko et al. 2009; Brunsing et al. 2013; Brunsing and Prossnitz 2011),

cardiovascular physiology (Haas et al. 2009; Meyer et al. 2014, 2015, 2016; Fredette et al. 2017), reproduction (Thomas et al. 2010; Wang et al. 2008), the nervous system (Srivastava and Evans 2013; Xu et al. 2009; Hazell et al. 2009), and cancer (Prossnitz and Barton 2011; Arias-Pulido et al. 2010; Lappano et al. 2014; Marjon et al. 2014; Petrie et al. 2013; Smith et al. 2007, 2009). Stimulation of GPER activates a multitude of cellular signaling pathways including MAPK, PKC, PI3K, adenylyl cyclase, eNOS, and Ca^{2+} mobilization (Prossnitz and Barton 2014; Prossnitz and Arterburn 2015; Revankar et al. 2005; Filardo et al. 2000, 2002).

Whereas the roles of nuclear ERs in metabolism are more established (Barros and Gustafsson 2011; Jia et al. 2015), the physiological or pathological roles of GPER in metabolic signaling are still emerging (Sharma and Prossnitz 2016; Nilsson et al. 2011; Sharma et al. 2017) (Fig. 1). The effects of E2 and its multiple receptors on metabolism, as in other systems (Hadjimarkou and Vasudevan 2017), may be direct or indirect and furthermore may exhibit synergism or

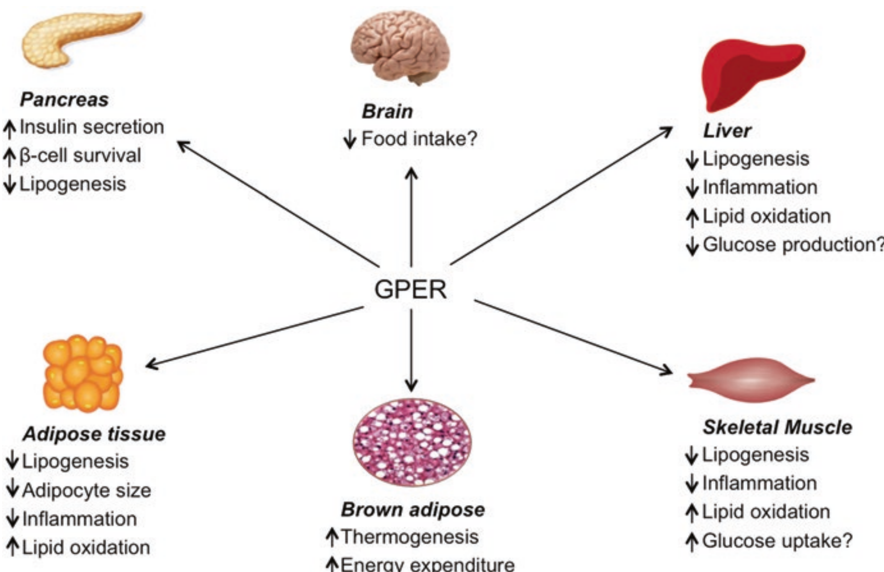

Fig. 1 Schematic representation of the metabolic roles of GPER. GPER exerts pleiotropic effects on metabolically active tissues such as the pancreas, adipose, brown adipose, liver, and muscle. GPER controls body weight by regulating food intake and increasing energy expenditure as well as thermogenesis in brown adipose tissue. GPER activation in the pancreas promotes β-cell survival and insulin secretion. Stimulation of GPER in the pancreas, adipose, liver, and skeletal muscle reduces lipid deposition by inhibiting lipogenesis and promoting lipid oxidation. GPER activation also attenuates inflammation in multiple tissues. GPER-mediated prevention of lipotoxicity and inflammation in non-adipose tissues may improve glucose homeostasis by increasing insulin secretion, improving glucose uptake, and reducing hepatic glucose production in the pancreas, skeletal muscle, and liver, respectively. Effects not clearly known are indicated by. See text for details

antagonism that may impact overall metabolic status. Thus, to assess the mechanisms involved in these complex interactions, the specific contribution of individual receptors must be assessed. Interestingly, mice lacking either ERα or GPER share similarities in metabolic phenotypes to varying degrees, such as increased adiposity, decreased insulin sensitivity, defective glucose/lipid homeostasis, and inflammation (Sharma et al. 2013; Martensson et al. 2009; Davis et al. 2014; Heine et al. 2000; Ribas et al. 2010; Prossnitz and Hathaway 2015). This suggests that both receptors might act cooperatively to mediate metabolic effects through similar or alternatively distinct mechanistic pathways. It is important to note, however, that in the chronic absence of an individual receptor, compensatory effects may take place, masking the role of a given receptor in normal physiology or disease.

Since E2 binds to multiple receptors, pharmacological and genetic approaches can be employed to discriminate the contributions of individual receptors. Through the use of GPER-selective pharmacological agents, such as the agonist G-1 or antagonists G15 and G36, it is now possible to investigate the specific functions of GPER compared with those of ERs in complex systems expressing multiple estrogen receptor types (Prossnitz and Arterburn 2015; Revankar et al. 2007; Prossnitz 2017; Bologa et al. 2006; Dennis et al. 2009, 2011). However, experiments employing selective estrogen receptor modulators (SERMs) and selective estrogen receptor downregulators (SERDs) should be carefully interpreted as multiple SERMs, such as tamoxifen and raloxifene, and the SERD fulvestrant have been shown to lack ER specificity, acting as GPER agonists (Prossnitz and Arterburn 2015; Revankar et al. 2005; Filardo et al. 2000; Petrie et al. 2013). In addition, the availability of various genetic tools such as GPER knockout (GPER KO) mice (Prossnitz and Hathaway 2015) and siRNA or shRNA directed against GPER has significantly advanced our knowledge of GPER function. GPER KO mice with a global gene deletion of GPER (of which four independent genetically modified strains have been developed as reviewed in Prossnitz and Hathaway 2015) have been employed to evaluate metabolic phenotypes, generally revealing weight gain and metabolic dysfunction (Sharma et al. 2013; Martensson et al. 2009; Davis et al. 2014). Pharmacological and genetic approaches have often complemented each other, wherein treatment of mice with the GPER-selective agonist G-1 results in the opposite effect observed in GPER KO mice (Prossnitz and Hathaway 2015; Sharma and Prossnitz 2011). Furthermore, treatment of GPER KO mice with G-1 lacks the stimulatory effect of G-1 in WT mice, thereby confirming the selectivity of this compound for GPER via the absence of off-target effects (Prossnitz and Hathaway 2015; Sharma and Prossnitz 2011). In many systems, the lack of effects upon stimulation of GPER KO mice with E2 further validates the importance of GPER signaling in the actions of E2 (Prossnitz and Hathaway 2015; Sharma and Prossnitz 2011). Thus, recent studies utilizing GPER-selective approaches have provided strong evidence of the contributions of GPER signaling to metabolic homeostasis.

GPER, Body Weight, and Energy Homeostasis

Multiple studies have examined whether GPER regulates overall body weight, fat content, and energy balance (Tables 1 and 2). In the first such study, in 2009, Barton and colleagues reported that both male and female mice lacking GPER exhibited increases in body weight and visceral adiposity compared to their WT counterparts (Haas et al. 2009). At about the same time, but contrary to these results, Leeb-Lundberg and coworkers reported that female GPER KO mice exhibited slightly lower body weights than the corresponding WT mice, whereas GPER deficiency in males had no effects on body weight (Martensson et al. 2009). In 2013, we reported increased adiposity in male GPER KO mice throughout life, from 6 to 24 months of age, compared to WT mice (Sharma et al. 2013). MRI analysis revealed an overall increase in fat content of GPER KO mice with increased fat deposition in subcutaneous depots as well as visceral fat depots, such as the epididymal and perirenal fat pads. The increase in adiposity of GPER KO mice occurred in the absence of altered food intake or locomotor activity. Furthermore, weight gain in male GPER KO mice was correlated with a significant increase in circulating levels of cholesterol, triglycerides, and LDL (Sharma et al. 2013), suggesting that GPER regulates key pathways involved in lipid homeostasis.

Consistent with these latter results, in a subsequent study in 2014, Clegg and colleagues observed that both male and female GPER KO mice demonstrated increased body weight and decreased energy expenditure in the absence of any changes in food intake (Davis et al. 2014). Furthermore, male and female GPER KO mice displayed a divergence in body weights compared to WT mice at different ages. Thus, while male GPER KO mice began to develop adiposity by 8 weeks of age, female GPER KO mice displayed detectable weight gain only at 14 weeks of age. Importantly, expression of two thermogenic genes, UCP1and β_3-adrenergic receptor, was reduced in brown adipose tissue of GPER KO mice. Interestingly, although expression of the β_3-adrenergic receptor was reduced in both male and female GPER KO mice, UCP1 expression was only decreased in male GPER KO mice. Furthermore, compared to WT mice, female GPER KO mice were less sensitive to the inhibitory effects of leptin on food intake and cholecystokinin (CCK) on satiety, whereas males did not reveal any differences. Interestingly, whereas E2 induced hypothalamic ERK activation in ovariectomized WT female mice, GPER KO mice failed to do so. Several studies have implicated hypothalamic ERK1/2 in the regulation of energy homeostasis (Rahmouni et al. 2009). Reduced E2-mediated ERK1/2 phosphorylation in hypothalamus may thus explain the diminished anorectic effects of leptin and CCK in female GPER KO mice. These findings strongly support the idea that the interaction of E2 with GPER is an important mediator of body weight regulation. Very recently and contrary to the multiple independent studies just outlined, Wang et al. reported that female GPER KO mice (the same as used in the previous studies by us and Clegg) were unexpectedly protected from diet-induced obesity, exhibiting lower body weight, decreased adipogenesis, and increased dark phase energy expenditure (Wang et al. 2016). The reasons behind these contradictory results are not clear. In general, factors such as chow, bedding,

Table 1 Effects of loss of GPER expression on metabolism. In vitro, ex vivo, and in vivo studies reveal that GPER regulates body weight, food intake, and energy expenditure. In addition, GPER modulates pancreatic cell survival and hormone secretion as well as glucose and lipid metabolism. Effects in GPER KO mice or islets are compared to WT control animals or islets. See the text for details

Cells/tissues/ mice	Treatment	Effect/(s)	Reference/year
GPER KO (F) mice	–	Slightly reduced body weight	Martensson et al. (2009)
		Hyperglycemia and impaired glucose tolerance	
	OVX + E2	No increase in serum insulin levels	
GPER KO (F) islets	Basal	Reduced insulin and glucagon secretion	
	E2	No increase in insulin or decrease in glucagon secretion	
GPER KO (M) mice	–	No changes in body weight and normal glucose homeostasis	
GPER KO (M) islets	Basal	Reduced insulin secretion but glucagon secretion unchanged	
	E2	No changes in insulin or glucagon secretion	
GPER KO (F) mice	STZ	Increased incidence of diabetes (vs. WT mice + STZ)	Liu et al. (2009)
		Lower insulin levels and pancreatic insulin content	
GPER KO (M) mice	STZ	Incidence of diabetes similar to WT mice	
GPER KO islets	STZ+E2	Increased pancreatic islet survival (possibly via ER)	
	STZ+G-1	No increase in pancreatic islet survival	
GPER KO (F) islets	E2 or G-1	Reduced insulin secretion under high glucose	Sharma and Prossnitz (2011)
GPER KO islets	E2	No decrease in lipid accumulation	Tiano et al. (2011)
GPER KO (M) mice	–	Increased body weight, fat content, and dyslipidemia	Sharma et al. (2013)
		Impaired glucose tolerance and insulin resistance	
		Increased fasting insulin	
GPER KO (F) mice	–	Increased body weight	Davis et al. (2014)
		Reduced energy expenditure and brown fat thermogenesis	
	OVX + E2	No decrease in body weight or improvement in glucose tolerance	
	Leptin or CCK	No decrease in food intake	
GPER KO (M) mice	–	Increased body weight	
		Reduced energy expenditure and brown fat thermogenesis	
	Leptin or CCK	Reduction in food intake	

(continued)

Table 1 (continued)

Cells/tissues/ mice	Treatment	Effect/(s)	Reference/year
GPER-lacZ (F) mice	HFD	No changes in body weight	Meoli et al. (2014)
		Increased liver fat accumulation, decreased HDL	
GPER-lacZ (M) mice		No changes in body weight, liver fat accumulation, or HDL levels	

or environment can confound an observed phenotype. Furthermore, differences between studies could also arise as a result of the method used to generate GPER KO mice (e.g., homologous recombination of embryonic stem cells vs. cre/loxP, where chromosomal translocations are possible due to cryptic or pseudo-loxP sites) as recently reviewed (Prossnitz and Hathaway 2015).

Because a number of studies examining GPER KO mice reported that loss of GPER resulted in adiposity, we hypothesized that selective activation of GPER in a mouse model of obesity might attenuate weight gain and alleviate other chronic disease states arising from obesity. To this end, we tested the therapeutic potential of selective GPER agonism, employing G-1 in a mouse model of metabolic dysfunction, namely, ovariectomy. This model mimics menopause in women and results in adiposity and metabolic dysfunction due to loss of endogenous E2, as revealed by the reversal of metabolic dysfunction by E2 supplementation. In this model, treatment of ovariectomized mice with G-1 resulted in attenuation of overall weight gain and fat content as revealed by DEXA and MRI scans, without effects on bone mineral density, bone mineral content, or lean mass (Sharma and Prossnitz, unpublished data). In addition, a significant reduction in the mass of multiple fat depots was observed upon G-1 treatment, as well as increased energy expenditure and higher expression of UCP1 in brown adipose tissue. Although G-1 exerted similar actions to those of E2 on the regulation of body weight and fat deposition, unlike E2, it did not increase uterine weight, reflecting a lack of the potent feminizing effects of E2 (Dennis et al. 2009). Thus, activation of GPER may represent a novel strategy to counteract weight gain and fat deposition.

GPER and Glucose Homeostasis

GPER is emerging as a key player in glucose homeostasis (Tables 1 and 2) (Sharma and Prossnitz 2016; Sharma et al. 2017). Evidence for an in vivo role of GPER in the regulation of glucose metabolism first emerged from studies of ERα/β double knockout mice exposed to streptozotocin (STZ). These mice did not exhibit a further increase in the incidence of diabetes when compared to either ERα KO or ERβ KO mice alone (Liu et al. 2009). In addition, ovariectomy increased the severity of insulin-deficient diabetes in ERα/β double KO mice following STZ treatment, an effect that was reversed by E2 supplementation, suggesting the presence of an

Table 2 GPER-mediated effects on metabolism. Selective pharmacological activation or genetic knockdown of GPER reveals that stimulation of GPER promotes human and murine pancreatic cell survival and insulin secretion, reduces lipid accumulation in adipocytes. In ovariectomized mice, GPER activation attenuates adiposity and improves glucose tolerance and lowers fasting glucose, insulin, and cholesterol

Cells/tissues/mice	Treatment	Effect/(s)	Reference/year
αβERKO (F) mice	OVX + STZ + E2	Lower incidence of diabetes (vs. OVX + STZ) (via non-ERs possibly GPER)	Liu et al. (2009)
		Higher insulin levels and pancreatic insulin content	
Mice	G-1	Increased pancreatic islet cell survival	Liu et al. (2009), Balhuizen et al. (2010), and Kumar et al. (2011)
Human islets			
MIN6 cells			
WT mice (F) islets	G-1	Increase in insulin secretion under high glucose	Balhuizen et al. (2010)
		Decrease in glucagon secretion under low glucose	
		Decrease in somatostatin secretion under high glucose	
		Inhibition of pancreatic cell apoptosis	
Human (F) islets	G-1	Increase in insulin secretion under high glucose	Kumar et al. (2011)
		Decrease in pancreatic cell apoptosis	
MIN6	E2 or G-1	Increased insulin secretion	Sharma and Prossnitz (2011)
	(± G15 or ± siRNA)	Inhibition of E2 or G-1-mediated insulin secretion	
WT mice (F) islets	E2 or G-1	Increased insulin secretion	
	(± G15)	Inhibition of E2 or G-1-mediated insulin secretion	
ZDF (M) islets	G-1	Reduced lipid accumulation	Tiano et al. (2011) and Tiano and Mauvais-Jarvis (2012)
INS-1 cells			
Human islets			
3T3-L1	E2 or G-1	Inhibition of lipid accumulation	Zhu et al. (2013)
	(± siRNA)	Reversal of GPER-mediated decrease in lipid deposition	
WT mice	OVX+G-1	Reduction in body weight and fat content	Unpublished data
		Improved glucose tolerance	
		Reduced insulin resistance	
		Lower fasting glucose, insulin, and cholesterol levels	
		Increased fatty acid oxidation in metabolic tissues	

additional distinct response mechanism to E2. Taken together, these studies suggested that even in the absence of ERα and ERβ, E2 continues to exert antidiabetic actions, consistent with the possible involvement of another estrogen receptor, such as GPER. Roles for GPER in glucose homeostasis have been established by a number of groups through the study of GPER KO mice. In 2009, Leeb-Lundberg and colleagues reported that deletion of GPER in mice resulted in impaired glucose tolerance and hyperglycemia with differential effects on male and female mice (Martensson et al. 2009). Whereas female GPER KO mice exhibited impaired glucose tolerance, increased plasma glucose, and defective GSIS compared to their WT counterparts, males did not reveal any differences between WT and GPER KO genotypes. In ovariectomized mice, GPER deficiency completely abolished E2-mediated increases in serum insulin. Furthermore, Mauvais-Jarvis and colleagues revealed that only female but not male GPER KO mice, upon exposure to STZ, exhibited a higher propensity toward insulin-deficient diabetes, displaying higher blood glucose levels, loss of β-cells, and a decrease in pancreatic insulin content (Liu et al. 2009). However, in contrast to the above reports, our observations revealed that male GPER KO mice exhibit age-dependent effects on glucose tolerance and insulin resistance (Sharma et al. 2013). At 6 months, although GPER KO mice were already insulin resistant as revealed by insulin tolerance tests (ITTs), they did not display any differences in glucose tolerance tests (GTTs), the latter consistent with the previous study by Leeb-Lundberg and colleagues (Martensson et al. 2009). However, at 12 months of age, GPER KO mice exhibited a trend toward impaired glucose tolerance, which was statistically significant by 18 months of age, with a concomitant exacerbation of insulin resistance (Sharma et al. 2013). The detection of higher fasting plasma insulin levels in GPER KO mice, with normal fasting glucose levels, further confirmed the presence of insulin resistance, in which elevated insulin levels are required to maintain normal glucose levels (Abdul-Ghani and DeFronzo 2009).

Weight gain, specifically visceral adiposity, is linked to a chronic inflammatory state and a decrease in the serum levels of adiponectin, an insulin-sensitizing adipokine that also exhibits anti-inflammatory properties (Bastard et al. 2006; Mangge et al. 2010). Consistent with these observations, we observed that adiposity in GPER KO male mice was accompanied by increases in systemic markers of inflammation, such as TNFα, MCP1, IL-1β, and IL-6, along with a decrease in adiponectin levels (Sharma et al. 2013). The glucose intolerance present in aged GPER KO mice could have resulted from the cumulative effects of adiposity, insulin resistance, dyslipidemia, and inflammation. The existence of a pro-inflammatory state in GPER KO mice was subsequently confirmed by Davis et al., who demonstrated that both male and female GPER KO mice exhibit systemic increases in levels of the inflammatory marker SAA3 and a decrease in adiponectin levels compared to WT mice (Davis et al. 2014). Furthermore, treatment of ovariectomized GPER KO mice with E2 did not yield any improvements in glucose tolerance as in ovariectomized WT mice, indicating a definitive requirement for GPER in E2-mediated glucose metabolism. Taken together, these studies clearly indicate that both male and female GPER KO mice, in a sex-specific manner, exhibit regulation of glucose metabolism via GPER.

To determine whether selective activation of GPER could alleviate the symptoms of metabolic dysfunction with respect to insulin resistance and glucose tolerance, we treated ovariectomized mice with the GPER-selective agonist G-1 (Sharma and Prossnitz, unpublished data). Our results revealed that G-1 treatment led to a significant improvement in glucose tolerance in ovariectomized mice, with lower fasting glucose and insulin levels. In addition, G-1 treated mice exhibited improved insulin sensitivity and reduced the levels of circulating pro-inflammatory cytokines and hormones leptin and resistin. Lower fasting blood glucose and insulin levels in the ovariectomized mice suggest beneficial effects of G-1 on glucose homeostasis in both the liver and skeletal muscle, leading to speculation that G-1 may directly modulate glucose production in the liver and glucose uptake in skeletal muscle. However, as discussed above, since treatment with G-1 also prevents weight gain and visceral fat deposition in ovariectomized mice, an improvement in glucose homeostasis could be due to either direct or indirect effects of GPER-mediated signaling events in the tissues involved in metabolic regulation, which exhibit substantial cross talk with respect to glucose homeostasis (Samdani et al. 2015; Kim 2016).

GPER and Pancreatic Function

Pancreatic β-cells produce, store, and release insulin, the critical hormone in glucose homeostasis. GPER promotes the survival and function of multiple cell types in islets, particularly β-cells, the mechanisms of which have been examined in some detail and exhibit definite sex differences (Tables 1 and 2) (Liu et al. 2009; Mauvais-Jarvis 2016; Ropero et al. 2012). GPER expression was considerably higher in islets from females compared to males, both in mice and humans (Balhuizen et al. 2010; Kumar et al. 2011). Under basal conditions, islets isolated from male and female GPER KO mice exhibited reductions in insulin secretion compared to WT controls, as well as in the presence of glucose or tolbutamide, a potassium channel blocker that causes insulin secretion by blocking potassium channels in pancreatic β-cells (Martensson et al. 2009). Interestingly, with respect to their WT counterparts, islets isolated from female GPER KO mice exhibited a greater reduction in insulin secretion compared to islets from male GPER KO mice. Furthermore, islets from female GPER KO mice under basal conditions exhibited a decrease in pancreatic insulin content compared to islets from WT mice, which may have resulted from a defective E2 signaling in the absence of GPER. Similarly, islets isolated from both male and female GPER KO mice completely lacked the E2-stimulated insulin secretion present in islets from WT mice, despite the use of supraphysiological concentrations of E2 (5 μM). Although E2 treatment of ovariectomized GPER KO mice failed to increase serum insulin levels, islets from treated mice exhibited higher pancreatic insulin content (presumably via ERα) compared to WT controls, suggesting that GPER may be important for insulin secretion from the pancreas. Finally, G-1 stimulation of both human and murine islets modulated hormone secretion and exerted antidiabetic effects in a dose-dependent manner similar to E2, with both agents

increasing insulin secretion while inhibiting glucagon and somatostatin secretion (Balhuizen et al. 2010; Kumar et al. 2011).

The mechanisms leading to insulin secretion upon GPER activation by E2 or G-1 involve increased signaling through the cAMP/PKA and PLC/IP3 pathways, as stimulation with both agents increased the formation of cAMP and IP3 in a dose-dependent manner in islets from human female donors (Kumar et al. 2011). Interestingly, G-1 was more potent in IP3 production, whereas E2 exhibited higher potency in cAMP generation. In cultured mouse insulinoma MIN6 cells, E2 and G-1 both stimulated insulin secretion that could be inhibited by pharmacologic GPER-selective antagonism with G15 and depletion of GPER by siRNA (Sharma and Prossnitz 2011). Similarly, insulin secretion in WT islets was inhibited by G15 in response to GPER activation by either E2 or G-1, both of which failed to induce insulin secretion in islets from GPER KO mice (Sharma and Prossnitz 2011). In MIN6 cells, stimulation of GPER results in intracellular calcium release as well as activation of the ERK and PI3K pathways (Sharma and Prossnitz 2011). As previously reported in some cancer and other cell lines, GPER-mediated ERK activation occurred via transactivation of the EGFR (Filardo et al. 2000; Sharma and Prossnitz 2011). Interestingly, whereas ERK activity exhibited a positive effect on insulin secretion, PI3K activity inhibited insulin secretion, as previously observed (Hagiwara et al. 1995; Longuet et al. 2005). Thus, whereas inhibition of either EGFR or ERK prevented E2- or G-1-induced increases in insulin secretion, inhibition of PI3K signaling led to an increase in insulin secretion compared to E2 and G-1 alone. These results suggest that E2- and G-1-mediated activation of the ERK and PI3K pathways oppose each other and may serve to balance the secretion of insulin in response to multiple signaling inputs.

Obesity and insulin resistance lead to the process of compensation in islets, increasing the biosynthesis and secretion of insulin as well as the number of β-cells to maintain normal blood glucose levels (Cerf 2013). Persistent cellular stresses associated with obesity and insulin resistance, such as cytokine-induced inflammation, mitochondrial dysfunction, oxidative stress, ER stress, and glucolipotoxicity, eventually lead to β-cell death and hyperglycemia. E2 is known to promote β-cell survival under these conditions (Prentki and Nolan 2006). Experimentally, activation of GPER by either E2 or G-1 promoted cell survival and counteracted apoptosis induced by pro-inflammatory cytokines and oxidative stress in both murine and human islets, as well as in MIN6 cells (Liu et al. 2009; Balhuizen et al. 2010; Kumar et al. 2011). In islets subjected to inflammatory injury, E2 or G-1 promoted islet cell survival via phosphorylation of pro-survival genes such as CREB, Akt, and ERK1/2 with concomitant suppression in the activity of stress proteins, such as SAPK/JNK and p38 (Kumar et al. 2011). Pretreatment with ER antagonists, ICI 182,780 or EM-652, did not inhibit the protective effects of E2, suggesting that E2 may function via GPER, consistent with the protective effects of G-1. In addition, islets from GPER KO mice exposed to oxidative stress lacked G-1-mediated protection, although survival mediated by E2 was maintained, suggesting a parallel effect of E2 via ERα (Liu et al. 2009). Interestingly, islets isolated from mice lacking both ERα and ERβ still exhibited protection against cell death when challenged with STZ,

suggesting the involvement of GPER or another unknown ER in mediating this response (Liu et al. 2009). In addition to the mechanisms described above, studies on isolated islets and cultured cells have shown that GPER activation also inhibits lipid accumulation, by suppressing the expression of important transcription factors involved in lipogenesis, such as chSREBP and SREBP1 via STAT3, potentially leading to even greater antiapoptotic effects as a result of reduced lipotoxicity (Tiano et al. 2011; Tiano and Mauvais-Jarvis 2012).

An additional physiological stressor, pregnancy, also leads to the expansion of pancreatic β-cell mass in order to compensate for maternal insulin resistance (Rieck and Kaestner 2010; Ernst et al. 2011). Increases in β-cell mass during pregnancy result from an increase in proliferation and survival of β-cells through the downregulation of islet-specific microRNA mi-338-3p (Jacovetti et al. 2012). In cultured β-cells and dissociated islets, downregulation of miR-338-3p increased proliferation and protected cells against pro-inflammatory cytokine-induced apoptosis. GPER expression increased in rat islets during pregnancy, peaking at day 14, with E2 repressing mi-338-3p in rat islets via GPER through a cAMP-dependent pathway (Jacovetti et al. 2012). Activation of GPER in rat islets reduced the expression of mi-338-3p, an effect that was reversed by treatment with GPER-targeted siRNA. These results indicate that E2 signaling via GPER suppresses the expression of miR-338-3p, which may be critical for the increase in β-cell mass during pregnancy.

Effects of GPER in Peripheral Metabolic Tissues

Although roles for GPER in overall body weight regulation and glucose homeostasis have been observed (Sharma et al. 2013; Martensson et al. 2009; Davis et al. 2014), little is known regarding the effects of GPER in the individual peripheral tissues that are actively involved in metabolism, such as the adipose, liver, and skeletal muscle, all of which act in a coordinated manner to maintain metabolic homeostasis. Although GPER is widely expressed in multiple insulin-sensitive tissues such as the liver, adipose, and skeletal muscle, female mice exhibited higher GPER expression in white adipose tissue compared to males (Davis et al. 2014). GPER expression was localized predominantly to adipocytes with little expression in the stromal vascular fraction (Davis et al. 2014). GPER expression has also been reported in 3T3-L1 preadipocytes, where it was upregulated during differentiation of preadipocytes into adipocytes (Zhu et al. 2013). Treatment of 3T3-L1 preadipocytes with E2 or G-1 during differentiation inhibited lipid accumulation in adipocytes, an outcome that was reversed by GPER knockdown with siRNA (Zhu et al. 2013). During adipogenic differentiation, after initial mitotic clonal expansion, cells arrest at the G_1 growth phase of the cell cycle and subsequently express adipogenic factors (Tang et al. 2003; Patel and Lane 2000). Treatment with G-1 in 3T3-L1 cells results in an aberrant differentiation process wherein most of the cells continue to divide even after 48 h of differentiation, whereas in the control group, the majority

of cells arrested in the G_0/G_1 state by 24 h following the induction of differentiation (Zhu et al. 2013). Furthermore, GPER activation increased the expression of cell cycle-regulating factors, such as CDK4, CDK6, and cyclin D. Thus, GPER inhibited lipid accumulation in adipocytes at least in part by preventing cell cycle arrest and subsequent differentiation (Zhu et al. 2013).

As described above for multiple studies, mice lacking GPER exhibit an increase in overall adiposity with increased fat deposition in subcutaneous, perigonadal, and perirenal fat depots compared to their WT counterparts (Sharma et al. 2013; Haas et al. 2009; Davis et al. 2014). In the gonadal fat pads of GPER KO mice, adipocytes were larger compared to WT mice, as a result of increased lipid storage (Davis et al. 2014). Interestingly, in GPER-lacZ mice (a mouse mutant that harbors a β-galactosidase (lacZ) reporter within the *Gper* locus, disrupting *Gper* expression), only female GPER KO mice exhibited increased lipid accumulation in liver along with a decrease in circulating HDL levels compared to WT mice, whereas male mice displayed no such differences (Meoli et al. 2014). Consistent with GPER KO mice, a study of a human cohort of the Northern European descent has revealed that individuals carrying a hypofunctional P16L genetic variant of GPER have increased plasma LDL cholesterol (Hussain et al. 2015). These observations were further extended using HepG2 liver cells in which activation of GPER with G-1 increased the expression of the LDL receptor (Hussain et al. 2015). This upregulation was blocked by either GPER antagonist G15 or knockdown of GPER expression by shRNA. These results imply that GPER plays a crucial role in modulating central pathways involved in lipid metabolism in multiple tissues, suggesting that selective GPER activation might be beneficial in lowering lipid levels. Thus, we examined the effects of GPER stimulation on lipid homeostasis in vivo in an ovariectomized mouse model (Sharma and Prossnitz, unpublished data). G-1 treatment lowered the levels of circulating lipids, reduced the expression of lipogenic and pro-inflammatory genes, and increased the expression of genes involved in lipid oxidation in the adipose, liver, and skeletal muscle. Thus, GPER exerts pleiotropic effects in metabolic tissues leading to reductions in both lipid accumulation and inflammation.

Conclusions

It has now become clear that GPER regulates not only body weight but also multiple aspects of metabolism in numerous tissues throughout the body, such as the pancreas, adipose, liver, and skeletal muscle. However, mechanisms of GPER-mediated effects remain poorly understood and merit further study. Global GPER KO mice have been used by a number of groups to investigate the functions of GPER in vivo (Prossnitz and Hathaway 2015), but due to cross talk between metabolic tissues and the possibility of compensatory effects during development, conclusions from such studies must be interpreted with caution. The use of pharmacological approaches to modulate GPER activity has largely supported the conclusions from GPER KO studies (Prossnitz and Arterburn 2015). With the epidemic prevalence of obesity and metabolic dysfunction, it is more critical than ever to identify new therapeutic

approaches to mimic the salutary effects of E2 without the feminizing and other side effects of estrogenic substances, particularly for men. The therapeutic targeting of GPER may represent one such approach to simultaneously treat multiple aspects of metabolic syndrome.

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Competing Interests GS and ERP are inventors on a US patent application for the therapeutic use of compounds targeting GPER. ERP is an inventor on US patent Nos. 7,875,721 and 8,487,100 for GPER-selective ligands and imaging agents.

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Sex-Dependent Role of Estrogen Sulfotransferase and Steroid Sulfatase in Metabolic Homeostasis

Wojciech G. Garbacz, Mengxi Jiang, and Wen Xie

Abstract Sulfonation and desulfation are two opposing processes that represent an important layer of regulation of estrogenic activity via ligand supplies. Enzymatic activities of families of enzymes, known as sulfotransferases and sulfatases, lead to structural and functional changes of the steroids, thyroids, xenobiotics, and neurotransmitters. Estrogen sulfotransferase (EST) and steroid sulfatase (STS) represent negative and positive regulation of the estrogen activity, respectively. This is because EST-mediated sulfation deactivates estrogens, whereas STS-mediated desulfation converts the inactive estrogen sulfates to active estrogens. In addition to the known functions of estrogens, EST and STS in reproductive processes, regulation of estrogens and other signal molecules especially at the local tissue levels has gained increased attention in the context of metabolic disease in recent years. EST expression is detectable in the subcutaneous adipose tissue in both obese women and men, and the expression of EST is markedly induced in the livers of rodent models of obesity and type 2 diabetes. STS was found to be upregulated in patients with chronic inflammatory liver diseases. Interestingly, the tissue distribution and the transcriptional regulation of EST and STS exhibit obvious sex and species specificity. EST ablation produces completely opposite metabolic phenotype in female and male obese mice. Adipogenesis is also differentially regulated by EST in murine and human adipocytes. This chapter focuses on the recent progress in our understanding of the expression and regulation EST and STS in the context of metabolic homeostasis.

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Estrogen Sulfotransferase (EST)

Local and systemic estrogenic activity can be regulated by ligand availability through the metabolic transformation of estrogens, which includes sulfation and desulfation. The sulfonation of drugs, xenobiotics, hormones, and neurotransmitters is a general biological mechanism of metabolism that can lead to dramatic structural and functional changes of the affected molecules (Strott 2002). Sulfonation is the transfer of a sulfonate group (SO_3^{-1}) from the sulfonate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a hydroxyl site on acceptor molecule (also referred as sulfoconjugation) (Strott 1996, 2002). PAPS is formed from ATP and inorganic sulfate, and it is the universal sulfonate donor for all sulfotransferase reactions in mammals (Lipmann 1958; Strott 1996). A large family of enzymes, known as sulfotransferases (SULTs), are responsible for transferring sulfate group of PAPS to a variety of endogenous and exogenous molecules, including steroids (Leiter and Chapman 1994; Song et al. 1995; Kakuta et al. 1997; Negishi et al. 2001). Sulfotransferases are both cytoplasmic and membrane-bound enzymes (Chapman et al. 2004), and their contributions in the regulation of biological processes are conducted by the modulation of signal molecules such as steroids, thyroids, and neurotransmitters by catalyzing their sulfonation (Kauffman 2004; Reinen and Vermeulen 2015). The sulfonation reaction usually results in inactivation of the substrates or substantially weakens the potency of the ligands (Strott 2002; Bjerregaard-Olesen et al. 2015), but the opposite might also be true. For example, some SULTs are capable of bioactivating pro-carcinogens to reactive electrophiles (Gamage et al. 2006). In most cases, however, the transfer of a sulfonate moiety to a molecule decreases its biological activity, increases its water solubility, and promotes its urinary excretion (Song et al. 1995; Falany 1997).

The estrogen sulfotransferase (EST or SULT1E1) is a member of the sulfotransferase family. It shares high amino acid homology with other sulfotransferase isoforms. However, EST is believed to have unique functions due to its distinct substrates and specific tissue distribution and sex-regulated expression (Leiter and Chapman 1994; Song et al. 1995; Gao et al. 2012). Estrogen sulfotransferase was initially described in 1958 during the formation of estrone sulfate in the rat liver (Nose and Lipmann 1958). It was cloned from the human liver in 1994 (Aksoy et al. 1994) and the mouse testis in 1995 as it is expressed abundantly in testicular Leydig cells (Song et al. 1995). The mouse Est shares 88% in amino acid sequence with the rat liver Est and 77% with human liver EST (Song et al. 1995). EST has a particularly high affinity for estrogens as substrates including estradiol (17β-estradiol), estrone, and a variety of synthetic estrogens, like diethylstilbestrol and tamoxifen (Song et al. 1995; Falany 1997). EST also exhibits a low affinity for thyroid hormones, testosterones, or glucocorticoids (Wada et al. 2011).

Consistent with the function of EST in estrogen sulfation and deactivation, EST has been shown to play a important role in protecting the reproductive tissue in both sexes. Male $Est^{-/-}$ mice developed age-dependent Leydig cell hypertrophy/hyperplasia, impaired steroidogenesis, had reduced total and forward sperm motility, and produced smaller litters compared with age-matched wild-type males (Qian et al.

2001; Tong et al. 2004). In female mice, ablation of the mouse Sult1e1 gene caused placental thrombosis and spontaneous fetal loss, which was associated with elevated free estrogen levels in the circulation and the amniotic fluid (Tong et al. 2005). Additionally, EST plays a role in inhibiting the estrogen-dependent growth of breast cancer cells both in vitro and in vivo through its activity in deactivating estrogens (Gong et al. 2007, 2008).

Emerging evidence suggests that EST also has functions outside the reproductive tissues and beyond estrogen metabolism. EST was found to be expressed in the human liver (Aksoy et al. 1994), white adipose tissue (Ahima et al. 2011; Ihunnah et al. 2014), kidney, brain, adrenal cortex, and epithelial cells of the gastrointestinal tract (Hobkirk 1985; Miki et al. 2002). In mice, under normal chow diet, the hepatic expression of EST is relatively low (Gong et al. 2007, 2008). However, the mRNA and protein expression of EST was highly induced in the livers of obese and diabetogenic leptin receptor deficient C57BL/KsJ-db/db mice. Interestingly, the db/db genotype does not change the constitutive expression of the enzyme in the testis (Song et al. 1995). The leptin-deficient ob/ob mice as well as wild-type mice fed with Western style high-fat diet also show increased expression of EST specifically in the liver (Gao et al. 2012). The expression of EST can also be stimulated in the liver by dexamethasone through the activation of the glucocorticoid receptor (GR) (Gong et al. 2008). The activation of GR can lead to hyperglycemia in genetically obese viable yellow (Avy) female mice due to an aberrant shift in hepatic androgen/estrogen balance (Gill et al. 1994). In mice, the expression of EST shows sex specificity. Male, but not female, mice express a high basal level of EST in the white adipose tissue (WAT). The expression of EST in the male epididymal fat pad is particularly highly. The expression of EST was also detected at a lower level in several other white adipose depots, but not in the brown adipose tissue (Khor et al. 2008). Interestingly, castration of male mice abolishes EST expression in the epididymal fat, whereas testosterone supplementation restores it, suggesting that EST expression in male WAT is testosterone dependent (Khor et al. 2008). Further analysis showed that EST is predominantly expressed in stromal vascular cells (pre-adipocytes) (Khor et al. 2008; Wada et al. 2011). Consistent with pre-adipocyte pattern of expression, the expression of EST was dramatically reduced in differentiated 3T3-L1 cells or mature primary adipocytes (Wada et al. 2011). Interestingly, although the basal expression of Est in the female mouse WAT is low, it can be induced in the parametrial fat by testosterone treatment. The induction of EST expression by testosterone in female mice is WAT specific, because testosterone treatment failed to induce the expression of EST in the liver (Khor et al. 2008). It might represent physiological conditions in postmenopausal women, where the circulating levels of testosterone and estradiol are not only low but also mostly formed locally in target tissues from a systemic source of androgens through aromatization to estrogens (Simpson et al. 2005). Overexpression of EST in 3T3-L1 cells or transgenic expression of EST in the WAT of female mice results in decreased differentiation of primary adipocytes and smaller adipocyte size, suggesting that conditional EST expression/activity in WAT has a profound effect on female adiposity (Khor et al. 2010; Wada et al. 2011). The inhibitory effect of EST on adipogenesis might

have resulted from the activation of ERK1/2 MAPK and inhibition of insulin signaling (Wada et al. 2011), which explains a blunted glucose uptake in parametrial adipose tissue during the hyperinsulinemic-euglycemic clamp in EST transgenic female mice (Khor et al. 2010).

Interestingly, the effect of EST on adipogenesis seems to be species specific. The anti-adipogenic activity of EST in mice was opposite to the pro-adipogenic effect of the same enzyme in human adipocytes (Ihunnah et al. 2014). The expression of EST in the abdominal subcutaneous fat of obese and nonobese female human subjects and in primary adipose-derived stem cells (ASCs) isolated from female subjects was low, but the expression of EST increased with the onset of differentiation (Ihunnah et al. 2014). The pro-adipogenic effect resulted from EST overexpression in ASCs could be mimicked by using an estrogen receptor (ER) antagonist or ER knockdown, suggesting that EST promoted adipogenesis by deactivating estrogens (Ihunnah et al. 2014). EST expression in whole fat was positively correlated with body mass index (BMI) in female patients (Ihunnah et al. 2014). It has also been reported that although the expression of EST can be positively associated with general adiposity or BMI scores, it does not correlate with the circulating levels of estradiol or testosterone in both men and women (Ahima et al. 2011). This particular result might reflect the heterogeneity or small size of the study group (Ahima et al. 2011). It is also possible that the expression of EST may affect only local concentrations of sex steroids rather than affecting the circulating levels of sex hormones. This notion was supported by the observation that only tissue-specific or local changes in insulin sensitivity were shown in the female murine model of WAT-EST overexpression (Khor et al. 2010).

Although EST expression can be induced in the parametrial fat of female mice, whole body ablation of EST did not cause any differences in fat indices (fat weight normalized to body weight) between female WT and $EST^{-/-}$ mice (Khor et al. 2008). However, the $EST^{-/-}$ male mice had their epididymal and inguinal fat indices significantly higher than those of the WT mice (Khor et al. 2008). Also, adipocyte size in $EST^{-/-}$ male mice was markedly larger than that of WT mice, but under chow diet, these changes were not accompanied by obvious metabolic abnormalities (Khor et al. 2008). However, the loss of Est dramatically worsens male metabolic performance under obesity condition. In the study by Gao and colleagues, male ob/ob mice deficient of Est (obe) exhibited aggravated diabetic phenotype (Gao et al. 2012). The obe males that had higher fasting glucose level and worse glucose tolerance performance also showed impaired glucose-stimulated insulin secretion. The white adipose tissue had elevated expression of several macrophage markers and higher density of crown-like structures indicating increased local inflammation (Gao et al. 2012). Consistent with impaired insulin secretion upon glucose stimulation, the obe male mice had reduced islet size, total islet area, and β-cell mass; however, Est expression is not detectable in islets (Gao et al. 2012). Therefore, the mechanism by which Est loss in obese males worsens metabolic phenotype remains to be better defined. Strikingly, female obe mice exhibited completely opposite metabolic phenotype. Whole body loss of Est expression in the ob/ob background decreased hepatic gluconeogenesis and lipogenesis, improved body composition and insulin sensitivity, and increased energy expenditure (Gao et al. 2012). This

effect was likely due to increased hepatic estrogens availability as a result of decreased estrogen deactivation, because this metabolic benefit was abolished in ovariectomized mice (Gao et al. 2012). The improved metabolic function in obe females was largely expected, because increased hepatic estrogen activity is known to be protective via suppressing hepatic gluconeogenesis and lipogenesis and by increasing hepatic insulin sensitivity (Mauvais-Jarvis et al. 2013). Sex-dependent effects became more apparent as estradiol was also found to regulate different pools of genes in site and sex-specific manner in visceral and subcutaneous adipose tissues of ob/ob mice. And this effect was independent of the amount of estrogen receptor expressed in given tissues (Shinozaki et al. 2007).

Ablation of Est in female ob/ob mice did not affect WAT inflammation (Gao et al. 2012), an effect somewhat expected, because female mice have little basal expression of Est in WAT (Khor et al. 2008). However, it is noted that unlike in mice, EST was detectable in the subcutaneous adipose tissue in both obese women and men, especially in the abdominal area (Ahima et al. 2011). Nevertheless, EST through controlling the local availability of the estrogens has a profound effect on adipocyte metabolism and growth (Cooke and Naaz 2004).

In another example of species-specific effect of EST, transgenic expression of EST in female WAT does not significantly change the mRNA expression levels of proinflammatory genes such as MCP1 and IL-1β (Khor et al. 2010). In contrast, elevated EST expression in WAT in obese men and women was significantly associated with the expression of tumor necrosis factor alpha (TNF-α) and suppressor of cytokine signaling-3 (SOCS3), a downstream target of TNF-α and a marker of inflammation (Ahima et al. 2011). This result points to potential inflammatory regulation of this enzyme, at least in human adipose tissues. Indeed, consistent with this notion, treatment of human vascular smooth muscle cells (VSMCs) with IL-1β significantly induces EST mRNA levels in vitro (Nakamura et al. 2003). Additionally, the expression of EST in the VSMCs was significantly higher in aortas with severe atherosclerotic changes than aortas with mild atherosclerotic changes (Nakamura et al. 2003). This further implicates a possible interaction between EST expression and inflammation, a state particularly common in obesity and metabolic diseases. Not surprisingly in this context, hepatic EST induction was reported in the mouse model of sepsis, where it participates in proper inflammatory responses (Chai et al. 2015). The induction of EST in acute inflammation in sepsis model is nuclear factor kappa-B (NF-κB) dependent, and EST is an NF-κB target gene (Chai et al. 2015). Another inducer of EST expression is hypoxia and oxidative stress as shown in the mouse model of liver ischemia and reperfusion (I/R) injury (Guo et al. 2015). Both hypoxia and oxidative stress are associated with inflammation and insulin resistance especially in WAT (Ye 2009; Lawler et al. 2016). Mechanistically, in I/R, inflammation and oxidative stress induce the activation of nuclear factor 2 (Nrf2), followed by the induction of EST who is a direct transcriptional target of Nrf2 (Guo et al. 2015). The interplay between inflammation, hypoxia, oxidative stress, and EST induction/activity represents a not entirely understood mechanism of endocrine regulation in the inflammatory response, which might play an important role in the clinical outcome of sepsis, ischemia/reperfusion, and chronic inflammation. Independently from inflammation, hepatic EST is also positively regulated by the

liver X receptor (LXR) (Gong et al. 2007), while the retinoid-related orphan receptor (ROR) negatively regulates the expression of EST (Wada et al. 2008; Kang et al. 2007), at least in mice. A confirmed repressor of EST gene in human primary hepatocytes and hepatocellular carcinoma Huh7 cells is pregnane X receptor (PXR), when the PXR was activated by rifampicin (Kodama et al. 2011). Aryl hydrocarbon receptor (AhR) activation has also been shown to suppress EST expression (Fu et al. 2011). Another xenobiotic nuclear receptor, constitutive androstane receptor (CAR), was reported to induce the expression of EST in the mouse liver in response to diallyl sulfide (DAS) treatment (Sueyoshi et al. 2011). Environmental pollutants such as dioxin, polychlorinated biphenyl 153, or bisphenol A have also been reported to induce the hepatic expression of EST in a sex-specific manner, but the mechanism of induction remains to be defined (Naville et al. 2013).

Steroid Sulfatase (STS)

Sulfonation decreases the biological activity of the estrogens, because estrogen sulfates are hormonally inactive. However, at the same time, the sulfation process creates a pool of estrogen reserves through the steroid sulfatase (STS)-mediated desulfation. This reaction is possible because estrogen sulfates have higher circulating concentrations than estradiol and a prolonged half-life (Reed et al. 2005). STS, also known as aryl sulfatase C, is an enzyme responsible for the cleavage of estrone sulfate to estrone and dehydroepiandrosterone sulfate (DHEA-S) to DHEA (Ferrante et al. 2002). Other important substrates for STS are pregnenolone sulfate and cholesterol sulfate. In fact, steroids appear mostly sulfated, as in the case of DHEA-S, which is present in concentrations up to 100-fold higher than unconjugated DHEA (Leowattana 2004). Similarly, estrone sulfate also has a long half-life and is a highly abundant steroid in human serum with concentrations that are 10–20-fold higher than estrone and estradiol (Pasqualini 2004). STS-mediated desulfation and EST-mediated sulfonation are therefore reversible reactions that tightly regulate steroid homeostasis.

STS is a membrane-bound protein localized in the lumen of the endoplasmic reticulum but also found in Golgi (Gande et al. 2008; Thomas and Potter 2013). STS gene spans over 10 exons and is located on the short arm of chromosome X and mapped in Xp22.3-Xpter (Conary et al. 1986; Yen et al. 1988). STS is expressed as a precursor with asparagine-linked oligosaccharide chains. These chains are cleaved by endoglucosaminidase H, and STS is formed with the final size of 61 kDa. The half-life of STS is approximately 4 days (Conary et al. 1986). STS activity was first shown in microsomes from rat liver (Dodgson et al. 1954). Although the organ and tissue distribution varies considerably between species, STS expression is virtually ubiquitous in small quantities. To date, STS expression has been confirmed not only in reproductive organs such the testis, ovaries, placenta, endometrium, and prostate but also in the adrenal glands, brain, fetal lung, lymphocytes, aorta, kidney, bone, and skin (Mueller et al. 2015). Because it is highly expressed in the human skin, inactivation of the STS gene, most often due to large deletions of the gene, results

in X-linked ichthyosis due to excess deposition of cholesterol sulfate in the skin (Webster et al. 1978). In general, STS gene deletion or mutation was reported to be associated with reproductive defects, such as cryptorchidism in males and failed labor progression in females, which is likely due to disrupted steroid hormonal homeostasis (Geyer et al. 2016).

Steroid metabolism is altered in many endocrine-related cancers (Purohit et al. 2011; Purohit and Foster 2012). Dysregulated androgen levels are associated with an adverse metabolic phenotype in both genders (Schiffer et al. 2017). Androgen excess can be associated with increased risk of obesity, insulin resistance, diabetes, or cardiovascular disease (Pasquali et al. 2008; O'Reilly et al. 2014). Among the molecular basis of androgen excess, one of them is disrupted sulfonation pathway that converts DHEA to DHEA-S and the other an excessive desulfation activity. Both of these events increase the DHEA reservoir for downstream conversion to active androgens. However, because STS has multiple substrates including estrone sulfate, the role of STS activity in metabolism is more complex and seems to be sex specific. In ob/ob mice as well as diet-induced obese WT mice, the liver expression of Sts was induced (Jiang et al. 2014). In the same study, Sts induction was also detected during the fed to fasting transition. Transgenic overexpression of STS in the liver mice alleviates some metabolic abnormalities in the obese and diabetic mice, including reduction of body mass, improved insulin sensitivity, and decreased hepatic steatosis and inflammation (Jiang et al. 2014). Interestingly ovariectomy abolished this protective effect, whereas the metabolic benefit in male STS transgenic mice was retained upon castration (Jiang et al. 2014). These results suggest that in obese female mice, STS, by converting biologically inactive estrogen sulfates to active estrogens, may have increased hepatic estrogen activity and consequently improved the metabolic performance. That seems to be confirmed by treatment of mice with estrone sulfate, an STS substrate, which also improved metabolic functions in both the diet-induced obesity and ob/ob models (Jiang et al. 2014). The mechanism for the metabolic benefit of the STS transgene in males is less obvious, but STS liver overexpression was associated with the male-specific decrease of inflammation in white adipose tissue and skeletal muscle as well as gene expression profile in muscle tissue that promotes energy expenditure (Jiang et al. 2014). However, STS transgene was neither targeted nor expressed in the adipose tissue or skeletal muscle. It is likely that the male liver-expressing STS transgene might have generated protective mediators to benefit those non-hepatic tissues. Serum biochemistry showed that serum DHEA, which can be converted from DHEA sulfate by STS, was increased in STS males (Jiang 2014; Jiang et al. 2014). STS mutant mice exhibited significantly lower DHEA serum levels without changes in the corticosterone levels (Trent et al. 2012).

DHEA was found to have multiple beneficial effects to attenuate metabolic syndrome. DHEA supplementation improves vascular endothelial function and insulin sensitivity in men (Kawano et al. 2003), reduced plasma triglycerides and the inflammatory cytokines IL-6 and TNFα in aging men and women (Weiss et al. 2011). In rodents, administration of DHEA significantly decreased the blood glucose level and the glucose 6-phosphatase (G6P) mRNA level in db/db mice (Aoki et al. 2000). In

rats, combination of DHEA administration and exercise training lowers fasting insulin and blood glucose levels and improves insulin sensitivity, which may reflect increased muscular DHEA and DHT concentrations (Sato et al. 2012).

STS is expressed at relatively high levels in both human and mouse brains where it may regulate DHEA/DHEA-S levels as they regulate brain functions (Mathur et al. 1993). Consistent with this notion, STS dysfunction has been associated with attention-deficit/hyperactivity disorder (ADHD). Deletion or mutation of the STS gene increases the risk of developing ADHD and other neurologic dysfunctions in human subjects (Brookes et al. 2008). Animal studies also confirmed that genetic loss or pharmacological inhibition of STS activity in mice leads to attention deficits, changed impulsivity, and increased aggressiveness, which resembles human ADHD phenotype (Davies et al. 2009).

STS is also expressed in the white adipose tissue of women where it plays an important role in the formation of biologically active sex hormones, especially in postmenopausal women. Both the expression and activity of STS were higher in postmenopausal adipose tissues than in premenopausal adipose tissues, which might suggest that the hydrolysis of circulating DHEA-S may have played an increasing role after menopause in local steroid biosynthesis in the adipose tissue (Paatela et al. 2016).

Increased estrogen levels have been reported in patients with chronic liver diseases as signs of endocrine disturbance (Adlercreutz 1970). Moreover, the serum estrogens levels are positively associated with the pathophysiology of the liver disease (Gavaler 1995). This estrogen excess has traditionally been reasoned to be due to compromised liver function in metabolizing estrogens. Whether or not the estrogen-reactivating STS also plays a role in estrogen excess is not known. We recently reported that the hepatic expression of STS was induced in patients with chronic inflammatory liver diseases, which was associated with increased circulating estrogen levels (Jiang et al. 2016). In human hepatic cells, the STS gene was induced by inflammatory stimuli through the activation of NF-κB, and STS was established as a direct target gene of NF-κB (Jiang et al. 2016). These results suggest a negative feedback loop in chronic inflammatory liver diseases, in which activation of NF-κB by inflammatory stimuli induces STS gene expression. Once induced, STS converts inactive estrogen sulfates back to active estrogens, which subsequently attenuate NF-κB-mediated inflammation (Jiang et al. 2016). From the metabolic perspective, liver STS activity is not only limited to desulfation of local estrogen sulfates. Patients with recessive X-linked ichthyosis due to STS deficiency also show elevated serum levels of oxysterol sulfates (Sanchez-Guijo et al. 2015). Apart from the accumulation of cholesterol sulfate, X-linked ichthyosis patients also manifest higher circulating levels of hydroxycholesterol sulfates, with 27-hydroxycholesterol-3-sulfate (27OHC3S) as one of the most abundant hydroxycholesterol sulfates (Sanchez-Guijo et al. 2015). 27OHC3S can be desulfonated to form 27OH, an endogenous ligand for the liver X receptor (LXR). The other sulfonated oxysterol found in the rat and human liver is 5-cholesten-3beta, 25-diol 3-sulfonate (25HC3S) (Ren et al. 2006).

Oxysterols are known to bind to LXR and upregulate hepatic de novo lipogenesis (Janowski et al. 1996). The expression of LXR was found to be correlated with the

degree of hepatic fat deposition, as well as with hepatic inflammation and fibrosis in NAFLD patients (Ahn et al. 2014). In this regard, STS activity might contribute to increased pool of endogenous LXR agonists and consequently increased the lipogenic activity of LXR in the liver. Interestingly, it has been proposed that the sulfated oxysterols are not simply blocked from binding to nuclear receptors but are actively inhibiting LXR signaling via an unknown mechanism. In one study, 25HC3S was found to decrease the levels of LXR and sterol regulatory element-binding proteins (SREBPs), inhibit SREBP processing and activation, and decrease the intracellular lipid accumulation in hepatocytes and THP-1-derived macrophages (Ren and Ning 2014). On the other hand, another report suggests that LXR can be negatively regulated in mouse gonadal adipose tissue by estrogens (Lundholm et al. 2004) and STS provides the unconjugated form of estrogens.

Induction and regulation of STS can be mediated by several factors. STS expression is often induced in breast tumors, which is often associated with higher estrogenic activity and poor prognosis (Utsumi et al. 1999; Shah et al. 2016). The direct induction of STS expression in tumor tissue could be mediated by cytokines and growth factors. From the analysis of STS gene transcription in ten human tissues including the ovary, adrenal cortex, uterus, thyroid, liver, pancreas, colon, mammary gland, and others, six different promoters were found to drive the expression of STS (Dalla Valle et al. 2007). These results suggest that the regulation of STS transcription appears to be complex and tissue dependent (Dalla Valle et al. 2007). It seems that STS can also be regulated in a transcription-independent manner. For example, cytokines such interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) are capable of increasing the activity of STS in a hormone-dependent human breast cancer cell line (MCF-7), without any change in STS mRNA expression levels (Newman et al. 2000).

In breast cancer cell lines, it was also shown that growth factors such as the insulin-like growth factor-1 (IGF-I) and basic fibroblast growth factor (bFGF) stimulate STS activity in a dose-dependent manner. The ability of cytokines and growth factors to induce STS activity even in postmenopausal women may explain the increased estrogen levels in breast tumor tissues even with low circulating estrogen concentrations (van Landeghem et al. 1985). Mechanistically, in the PC-3 human prostate cancer cells and human keratinocyte (both expressing relatively high levels of STS), the phosphatidylinositol 3-kinase/Akt pathway has been found to mediate the induction of STS expression and activity by growth factors and cytokines (Suh et al. 2011; Hattori et al. 2012). This pathway was also found to promote cell survival and tumor progression (Courtney et al. 2010). STS is also induced or activated by steroids. STS induction, at least in part, could be controlled by the exogenous testosterone treatment in male mice (Lam and Polani 1985). Castration significantly reduces STS activity in the adrenal, heart, and liver in rats, confirming the androgen influence in STS activity (Snyder et al. 2000). In contrast, ovariectomy was not found to be not necessary for estrone sulfatase-inhibiting studies in female rats (Barth et al. 2000). Injections of exogenous estrone sulfate enhanced the STS activities in the liver and white blood cells in female rats, suggesting that STS can be induced directly through substrate supply (Barth et al. 2000). Additionally, STS

activity has been reported to be induced by retinoids and 1,25-dihydroxy vitamin D3 in HL-60 promyelocytic cells (Hughes et al. 2001) and by stress-stimulated adrenocorticotropic hormone (ACTH) secretion in rat adrenals (Dominguez et al. 1975). In contrast, the progestagen promegestone (R-5020) can decrease the mRNA expression of STS in MCF-7 cells, as well as inhibit the enzyme activity of STS in the same cell line (Pasqualini et al. 1994).

Summaries and Perspectives

Understanding of how sulfonation and desulfation processes are regulated and dysregulated in metabolic homeostasis and disease models provides key insights into the endocrine control of physiology and pathophysiology. The induction or inhibition of EST and STS in the context of metabolic disease is complex. The two enzymes not only represent opposite enzymatic functions, but they also differ in their substrate preferences, and they are expressed in a sex- and tissue-specific manner. Figure 1 shows an example of the opposite and diverse function of EST and STS in metabolic disease and chronic inflammatory diseases.

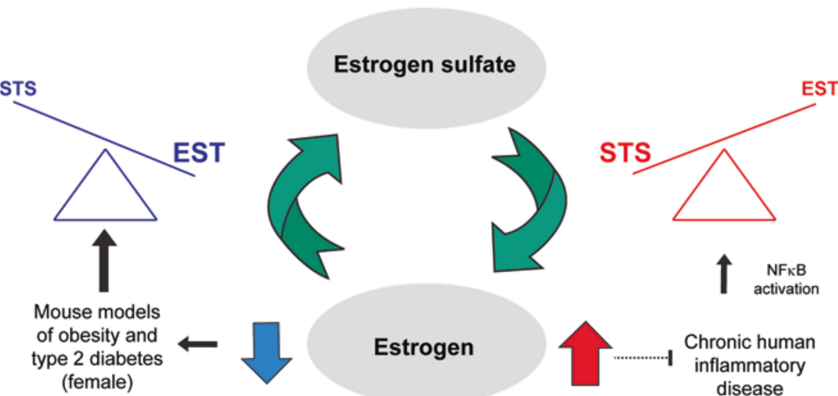

Fig. 1 Schematic illustration of the role of the STS-mediated estrogen homeostasis in energy homeostasis and inflammation. Under physiological conditions, estrogen homeostasis is maintained by the balanced sulfation and desulfation reactions catalyzed by EST and STS, respectively. However, in mouse models of type 2 diabetes, EST is dramatically induced in the liver, which overrides the small increase of STS, resulting in increased estrogen deprivation and contributing to the development of insulin resistance. During chronic inflammation and at least in humans, activation of NF-κB induces the expression of STS, but the expression of EST was downregulated, which together increase estrogen level and bioactivity in the circulation. STS-mediated activation of estrogen signaling may suppress the NF-κB response and inhibit inflammation (Adapted from Jiang 2014)

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Part III
Impact of Androgens in Metabolic Homeostasis and Disease

Negative Impact of Testosterone Deficiency and 5α-Reductase Inhibitors Therapy on Metabolic and Sexual Function in Men

Abdulmaged M. Traish

Abstract Androgens are steroid hormones with pleotropic and diverse biochemical and physiological functions, and androgen deficiency exerts a negative impact on human health. Testosterone (T) either directly or via its transformation into the more potent metabolite 5α-dihydrotestosterone (5α-DHT) or via aromatization into estradiol (E_2) modulates important biochemical signaling pathways of human physiology and plays a critical role in the growth and/or maintenance of functions in a host of tissues and organs. T and 5α-DHT play an important role in regulating physiology of the muscle, adipose tissue, liver, bone, and central nervous system, as well as reproductive and sexual functions. Thus, androgen deficiency (also referred to as hypogonadism) is a well-recognized medical condition and if remained untreated will have a negative impact on human health and quality of life.

In this chapter, we have summarized the negative impact of T deficiency (TD) on a host of physiological functions including reduced lean body mass (LBM), increased fat mass (FM), increased insulin resistance (IR), metabolic syndrome (MetS) and adiposity, reduced bone mineral density (BMD), anemia, sexual dysfunction, and reduced quality of life and increased mortality. In addition, we discuss another critical aspect of unrecognized form of androgen deficiency resulting from inhibition of 5α-reductases with drugs, such as finasteride and dutasteride, to block transformation of T into 5α-DHT in the course of treatment of benign prostatic hyperplasia (BPH) and male pattern hair loss, also known as androgenetic alopecia (AGA). The negative impact of drugs that inhibit transformation of T to 5α-DHT by 5α-reductases on metabolic function is manifested in fat accumulation in the liver, which may predispose to nonalcoholic fatty liver disease (NAFLD). Also, inhibition of 5α-DHT formation increases glucose synthesis and reduces glucose disposal potentially contributing to hyperglycemia, IR, and elevated activities of liver function enzymes concomitant with reduction in circulating T levels, worsening erectile dysfunction (ED), and reduced quality of life.

Although we have attempted to summarize the current literature pertaining to this critical topic "androgen deficiency" and its impact on men's health and quality

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of life, there remain many gaps in the knowledge regarding the biochemical pathways that are involved in the pathophysiology of androgen deficiency. We wish to clearly state that there are areas of controversies, including whether age-related androgen deficiency (functional hypogonadism) merits treatment and whether T therapy provided real proven benefits. Finally, considerable debate exists with respect to the potential and purported cardiovascular (CV) risks of treating TD with exogenous T. For brevity sake, we will not discuss in detail the benefits of T therapy in men with TD since this topic is comprehensively covered by Dr. F. Saad's chapter in this book, entitled "*Testosterone Therapy and Glucose Homeostasis in Men with Testosterone Deficiency (Hypogonadism).*"

We have made a concerted effort to address the controversy of T therapy in men with TD in the discussion. However, we wish to acknowledge that these issues will remain a matter of debate for some time to come. Only with advances in fundamental basic science and clinical research, some of these controversial issues may be laid to rest. Nevertheless, we believe that there is considerable body of credible evidence to suggest that T therapy of men with TD is safe and effective and provides a host of health benefits and therefore merits considerations in men with TD, irrespective of the underlying cause or etiology. An additional aspect of androgen deficiency is the drug-induced reduction in 5α-DHT levels by the use of 5α-reductase inhibitors. We also believe that physicians prescribing 5α-reductase inhibitors (i.e., finasteride or dutasteride) for relief of BPH symptoms or treatment of hair loss should engage their patients in a productive discussion regarding the potential adverse side effects of these medications on their overall health and quality of life.

List of Abbreviations

5α-DHT	5α-Dihydrotestosterone
ALT	Alanine aminotransferase
AR	Androgen receptor
ARKO	Androgen receptor knockout mouse
BMI	Body mass index
CHF	Congestive heart failure
CPT1	Carnitine palmitoyltransferase 1
E_2	Estradiol
ER	Estrogen receptor
ERKO	Knockout mouse for ER
FAS	Fatty acid synthase
HDL-c	High-density lipoprotein cholesterol
HFD	High-fat diet
HgA1c	Hemoglobin A1c

HOMA-IR	Homeostatic model of insulin resistance
IR	Insulin resistance
LDL-c	Low-density lipoprotein cholesterol
MetS	Metabolic syndrome
MMP	Mitochondrial membrane potential
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
P-ACC	Acetyl coenzyme A carboxylase
P-HMGCR	3-Hydroxy-3-methyl-glutaryl-CoA reductase
T	Testosterone
T2DM	Type 2 diabetes mellitus
TD	Testosterone deficiency (hypogonadism)
TG	Triglyceride
TGs	Triglycerides
UCP2	Uncoupling protein 2
WC	Waist circumference

Introduction

Testosterone (T) is a metabolic, vascular, reproductive, and sexual hormone, which regulates a multitude of physiological processes, including energy utilization; protein, carbohydrate, and fat metabolism; muscle differentiation; growth and function; and bone metabolism, and influences body composition, sexual function, and quality of life (Kelly and Jones 2013a, b, 2014, 2015; Kelly et al. 2014, 2016; Traish et al. 2009a, b, c,d 2011a, b, Traish 2014a, b, c,Traish et al.2014a, 2017a; Traish 2016a, b; Rao et al. 2013; Traish and Guay 2006; Snyder et al. 2016). T serves as substrate for 5α-reductases (5α-Rs) in the formation of 5α-dihydrotestosterone (5α-DHT), a potent androgen hormone with a high affinity for the androgen receptor (AR). T also serves as the substrate for aromatization of T into estradiol (E_2). T and its metabolites 5α-DHT and E_2 are critical signaling molecules in regulating metabolic, reproductive, and sexual functions. Furthermore, T, directly or via conversion to 5α-DHT or E_2, regulates the differentiation of a host of pluripotent stem cells, in various tissues and organs, thus contributing to tissue remodeling and maintaining physiological function (Carruthers et al. 2008).

It is well recognized that T and 5α-DHT are key signaling molecules in the differentiation of muscle progenitor cells into smooth muscle, striated muscle, and cardiac muscle. Also, T and 5α-DHT inhibit differentiation of pre-adipocytes into adipocytes and therefore play an important role in obesity (Singh et al. 2003, 2006; Gupta et al. 2008; Chazenbalk et al. 2013). T also modulates the differentiation of hemocytoblasts to hematoblasts and osteoblastic progenitor cells differentiation into osteoblasts. Moreover, differentiation of epithelial progenitor cells into vascular

endothelium is also subjected to T and 5α-DHT regulation (Carruthers et al. 2008; Traish and Galoosian 2013; Xu et al. 2010; Zhang et al. 2016a; Giatti et al. 2015; Bolduc et al. 2007). Thus, it is important to recognize the critical physiological role of T and its metabolites (5α-DHT and E_2) in maintaining physiological processes and overall health and quality of life. Reductions in physiological T levels as well as 5α-DHT and E_2, irrespective of the underlying cause or etiology, are associated with a host of recognized clinical signs and symptoms, and if remained untreated, TD will negatively impact overall health and quality of life (Buvat et al. 2010, 2013; Traish 2014a, b, c; Morgentaler et al. 2015a, b, 2016; Kelly and Jones 2013a, b, 2014, 2015).

Negative Impact of Testosterone Deficiency (TD) on Metabolic and Sexual Function

Testosterone Deficiency (TD) and Its Impact on Human Health

For clarity sake, and throughout this chapter, we will use the following terminology when referring to older terms commonly used in the literature. Henceforth, we will use the term T deficiency (TD) instead of the older term "hypogonadism," irrespective of etiology. We will also use the term T therapy instead of the older term "testosterone replacement therapy (TRT)." The term low T will be used interchangeably with TD, since it is commonly used in the literature and in the clinical setting when referring to TD.

TD has recently been propelled to the forefront of clinical debate and discussions and has attracted considerable media and public attention after being ignored for decades. Nevertheless, TD and its treatment also provoked considerable controversies even though treatment of this medical condition has significant benefits in men's health (Traish 2014a, b; Morgentaler et al. 2015a, b, 2016; Snyder et al. 1999a, b, 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017). TD is characterized by a host of clinical signs and symptoms and reduced biochemical levels of circulating plasma T (Buvat et al. 2010, 2013; Wang et al. 2000, 2001, 2004, 2008, 2011; Bhasin et al. 2010). As early as the 1940s, TD is recognized as an important medical condition that merits treatment (Aub 1940; Aub and Kety 1943). TD is a well-established medical condition that negatively impacts men's health with regard to physical, reproductive, and sexual function and affects mood, energy, and well-being, with important implication on the quality of life (Morgentaler et al. 2016).

The signs and symptoms of TD include sexual dysfunction, reduced BMD, diminished muscle mass and strength, gynecomastia, anemia, frailty, increased body fat and body mass index (BMI), fatigue, and IR. TD also impacts psychological function by increasing depressed mood, diminishing energy and sense of vitality and well-being, and impairing cognition and memory. Furthermore, TD negatively

impacts sexual function resulting in diminished libido, difficulty achieving erections, and reduced spontaneous and nocturnal erections (Yu and Traish 2011; Traish 2014a; Buvat et al. 2010, 2013; Wang et al. 2008; Bhasin et al. 2010; Snyder et al. 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017).

Lessons from ADT Pertaining to TD and Human Pathology

Androgen deprivation therapy (ADT) in the treatment of patients with metastatic prostate cancer (PCa) either by surgical or medical castration produces significant changes in metabolic function and alters body composition with concomitant increases in body weight and body fat and loss of LBM (Smith et al. 2001, 2002, Smith 2004, Smith et al. 2006, Smith 2007a, b, Smith 2008). In men without diabetes, ADT decreased insulin sensitivity and increased fasting plasma insulin and elevates fasting glucose and glycated hemoglobin (HbA1c) levels (Smith et al. 2006; Keating et al. 2006, 2012, 2014; Dockery et al. 2003; Nishiyama et al. 2005; Yannucci et al. 2006). In nondiabetic men with PCa, short-term ADT, together with administration of AR antagonist, significantly increased FM and decreased insulin sensitivity (Smith et al. 2006; Keating et al. 2006). Men receiving ADT had significantly higher fasting glucose, HbA1c, insulin, leptin levels, and homeostatic model assessment (HOMA) index compared with men with prostate cancer but who were not treated with ADT or healthy controls (Basaria and Dobs 2001, 2007; Basaria et al. 2006, 2008). These findings demonstrate significant negative correlation between total and free T levels with fasting glucose, insulin, leptin, and HOMA-IR and suggested that long-term ADT increases the prevalence of type 2 diabetes (T2DM) and metabolic syndrome (MetS) and increased cardiovascular mortality (Basaria et al. 2006, Basaria 2008). The estimated risk of incident diabetes associated with ADT approaches 1.36-fold, and patients are more likely to develop T2DM within 1 year, even after adjustments for age, poor health, and hypertension (Lage et al. 2007). Long-term ADT produces unfavorable hormonal and metabolic profiles, including insulin resistance (IR) and hyperglycemia, independent of age and BMI and increases the risk of T2DM (Derweesh et al. 2007).

Nguyen et al. (2015a) reported that ADT increased IR, altered glycemic control, and contributes to development of T2DM and MetS, corroborating findings reported in other studies (Rubinow et al. 2012; Shahani et al. 2008). Glycemic control was worsened substantially in men treated with ADT with concomitant increases of serum glucose and HbA1c levels (Haider et al. 2007). ADT is associated with increased risk and worsening of diabetes, coronary heart disease (CHD), myocardial infarction (MI), and sudden death (Saylor & Smith 2009; Tsai et al. 2007, 2015; Keating et al. 2012, 2014).

Keating et al. (2006, 2012, 2014) evaluated the impact of ADT on diabetes, CHD, MI, and sudden cardiac death in a population-based cohort of 73,196, aged

66 years or older who were diagnosed with locoregional prostate cancer. ADT was associated with increased risk of incident diabetes, CHD, MI, and sudden cardiac death. ADT decreases LBM and increases FM and reduces insulin sensitivity. It was concluded that ADT is associated with higher incidence of diabetes and cardiovascular disease (CVD) (Keating et al. 2006, 2012, 2014; Saigal et al. 2007; Pilepich et al. 2005; Tsai et al. 2007).

Hamilton et al. (2011) reported that ADT increased visceral and subcutaneous abdominal fat areas. Fat mass increased by 14%, lean tissue mass decreased by 3.6%, and IR (as assessed by HOMA-IR) increased by 12%. Based on these findings, Hamilton et al. (2011) proposed that ADT-induced changes in body composition contribute to increased IR, hyperglycemia, and onset of T2DM. Patients treated with ADT had elevated glucose and increased IR, as measured by HOMA index levels; these findings were independent of age and BMI. In a large number of Japanese patients with TD and T2DM who received T therapy, increased insulin sensitivity and reduced IR and atherosclerosis were reported when compared with healthy men (Fukui et al. 2007, 2008). These findings strongly suggest that induced TD contributes to the pathophysiology of MetS, including T2DM, IR, and obesity (Traish et al. 2009a, b, c, 2014a, Traish et al 2011a), and T therapy has substantial benefits.

TD and Role of β-Cell Function in Hyperglycemia, IR, and Diabetes

A number of large observational studies have reported that androgen depletion therapy (ADT) in prostate cancer patients produced profound TD and increases the risk of T2DM (Mauvais-Jarvis 2016; Mauvais-Jarvis et al. 2002) suggesting that T is important for insulin secretion in men. Therefore, a new paradigm may be considered to explain the impact of ADT and the resulting TD in predisposing individuals treated with ADT to hyperglycemia and diabetes. As proposed by Mauvais-Jarvis (2016), the clinically documented IR and associated increase in adiposity subsequent to ADT may not contribute to the observed hyperglycemia in the absence of some degree of β-cell failure (Polonsky 1995; Prentki and Nolan 2006; U.K. Prospective Diabetes Study Group 1995; Weyer et al. 1999). Inaba et al. (2005) reported significant hyperglycemia and diminished β-cell function in PCa patients receiving ADT, suggesting that TD predisposes β-cell to dysfunction and failure. It should be noted that IR and/or adiposity in men with TD may not contribute to frank diabetes if adequate β-cell compensation remains (Mauvais-Jarvis et al. 2002). Similarly, obese men may not develop diabetes if they develop β-cell hyperfunction to compensate for IR (Prentki and Nolan 2006). Thus, reports of hyperglycemia in patients with ADT indicate that severe TD may result in β-cell failure in order to compensate for IR. It is critical to point out that in β cells the AR exhibits

an extranuclear location and mediates glucose-stimulated isulin secretion (GSIS) by increasing cAMP production and activating the cAMP-dependent protein kinase A in a manner similar to glucagon-like peptide 1 (GLP-1) (Navarro et al. 2016). In contrast, it is believed that in the prostate AR mediates its function via translocation into the nucleus and binding to androgen response elements (ARE) activating AR-specific genes. In vitro experiments using cultured islets suggested that the insulinotropic effect of T depends on activation of the GLP-1 receptor by islet-derived GLP-1. In addition GSIS and glucose intolerance in response to parenteral glucose were remarkably attenuated in βARKO mice indicating that T improves insulinotropic signaling of islet-derived GLP-1 in vivo (Smith et al. 2014). Therefore, a new paradigm is proposed in which T facilitates insulin secretion mediated by GLP-1 action.

The underlying biochemical bases of TD and diabetes are supported by a body of evidence from clinical and preclinical studies; however, the exact molecular mechanisms of TD and increased onset of diabetes, IR, and hyperglycemia remain unknown. Marked hyperglycemia and diminished β-cell function are reported in PCa patients receiving ADT (Inaba et al. 2005). These findings suggest that TD brings about impairment in β-cell function and contributes to failure to compensate for IR (Mauvais-Jarvis 2016; Navarro et al. 2016; Xu et al. 2017). Animal experimentation has provided evidence for the role of androgens in maintaining β-cell function. In adult male β-cell AR knockout (βARKO) mice, it was shown that decreased GSIS resulted in glucose intolerance (Navarro et al. 2016). βARKO mice exposed to a Western diet developed hypoinsulinemia and hyperglycemia in the fed or fast states. T facilitated GSIS in human and mouse islets in culture. This effect is inhibited by flutamide, an AR antagonist in βARKO islets and in human islets, suggesting that T mediates its action on GSIS directly on AR in islet β cells. Based on findings from in vivo and in vitro experimentations, a novel non-genomic mechanism was postulatled by which the extranuclear AR and its paracrine interactions with β-islets regulate the GLP-1 receptor, which enhances β-cell function. In β-cells, the AR stimulates GSIS by increasing intracellular cAMP biosynthesis, thus activating the cAMP-dependent protein kinase A (PKA). Furthermore, the insulinotropic effect of T was attributed to the activation of the GLP-1 receptor by islet-derived GLP-1 via paracrine mechanisms (Mauvais-Jarvis 2016; Navaro et al. 2016; Xu et al. 2017). These novel mechanisms shed new light on the potential role of androgen deficiency in increasing the risk of diabetes but also provide new therapeutic approaches that may help in prevention and/or treatment of diabetes. The biochemical and physiological mechanisms proposed in this new paradigm may explain in part why ADT leads to a 30% increased risk of type 2 diabetes, which suggests that in addition to IR, ADT predisposes to β-cell failure since hyperglycemia does not develop with IR alone. Thus, IR can only result in hyperglycemia if β-cell failure occurs. Because AR is an important signaling molecule in promoting insulin secretion in β-islets in rodents and humans, it is imperative to appreciate the importance of TD in IR, hyperglycemia, and diabetes.

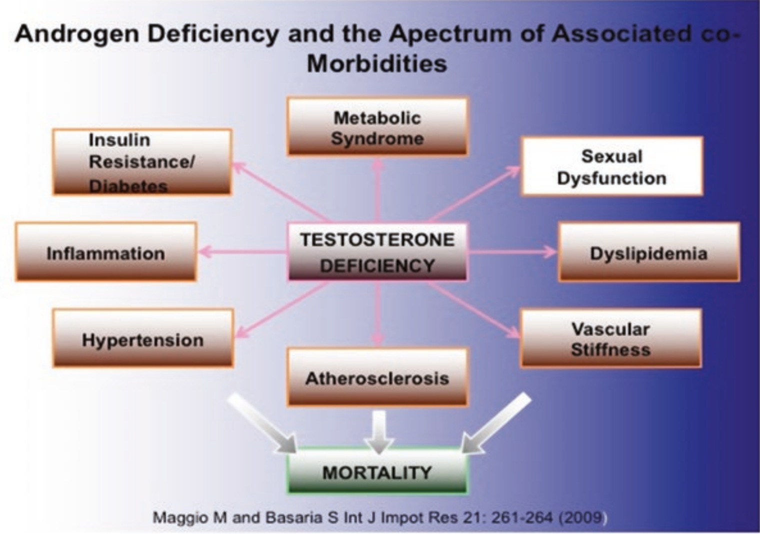

Fig. 1 Negative impact of testosterone deficiency on human physiology and its related comorbidities (Adapted from Maggio and Basaria 2009 (with permission from the publisher))

Prevalence of TD and Associated Comorbidities

The prevalence of TD increases with age, and almost 55% of individuals between 70 and 79 years of age exhibit TD as compared with 24% of men at 50–59 age (Harman et al. 2001; Dhindsa et al. 2004, 2010). As reported by Harman et al. (2001), the prevalence of TD is more pronounced when free T is correlated with signs and symptoms of TD. Among the major prevalent risk factors for TD are obesity (52%), type 2 diabetes mellitus (T2DM) (50%), hypertension (42%), hyperlipidemia (40%), and asthma or COPD (43%) (Mulligan et al. 2006). Data from 3825 patients in 20 studies showed that T levels were significantly lower in men with T2DM (Ding et al. 2006). The prevalence of TD in patients with T2DM ranges from 30% to 50% (Kapoor et al. 2006, 2007; Dhindsa et al. 2004). Increased waist circumference (WC) and obesity are also thought to contribute to reduced T levels (Svartberg et al. 2004; Camacho et al. 2013; Corona et al. 2013). Approximately 40% of obese nondiabetic men and 50% of obese diabetic men aged >45 years exhibited TD (Dhindsa et al. 2010). Corona et al. suggested that TD is more prevalent in men with T2DM and 34% of men with erectile dysfunction (ED) exhibited TD (Corona et al. 2004, 2006, 2007, 2011a, b). The prevalence of TD and its negative impact on men's health is far greater than previously recognized (Traish 2014a, b, 2016a, b; Morgentaler et al. 2016; Jones 2010; Jones et al. 2011; Hackett et al. 2013, 2014, 2016a, b). TD negatively impacts overall health and quality of life, since it is associated with increased incidence of MetS, IR, T2DM, CVD, inflammation, and ED (Fig. 1). In addition, TD is associated with reduced BMD and increased

incidence of anemia in the elderly, and T therapy exerts a host of benefits on overall health (Snyder et al. 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017; Saad et al. 2016; Traish 2016a, b). For more details on the benefits of T therapy on glucose homeostasis, the readers are referred to Dr. Saad's chapter in this current book.

TD and Adipose Tissue Pathophysiology

It is well known that androgens exert a critical role in differentiation of pre-adipocytes into mature adipocytes and also regulate fat metabolism and visceral fat accumulation (Singh et al. 2003, 2006; Gupta et al. 2008; Chazenbalk et al. 2013; Maneschi et al. 2012). In an animal model of the MetS, Maneschi et al. (2012) reported striking changes in visceral adipose tissue histology and histopathology in castrated rabbits fed high fat diet (HFD) treated with or without androgens (Fig. 2). What was profoundly of interest is that T treatment in this animal model resulted in reducing the size of adipocytes and normalized the histology of the visceral adipose tissue in the HFD.

This treatment also restored membrane glucose transporter type 4 (GLUT4), phosphorylated protein kinase b (pAKT) and protein kinase AKT ratio (pAKT/AKT), and perilipin expression (Maneschi et al. 2012). The accumulation of a dysfunctional visceral adipose tissue in androgen-deficient animals was attributed to increased differentiation of pre-adipocytes into mature adipocytes due to impaired insulin signaling (Maneschi et al. 2012). T treatment appears to preserve visceral adipose tissue histological and physiological function, likely through maintenance of insulin sensitivity, which regulates pre-adipocyte commitment to normal turnover and reduced differentiation into adipocytes (Maneschi et al. 2012). More importantly, T therapy appeared to protect the lower urinary tract from MetS-induced histopathological changes, suggesting that TD alters detrusor function, while androgen therapy protects against these pathological changes (Vignozzi et al. 2012). Similarly, Zhang et al. (2016b) investigated the effects of TD on visceral adipose tissue (VAT) deposition in castrated male pigs. The authors reported that TD altered expression of a number of key genes involved in biological processes of VAT accumulation under high fat and cholesterol (HFC) diet. T treatment reversed the changes brought about by TD. This study provided a novel genome-wide view on the potential role of T in regulating genes involved in VAT deposition under HFC diet. Dubois et al. (2016) examined the combined effect of TD and high fat diet (HFD) on body composition and glucose homeostasis in two rodent models of TD (castration and global androgen receptor AR knockout mice (ARKO)). Both castration and ARKO exacerbated HFD-induced glucose intolerance by impairing insulin action in the liver and skeletal muscle, as determined by the increased triglyceride and decreased glycogen content in these tissues. In addition, in castrated animals, HFD resulted in adipocyte hypertrophy, with concomitant reduction in mitochondrial content and increased lipogenesis and decreased lipolysis as inferred from upregulation of fatty

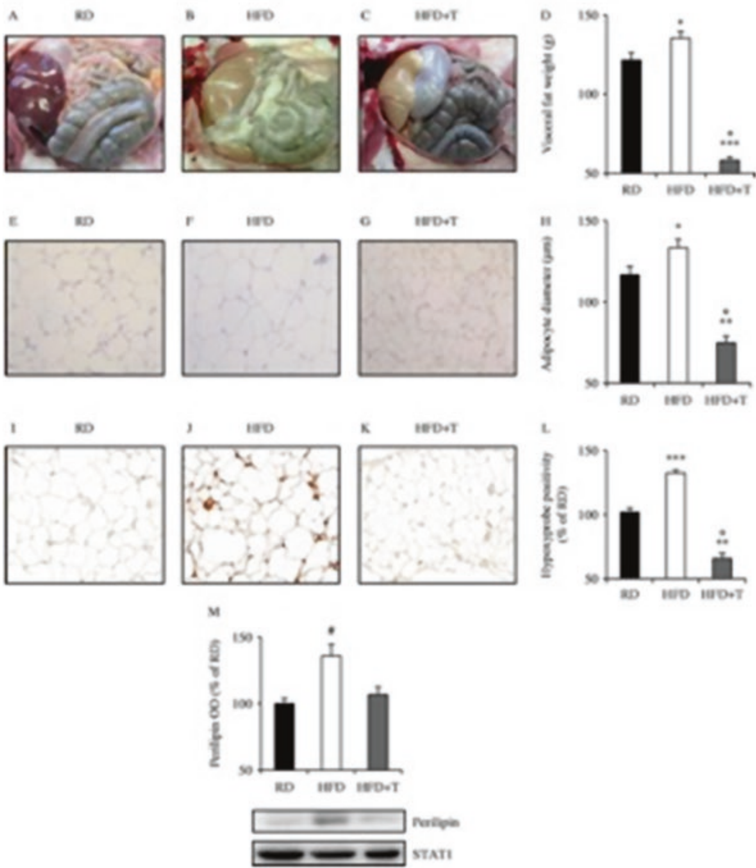

Fig. 2 Effects of testosterone treatment on VAT weight, adipocyte size, and hypoxia in experimental rabbits. (**a**–**c**) Pictures show fat accumulation at the visceral level within the intestinal loops. (**d**) VAT weight was significantly increased in HFD rabbits ($n = 32$), when compared with RD ($n = 35$) and testosterone-treated HFD rabbits (HFD+T; $n = 19$). (**e**–**g**) Representative images of hematoxylin/eosin-stained VAT sections showing different adipocyte size among experimental groups (magnification 20×). (**h**) Histomorphometric analysis of adipocyte diameter (μm) in the different experimental groups ($n = 3$ for each group). (**i**–**l**) Immunohistochemical staining of hypoxyprobe adducts in VAT sections. Hypoxyprobe adducts were revealed in hypoxic cells (PO2 <10 mmHg) of VAT transverse sections by a MAB (magnification 12.5×). Only scanty positive labeling is present in RD (**i**) and testosterone-treated HFD (**k**) VAT, while intense hypoxyprobe positivity is detected in HFD VAT (**j**). (**l**) Computer-assisted quantitative image analysis of three independent experiments ($n = 3$ for each group). RD optical density was taken as 100%. (**m**) Protein expression of perilipin in VAT extracts from experimental rabbits. Representative immunoblots with anti-perilipin and anti-STAT1 primary antibodies and the corresponding graphical representation of optical density (OD) analysis of perilipin band intensity normalized over STAT1 ($n = 3$ for each group) are reported. Data are mean ± s.e.m. expressed as percentage of RD values $*P < 0.05$; $**P < 0.001$; $***P < 0.0001$ vs RD, $P < 0.0001$ vs HFD and $^{\#}P = 0.001$ vs all groups (Maneschi et al. 2012 (with permission from the publisher))

acid synthase and downregulation of hormone-sensitive lipase. In castrated animals on HFD, histological analysis of subcutaneous white adipose tissue showed increased adipocytes size compared with intact animals on HFD. In addition, pancreatic insulin secretion was impaired in castrated animals. It was suggested that TD potentiates HFD-induced metabolic alterations, including increased adiposity, impaired glucose tolerance, and decreased insulin secretion and sensitivity.

It should be pointed out that in all studies reported to date, TD is associated with increased fat mass and reduced lean body mass and T therapy results in increased lean body mass and reduced fat mass (reviewed in Traish et al. 2014a; Traish 2014 a, b, c; Traish and Zitzmann 2015; Kelly and Jones 2015). Increased visceral obesity results in increased inflammation and promotes a vicious cycle that would exacerbate TD and may increase insulin resistance and predispose to diabetes. Therefore, clinical treatment of TD is of significance with regard to ameliorating components of the MetS and improving cardio-metabolic function (Saad et al. 2016; Traish 2016a, b; Traish et al. 2017a).

TD and Dyslipidemia

Studies on the effects of androgen on lipid profiles in animal models and in humans have suggested that TD alters lipid profiles and may contribute to increased cholesterol, LDL, and triglycerides (Traish et al. 2009d). Khaw et al. (2007) demonstrated that higher endogenous T levels were associated with reduced triglycerides and increased HDL, LDL, and TC. However, Simon et al. (1997) reported that higher T levels are associated with reduced triglycerides, TC, and LDL cholesterol and increased HDL. Studies utilizing ADT in men with PCa showed that triglycerides, TC, LDL cholesterol, and HDL cholesterol all increased in response to ADT (Smith et al. 2006; Nishiyama et al. 2005). Data from interventional studies provided mixed results. For instance, several studies showed that T therapy reduced triglycerides, TC, and LDL (Saad et al. 2008a, b; Zitzmann and Nieschlag 2007; Page et al. 2005a, b; Malkin et al. 2004; Dobs et al. 2001); however, other studies showed that there were no significant changes in the lipid profile (Emmelot-Vonk et al. 2008; Allan et al. 2008). These discrepancies could be attributed to the modality of treatment, T formulations, duration of treatment, and other comorbidities in the study populations. Recent studies have shown that long-term T therapy for up to 8 years has clearly demonstrated significant reductions in TC, LDL, and triglycerides with concomitant increase in HDL (Saad et al. 2013, 2016; Traish et al. 2014a, 2017a; Permpongkosol et al. 2016). A recent study reported a negative correlation between total T levels and glucose ($p < 0.001$), HOMA-IR (13 $p < 0.001$), HbA1c ($p < 0.01$), triglycerides ($p < 0.001$), non-HDL-c ($p < 0.01$), ApoB ($p < 0.05$), and AIP ($r = -0.393$; $p < 0.001$). T concentration was positively correlated with HDL-c levels ($p < 0.001$) and ApoA1 ($p < 0.001$). Thus, it was concluded that reduced T levels are related to hyperglycemia and IR (Rovira-Llopis et al. 2017a, b). We believe that T plays a critical role in regulating lipid metabolism (Table 1) and adipogenesis and

Table 1 Influence of testosterone on targets of lipid and glucose homeostasis (From Kelly and Jones 2013a, b (with permission from the publisher))

Testosterone action	Tissue	Target function
Glucose homoeostasis		
↑ Glut4	Muscle[a,b,c,d], liver[c] and adipose[a,b,c]	Glucose transporter protein involved in cellular glucose uptake
↑ IR	Liver[e,f] and larynx[g]	Insulin signalling
↑ IRS1	Muscle[a], adipose[a] and liver[f]	Insulin signalling
↑ IRS2	Muscle[h]	Insulin signalling
↑ Akt	Muscle[b]	Insulin receptor signalling pathway
↑ Protein kinase C	Muscle[b]	Insulin receptor signalling pathway
↑ Phosphofructokinase	Muscle[b]	Key regulatory enzyme in glycolysis
↑ Hexokinase	Muscle[b,d,i]	Key regulatory enzyme in glycolysis
↑ UQRCB	Muscle[j]	Oxidative phosphorylation in mitochondrial respiration
↑ Glycogen synthase	Muscle[k]	Glycogenesis
↓ Glycogen phosphorylase	Muscle[k]	Glycogen breakdown
↑ G6PD	Muscle[l,m]	Rate-limiting enzyme in the pentose phosphate pathway
Lipid homoeostasis		
↓ ACS	Adipose[n]	De novo lipogenesis
↓ ACC	Liver[o,p], sebaceous gland[q,r] and adipose[s]	FA synthesis
↑ ACC	Prostate cancer cells[t]	
↔ ACAT1	Sebaceous gland[r]	Conversion of cholesterol to cholesteryl esters
↔ LDLr	Sebaceous gland[r]	Receptor-mediated endocytosis of LDL
↑ ABC1	Sebaceous gland[r]	Cholesterol efflux
↓ FAS	Liver[p,u] and adipose[s,v]	FA synthesis
↔ FAS	Sebaceous gland[r]	
↑ FAS	Prostate cancer cells[t]	
↑ HMG-CoA	Sebaceous gland[q,r] and prostate cancer cells[t]	Cholesterol synthesis
↑ GPAT	Sebaceous gland[q,r]	Cholesterol synthesis
↓ HSL	Adipose[v,w]	Triglyceride breakdown
↑ β-Adrenergic receptor	Adipose[x]	Noradrenaline-stimulated lipolysis
↓ ATGL	Adipose[v]	Lipolysis
↓ LPL	Adipose[v,y,z,AA]	Triglyceride uptake
↑ ApoE	Liver[p]	Cholesterol efflux
↓ RBP4	Adipose[v]	Role in insulin resistance and lipid metabolism

(continued)

Table 1 (continued)

Testosterone action	Tissue	Target function
↓ Scd1	Liver[o,u]	Key enzyme in FA metabolism
↑ SCD1	Liver[BB]	
↓ C7AH	Liver[BB]	Key enzyme in cholesterol conversion to bile acid
SR-1B	Liver[BB]	Selective uptake of cholesterol esters from HDL
MTTP	Liver[BB]	Central role in lipoprotein assembly; ApoB and VLDL secretion
Master regulators		
↑ LXR	Liver[p]	Regulator of glucose and cholesterol metabolism, FA synthesis and inflammation
↓ PPARγ	Liver[o] and stem cells[CC]	Whole-body energy homoeostasis adipogenesis and inflammation
↑ SREBP-1c	Sebaceous gland[r] and prostate cancer cells[DD,EE]	Regulator of *de novo* lipogenesis, cholesterol homoeostasis and glucose homoeostasis
↑ SREBP-1a	Sebaceous gland[r]	
↓ SREBP-1c	Liver[o]	

ABC1 ATP-binding cassette 1, *ACAT1* acyl-CoA cholesterol acyl transferase-1, *ACC* acetyl-CoA carboxylase, *ACS* acyl-CoA synthetase, *ApoE* apolipoprotein E, *ATGL* adipose triglyceride lipase, *C7AH* cholesterol 7α-hydroxylase, *FAS* fatty acid synthase *GLUT4* glucose transporter-4, *GPAT* glycerol 3 phosphate acyl transferase *HMG-CoA* hydroxymethylglutaryl coenzyme A, *HSL* hormone-sensitive lipase, *IR* insulin receptor, *IRS* insulin receptor substrate, *LDLr* LDL receptor, *LPL* lipoprotein lipase, *LXR* liver X receptor, *MTTP* microsomal triglyceride transfer protein, *PPARγ* peroxisome proliferator-activated receptor γ, *RBP4* retinol-binding protein-4, *Scd1* stearoyl-CoA desaturase 1, *SR-1B* scavenger receptor class B member 1, *SREBP-1c* sterol regulatory element binding protein 1c, *UQRCB* ubiquinol cytochrome *c* reductase-binding protein

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Table 1 (continued)

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in visceral fat accumulation and adiposity in males (Traish et al. 2009c, d, a; Kelly and Jones 2013a, b, 2015; Traish and Zitzmann 2015).

TD, Obesity, and MetS

Most importantly, the link between TD, obesity, MetS, and cardio-metabolic dysfunction is recognized in several recent reports in the medical literature (Morgentaler et al. 2015a, b, 2016; Traish 2014a, b, 2016a, b; Laaksonen et al. 2003, 2004, 2005; Antonio et al. 2015). Low T is thought to be an independent risk factor for MetS (Laaksonen et al. 2004; Antonio et al. 2015), and a complex and multifactorial relationship has been proposed between TD and obesity (Kelly and Jones 2015; Traish and Zitzmann 2015). Obesity reduces circulating T levels, most likely via a negative effect on the hypothalamic-pituitary-gonadal axis (HPGA) (Camacho et al. 2013). Interestingly, weight loss (WL) via lifestyle changes or via bariatric surgery increases circulating T levels. Camacho et al. (2013) reported that WL is associated with increased T levels and weight gain is associated with reduced T levels. Kelly and Jones (2015) proposed that relationship between obesity and TD is bidirectional with multiple underpinning causes and it may involve the hypogonadal-obesity cycle hypothesis (Cohen 1999).

As depicted in Table 1 (Kelly & Jones 2013), T modulates carbohydrate and lipid metabolism by regulating a number of key enzymes that play a critical role in lipid and glucose homeostasis and mitochondrial function (Traish et al. 2011b; Kelly & Jones 2013, Kelly et al. 2016). It is likely that reduced T levels negatively

impact lipid and carbohydrate metabolism, resulting in lipid accumulation and therefore contributing to weight gain and visceral obesity (Traish and Zitzmann 2015; Kelly and Jones 2015).

TD, IR, and Diabetes

Approximately six million people were reported to have diabetes in the USA in the 1980s, and by 2004 this number had increased to approximately 15 million people (Steinbrook 2006). It is estimated that the incidence of diabetes will continue to rise. The observed increase in diabetes parallels increases in obesity, reported in the USA over the same period.

TD is prevalent in men with obesity, MetS, and T2DM (Ding et al. 2006; Kapoor et al. 2007; Dhindsa et al. 2004; Svartberg et al. 2004; Hofstra et al. 2008; Singh et al. 2011), and TD predicted increased adiposity and overall obesity (Couillard et al. 2000). Both subcutaneous and visceral fat are increased in men with TD (Hofstra et al. 2008; Blouin et al. 2005, 2006). A study of 1896, nondiabetic middle-aged men according to the presence of MetS (Laaksonen et al. 2003) showed that fasting insulin levels increased with TD, as compared with controls. In contrast, increased T and SHBG levels were correlated with increased insulin sensitivity and reduced risk of MetS (Muller et al. 2005). Pagotto et al. (2003) suggested that TD is a stronger risk factor for development of elevated insulin and glucose levels when compared with overweight/obesity. In men with MetS, approximately 30% exhibited signs and symptoms of TD, and only about 3.1% in healthy men had signs and symptoms of TD. Patients with T2DM had higher prevalence of TD compared to nondiabetic men (Corona et al. 2011b). The Rancho Bernardo study showed that T levels predicted IR and incident T2DM in older adults (Oh et al. 2002). Similarly, the Massachusetts Male Aging Study and the NHANES III study demonstrated increased risk of MetS and T2DM in men with low T, even in nonobese men (Stellato et al. 2000; Selvin et al. 2007). In the cohort of the Massachusetts Male Aging Study, diabetes at follow-up was predicted jointly and independently by lower baseline levels of free T and SHBG suggesting that low levels of T and SHBG play a role in the development of IR and subsequent T2DM (Stellato et al. 2000). In a study of 294 men 55–89 years, who did not have diabetes, low T levels predicted IR and incident T2DM (Oh et al. 2002). Colangelo et al. (2009) reported that T2DM and fasting blood glucose were inversely associated with total T levels, even after adjusting for age, ethnicity, BMI, and WC. Interestingly, Vikan et al. (2010) noted a significantly lower risk for diabetes with higher normal total T levels. These findings suggest that men with TD are at a higher risk for IR, T2DM, and obesity.

A considerable body of evidence suggests that men with TD are at a greater risk of developing IR and T2DM, and TD predicts the onset of diabetes (Haffner et al. 1996, 1997; Stellato et al. 2000; Oh et al. 2002; Rhoden et al. 2005a, b; Shores et al. 2006; Selvin et al. 2007). TD is linked with IR, hyperglycemia, hypertension, dyslipidemia, and an increased risk of CVD (Simon et al. 1997; Stellato et al.

2000; Oh et al. 2002; Dhindsa et al. 2004; Pitteloud et al. 2005a, b, 2008; Corona et al. 2006; Kapoor et al. 2006, 2007; Fukui et al. 2007, 2008; Selvin et al. 2007; Andersson et al. 1994; Osuna et al. 2006; Rhoden et al. 2005a, b; Basaria et al. 2006; Malkin et al. 2007; Navarro et al. 2015). For instance, Tsai et al. (2004) demonstrated an inverse relationship between T levels and IR, irrespective of whether this was related to free T, bioavailable T or total T, or reduced SHBG levels. It was suggested that this inverse relationship was mediated by increased visceral obesity. Yeap et al. (2009, 2012) also suggested that low T is independently associated with increased IR in nondiabetic older men. The prevalence of obesity and T2DM in men with TD necessitates a careful discussion of the link between TD and IR and T2DM and obesity.

Reduced T levels were commonly found in men with T2DM and IR (Grossmann et al. 2008). T concentrations were significantly associated with fasting plasma insulin, 2-h plasma insulin levels, and glucose levels (Simon et al. 1997). In a systematic review of 43 studies with 6427 men, normal physiological T levels were thought to be associated with reduced risk of T2DM, and reduced T levels are associated with increased risk of T2DM (Ding et al. 2006). Furthermore, reduced levels of sex hormone-binding globulin (SHBG) and low T predicted hyperglycemia, increased insulin levels, and obesity (Haffner et al. 1996, 1997). The aforementioned findings are supported by the Massachusetts Male Aging Study (MMAS) which also postulated that TD and SHBG play a role in development of IR and T2DM (Stellato et al. 2000).

TD and T2DM are often diagnosed in the same patient (Dhindsa et al. 2004; Kapoor et al. 2007), and TD is highly prevalent in obese T2DM patients. In a large cohort of more than 1100 men, Corona et al. (2007) reported that TD correlated significantly with visceral fat and diabetes. Men with T2DM are often obese with significantly reduced T levels concomitant with elevated fasting insulin levels (Pasquali et al. 1991, 1997). This observation was further confirmed in obese individuals with BMI > 30 who exhibited greater fasting plasma insulin and IR index and reduced T than subjects with BMI < 30 (Isidori et al. 2000). In addition, Osuna et al. (2006) reported a significant negative correlation between T levels and WC, BMI, insulin, and higher HOMA-IR. These findings strongly support a strong link between TD, IR, T2DM, and obesity.

Pitteloud et al. (2005a, b, 2008) investigated the effect of T suppression followed by sequential stimulation of the pituitary and testes with GnRH and human chorionic gonadotropin (hCG), respectively. Interestingly, hCG-stimulated T levels at 48 h were positively correlated with insulin sensitivity as well as with baseline serum T levels. On the other hand, Pitteloud et al. (2005a, 2008) did not observe a correlation between insulin sensitivity and parameters of LH secretion or LH response to exogenous GnRH, suggesting that low T levels associated with IR are not attributable to a major decrease in hypothalamic or pituitary hormone secretion. Furthermore, Pitteloud et al. (2005b) demonstrated that acute androgen withdrawal is not dependent on changes in body composition in impairing insulin sensitivity. We have speculated that since T plays a critical role in mitochondrial biogenesis and regulates the expression and activities of a host of mitochondrial enzymes and fatty

acid metabolism, it is possible that TD contributes to mitochondrial dysfunction and IR (Traish et al. 2011b). In an animal model of post-infarct myocardium, androgen deprivation by castration aggravated mitochondrial damage, including mitochondrial swelling and disordered arrangement, loss of cristae, reduced mitochondrial length, and decreased ATP levels leading to cardiomyocyte apoptosis in ischemic myocardium (Wang et al. 2015). It was suggested that castration downregulated peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) and mitofusin 2 (MFN2) but upregulated dynamin-related protein-1 (DRP1). More importantly mitochondrial damage was reversed by T treatment, which also elevated the upstream AMP-activated protein kinase (AMPK) activation of PGC1-α (Wang et al. 2015). Usui et al. (2014) reported that T treatment in the animal model increased expression of PGC1-α and increased mitochondrial biogenesis in skeletal muscle. In the ARKO the authors reported reduced expression of PGC1-α and mitochondrial biogenesis, suggesting a critical role for androgens in mitochondrial function.

Yialamas et al. (2007) demonstrated that acute sex steroid withdrawal for 2 weeks reduced insulin sensitivity in young healthy men with TD (idiopathic hypogonadotropic hypogonadism, IHH) suggesting that T modulates insulin sensitivity directly and further suggesting that this pathway is not mediated by changes in body composition. On the contrary, Chen et al. (2006) argued that TD is a consequence of, and is not a cause of, poor metabolic status. Thus, it is possible that such interplay is bidirectional as proposed previously by Traish et al. (2009a, b, c). Thus, it is of critical importance to investigate the relationship between TD, mitochondrial dysfunction, and IR in order to have a better understanding of the role of T in regulating carbohydrate and lipid homeostasis.

In summary, TD, irrespective of its underlying cause or etiology, reduces lean body mass and increases FM with increased incidences of gynecomastia and hot flushes. TD also contributes to MetS with increased IR, hyperglycemia, and altered lipid profiles characterized by increased total cholesterol (TC), LDL, and triglycerides. TD contributes to sexual dysfunction, diminished libido, and ED. TD contributes to increased incidence of anemia and reduces red blood cell counts, hemoglobin, and hematocrit and increases arterial stiffness and arterial pressure which may contribute to increased CVD and mortality. TD as a result of ADT also negatively impacts energy and well-being, increases fatigue and reduces cognition, and diminishes quality of life (Shastri and Yaturu 2011; Nguyen et al. 2015a; Shahani et al. 2008; Basaria et al. 2008; Saad 2015; Yu and Traish 2011; Traish et al. 2011a, b). TD is also associated with increased risk of cardiovascular and overall mortality, and T therapy is associated with reduced mortality. For a comprehensive discussion of this topic, the readers are encouraged to consult the review by Morgentaler et al. (2015a, b). We should point out that discussion of the benefits of T therapy on the various signs and symptoms of TD is not addressed here. For more comprehensive discussion of the effects of T therapy on glucose homeostasis, the readers are directed to Dr. Saad's chapter in this book.

Negative Impact of 5α-Reductase (5α-Rs) Inhibitors Therapy on Metabolic and Sexual Function

In this forthcoming section, we wish to focus on the often-ignored and very important dimension of induced androgen deficiency due to blunting 5α-DHT levels, as a result of inhibiting 5α-reductases (5α-Rs) in the course of treatments of BPH and AGA with finasteride or dutasteride (Roehrborn et al. 2002, 2008, 2015; Andriole & Kirby l. 2003, Andriole et al. 2010; Gupta & Charrette 2014; Mella et al. 2010). Although 5α-DHT is recognized as a potent hormone, binds to AR with greater affinity than T, and also plays a critical role in development and maintenance of reproductive and sexual function, most often plasma 5α-DHT levels are neither measured nor evaluated when clinically diagnosing androgen deficiency. Several recent studies have suggested that 5α-DHT is a critical signaling molecule and regulates mitochondrial function and lipid metabolism (Zhang et al. 2013a; Wang et al. 2015; Joyce et al. 2017). TD is expected to be associated with reduced T and 5α-DHT levels, and T therapy is associated with increased T and 5α-DHT levels. Thus, the critical importance of this potent androgen (5α-DHT) in human physiology and in the clinical management of androgen deficiency remains, at best, poorly understood. Here we provide new findings and information on the potential role of 5α-DHT in regulating metabolic and sexual function.

Role of 5α-Rs in Human Physiology

5α-reductases (5α-Rs) are a family of enzymes, widely expressed in a large number of tissues, which regulate steroid metabolism and metabolic function (for review cf. Traish et al. 2011c, 2014b, c, 2015b, a, b, 2017b ; Traish 2012). 5α-Rs catalyze the reduction of the double bond in the A-ring at Δ4,5 position in C-19 and C-21 steroids (Traish et al. 2011c, 2015a, b; Traish 2012). 5α-Rs transform multiple gonadal-, adrenal-, and central nervous system (CNS)-produced steroid precursors into active hormonal ligands (reviewed in: Traish et al. 2011c, 2014b, c, 2015b; Traish 2012). The substrates for 5α-Rs include T, progesterone, deoxycorticosterone (DOC), corticosterone, cortisol, and aldosterone (Traish et al. 2011c, 2015b; Traish 2012). For example, conversion of T into 5α-DHT is prerequisite for the development and maintenance of male sexual organs, and individuals with 5α-Rs deficiency exhibit ambiguous genitalia. To date there are three well-characterized isoforms of 5α-Rs, with varying biochemical characteristics and tissue distributions, suggesting diverse physiological functions (Traish 2012; Traish et al. 2015a, b). 5α-Rs regulate steroid action in the skin, muscle, liver, adipose tissue, and CNS (Traish et al. 2015a, b). For a comprehensive discussion, the readers are encouraged to consult a recent review, which summarized the current state of knowledge regarding 5α-Rs and their role in human physiology (Traish et al. 2015a, b).

Therapeutic Applications of 5α-Rs Inhibitors for Treatment of Benign Prostatic Hyperplasia (BPH) and Androgenetic Alopecia (AGA)

5α-DHT is a critical hormone for the development and growth of normal prostate tissue. Berry and Isaacs (1984) reported that in untreated rats and dogs, there is a species-specific time period when proliferative growth of the prostate normally occurs during which the gland grows to its maximum normal cellular content. In both species, after the prostate has reached its maximum normal cellular content, which requires 1 year for the rat and 2 year for the dog, net proliferative prostatic growth ceases and is replaced by a steady-state maintenance of the gland even though the circulating androgens are maintained at physiological levels. Thus, although in the young adult animal circulating physiological T and 5α-DHT levels are normal, no additional prostate growth is observed. It can be said that once the prostate reaches its mature size in the adult, the prostate remains in a dynamic state where cellular growth and apoptosis maintain normal prostate size. Interestingly, with advancing age, physiological levels of T and 5α-DHT begin to decline; however, prostate volume increases resulting in the onset of BPH. Thus, while 5α-DHT plays a role in the normal growth of prostatic epithelial and stromal cells, the age-dependent growth of the prostate in later years is not specifically due to increased androgenic activity (Marks et al. 2006; Haider et al. 2017). Findings from cross-sectional and longitudinal studies do not support the contention that increased 5α-DHT physiological levels increase the risk of prostate disease (Idan et al. 2010; de Lignieres 1993; Choi et al.1993). Nevertheless, observations from individuals with 5α-Rs deficiency supported the hypothesis that inhibition of 5α-Rs with synthetic drugs would result in reducing prostate volume and ameliorate the lower urinary tract symptoms (LUTS) secondary to BPH. To this end, synthetic chemical inhibitors were developed to block 5α-DHT formation in order to treat BPH symptoms. Two agents have been on the market for the past two and half decades, namely, finasteride, which is a selective inhibitor of 5α-R type 2 and a weak inhibitor of 5α-R type 1, and dutasteride, which is a potent inhibitor of both 5α-R type 1 and type 2 (Traish et al. 2015a, b).

Finasteride and dutasteride were utilized as therapeutic agents for the treatment of lower urinary tract symptoms (LUTS) in patients with BPH. In the prostate, finasteride and dutasteride inhibit the conversion of T to 5α-DHT resulting in reduction of intraprostatic 5α-DHT levels and inhibition of epithelial cell growth and therefore reducing prostate volume and attenuating obstruction of urine flow, providing symptomatic relief (Bechis et al. 2014; Roehrborn et al. 2002, 2008, 2015; Andriole & Kirby 2003; Andriole et al. 2010). This treatment minimizes incidence of prostate surgery due to large prostate volume in cases of urinary retention. Also since 5α-DHT plays a role in the hair follicle cycle, finasteride was introduced to impede hair loss, as a treatment of androgenetic alopecia (AGA). Although these medications are proven useful in the management of LUTS and reducing the need for surgery, there is a growing concern that these agents are also associated with

adverse metabolic and sexual side effects, which have been recognized by the medical community (Traish et al. 2015a, b, 2017b).

Effects of 5αR Inhibitors Therapy on Metabolic Function

The potential negative impact of 5α-Rs inhibition on metabolic function is a critical area of scientific and clinical discussion that has yet to receive adequate attention in the scientific literature. For example, recent studies have suggested that 5α-Rs regulate glucose and lipid metabolism and inhibition of these enzymes may result in increased IR and diabetes (Zhang et al. 2013a; Livingstone et al. 2009, 2014, 2015, 2017; Upreti et al. 2014; Hazlehurst et al. 2016; Dowman et al. 2013; Nasiri et al. 2015). In addition, 5α-Rs inhibition by finasteride and dutasteride exerts serious negative effects on sexual function (Roehrborn et al. 2015; Erdemir et al. 2008; Traish et al. c; Traish 2012; Traish et al. b, c, 2015a, b; Irwig & Kolukula 2011, Irwig 2012a, b, 2013, 2014; Gur et al. 2013; Ganzer et al. 2015; Fwu et al. 2013, 2014; Guo et al. 2016; Pinsky et al. 2011; Oztekin et al. 2012; Park & Choi 2014).

It is well established that glucocorticoids (GCs) elicit their physiological responses via high-affinity glucocorticoid receptors (GR) regulating gene expression and protein synthesis pathways involved in carbohydrate metabolism, gluconeogenesis, and lipolysis in the liver and adipose tissues. (reviewed in Traish et al. 2014c). We have previously postulated that inhibition of 5α-Rs interferes with signaling by androgens and glucocorticoids (Traish et al. 2014c). Inhibition of 5α-Rs activities reduces 5α-DHT levels as well as clearance of glucocorticoids and mineralocorticoids. This inhibition of steroid transformation may potentiate IR and diabetes.

The majority of cortisol is inactivated, principally via the A-ring reduced metabolites, on a single pass through the liver (Walker et al. 1993). However, this inactivation is offset by reactivation of cortisone into cortisol by hepatic 11β-HSD type 1; thus, the overall changes in the gradient of cortisol in liver may be relatively small. An increase in the level of circulating cortisol may be related to stimulation of 11β-HSD type 1 or by inhibition of 5α- or 5β-R due to administration of 5α-R inhibitors (5α-RI) in patients with BPH or androgenetic alopecia (AGA). The balance between the activities of these enzymes maintains the physiological concentration of active GCs (Walker et al. 1993; Hellman et al. 1971; Bamberger et al. 1996; DeRijk et al. 2002). Increased circulating cortisol is associated with intra-abdominal fat and IR (Purnell et al. 2009). Increased cortisol levels antagonize insulin action and increase peripheral glucose, as well as gluconeogenesis in the liver, resulting in hyperglycemia (Fig. 3) (Ferris & Kahn 2012; Di Dalmazi et al. 2012). It is possible that 5α-RI therapy may produce an imbalance in circulating GCs and their 5α-reduced metabolites, resulting in adverse metabolic effects and inflammatory responses and increased insulin resistance.

Tomlinson et al. (2008) proposed that fat mass correlates with GC secretion rate and GC secretion rate was inversely related to insulin sensitivity. Decreased GC

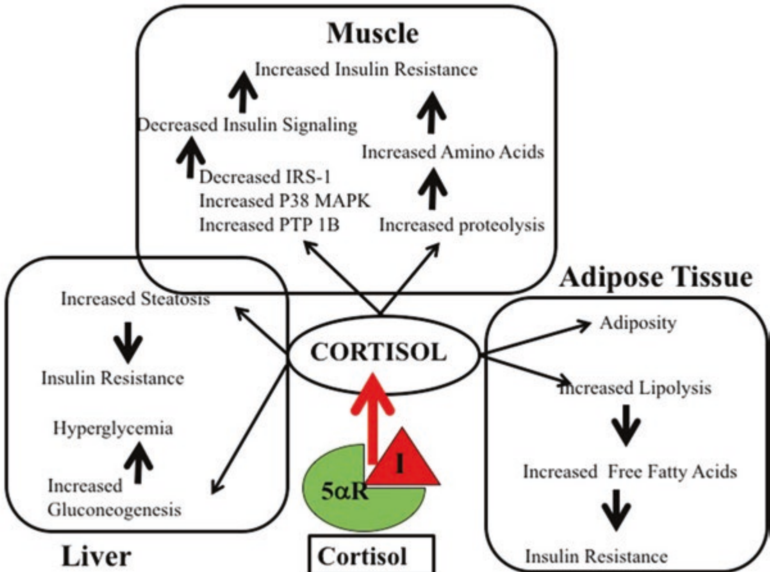

Fig. 3 Putative mechanisms of 5α-reductase inhibition of cortisol ring A reduction and metabolism. Inhibition of 5α-reductase by finasteride or dutasteride (I) blocks the reduction of ring A in the cortisol molecule and inhibits its metabolism and clearance. The increased levels of cortisol due to 5α-reductase inhibition increase the levels of active cortisol. In adipose tissue, the increased levels of cortisol result in increased adiposity and lipolysis, thus producing high levels of free fatty acids in the circulation and potentiating insulin resistance. In the "muscle," protein breakdown into peptides and amino acids due to proteolysis also increased insulin resistance. In the "liver," abnormalities in downstream, post-receptor insulin signaling such as reduced expression of insulin receptor substrate 1 (IRS-1) and increased expression of P38 mitogen-activated protein kinase (P38 MAPK) lead to insulin resistance. Increased liver steatosis causes insulin resistance, coupled with increased gluconeogenesis and hyperglycemia (Traish et al. c; Hormone Molecular Biology and Clinical Investigation; with permission from the publisher)

secretion subsequent to weight loss results in increased insulin sensitivity. These observations support the notion that obesity is associated with IR and increased cortisol secretion rates by adipose tissue.

It is postulated that augmented GC inactivation by 5 α-Rs may serve as a compensatory, protective mechanism to preserve insulin sensitivity (Tomlinson et al. 2008). Studies with obese Zucker rats showed that the increased levels of circulating GCs overstimulate the glucocorticoid receptors (GR), promoting fat deposition (Livingstone et al. 2009). Hypercortisolemia generally results in the preferential expansion of central, especially visceral, fat depots (Björntorp & Rosmond 2000; Lee et al. 2014). Interestingly, the activities of 5α-Rs are significantly reduced in this animal model, which may explain in part why there is marked increase in cortisol buildup. Obesity is associated with IR and dyslipidemia, and hypercortisolemia may provide a link between GCs and fat deposition (Lee et al. 2014; Boonen et al. 2013).

The potential role of 5α-reductase type 1 (5α-R type 1) in carbohydrate and lipid metabolism was inferred from studies in animals lacking 5α-R type 1 (knockout, KO). When these animals were fed a high-fat diet, they exhibited increased susceptibility to weight gain, hyperinsulinemia, fasting hyperglycemia, increased ratios of insulin to glucose, and liver fat accumulation (Livingstone et al. 2015, 2017). Furthermore, in 5α-R type 1-KO mice, liver fatty acid β-oxidation and gluconeogenesis were impaired, and gene expression for enzymes catalyzing triglyceride esterification and cholesterol synthesis and excretion were elevated. Animals lacking 5α-R type 1 also exhibited greater hepatic fibrosis than wild-type mice. Furthermore, inhibition of 5α-Rs type 1 and type 2 in male Zucker rats increased plasma glucose and insulin levels (Livingstone et al. 2015, 2017). These findings suggest that pharmacological inhibition of 5α-R type 1 may contribute to onset of metabolic diseases and may lead to hepatic steatosis, which increases the susceptibility to fibrotic liver injury and accelerated progression to nonalcoholic fatty liver disease (NAFLD). These findings emphasize the potential adverse metabolic function in men treated with 5α-reductase inhibitors (e.g., finasteride or dutasteride).

Livingstone et al. (2015) proposed that 5α-RIs influence predisposition to metabolic disease contributing to hepatic steatosis and body fat distribution and reduced insulin sensitivity with accelerated progression of NAFLD. In rats, after only 3 weeks of finasteride treatment, which inhibits both 5α-R type 1 and type 2 isoforms (Thigpen et al. 1992; Azzolina et al. 1997), steatosis was a highly robust observation (Livingstone et al. 2014, 2015). Moreover, IR was also observed after finasteride treatment (Livingstone et al. 2014, 2015).

Dowman et al. (2013) reported that the 5α-R type 1-KO mice are susceptible to hepatocellular carcinoma on prolonged feeding (12 months) of the American lifestyle-induced obesity syndrome (ALIOS) diet. This was attributed to increased local glucocorticoid concentrations, which play a role in hepatic steatosis due to 5α-R1 deficiency or inhibition (Livingstone et al. 2014, 2015, 2017).

The inhibition of 5α-Rs has several potentially clinically important implications. Dutasteride, a dual 5α-R inhibitor, increases IR most likely via impaired glucose disposal in the muscle (Upreti et al. 2014). Dowman et al. (2013) cautioned about the potential adverse metabolic consequences in men treated with dual 5α-R inhibition, given the long-term nature of treatment and the age of the patient group affected, in whom risk factors for MetS are most prevalent. Nasiri et al. (2015) also suggested that glucocorticoids and androgens are implicated in the pathogenesis of NAFLD. 5α-reductases are critical for inactivation and clearance of glucocorticoids as well as in the transformation of T to the more potent 5α-DHT. It is proposed that 5α-Rs regulate glucocorticoid and androgen action by determining the pre-receptor concentrations of such ligands. Reduced T concentrations are thought to be associated with increased hepatic steatosis (Kim et al. 2012; Völzke et al. 2010) and are consistent with findings in rodent models, suggesting that 5α-DHT treatment decreases hepatic lipid accumulation (Fig. 4) (Zhang et al. 2013a).

In a prospective cohort study, Joyce et al. (2017) evaluated baseline levels of SHBG, T, and 5α-DHT in 852 men free of diabetes and CVD and reported that

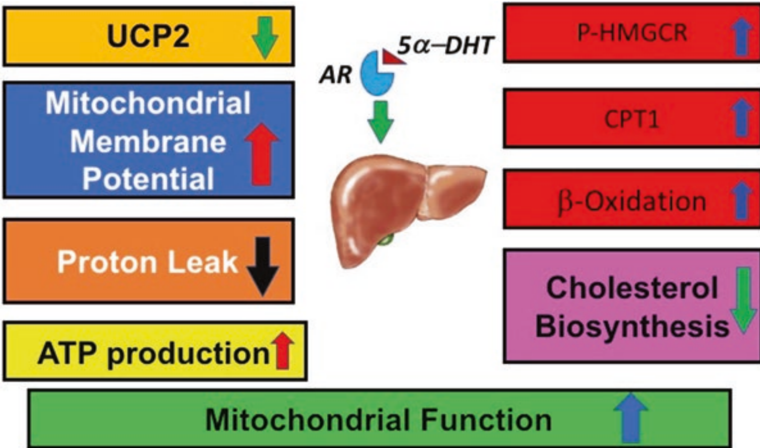

Fig. 4 Potential mechanisms of androgen action in the prevention of NAFLD/NASH. The postulated 5α-DHT/AR signaling pathway likely suppresses hepatic steatosis by increasing CPT-1-mediated b-oxidation and phosphorylation of P-HMGCR and also increasing ATP production and mitochondrial membrane potential and decreasing proton leak and cholesterol synthesis, thus improving mitochondrial function. 5α-DHT modulates hepatic lipid metabolism and subsequently normalizes the mitochondrial function (Adapted from Zhang et al. 2013a)

baseline 5α-DHT levels were strongly associated with reduced risk of diabetes and IR but no statistically significant relationship of SHBG levels or T levels, consistent with previous reports (Mather et al. 2015; Vandenput et al. 2007). The aforementioned findings suggest that 5α-DHT may play an important role in regulating glucose homeostasis and insulin sensitivity.

Recently, Upreti et al. (2014) reported that, in men, inhibition of 5α-Rs type 1 and 2 by dutasteride reduced stimulation of glucose disposal by high-dose insulin and reduced suppression of nonesterified fatty acids (NEFA). These findings suggested that dutasteride inhibition of critical biochemical pathways mediated by 5α-reductases negatively alters metabolic function (Upreti et al. 2014). Dutasteride treatment also increased fasting HOMA-IR and increased plasma insulin levels. Dutasteride increased body fat and reduced insulin-mediated suppression of nonesterified fatty acids (NEFAs) (Upreti et al. 2014). These findings are consistent with the findings of Joyce et al. (2017) who reported that among older men, levels of 5α-DHT were inversely associated with IR and risk of diabetes.

5α-RI therapy has important clinical implications not only in terms of predisposing individuals to development of hepatic steatosis and NAFLD but also due to the large numbers of patients prescribed 5α-RIs. Since the long-term metabolic consequences of these medications have not been fully assessed, it is imperative to determine the long-term impact of such agents in men with BPH on glucose, HbA1c, lipid profiles (TC, LDL cholesterol), and T levels and the impact on liver function and overall health.

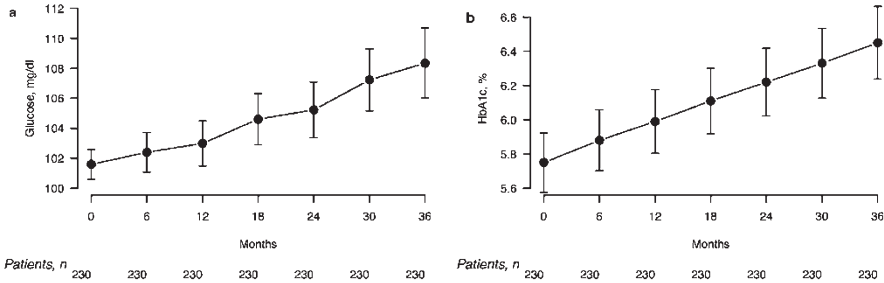

Fig. 5 Effects of long-term dutasteride therapy on fasting blood glucose levels (**a**) and HbA1c levels (**b**) in men treated for BPH

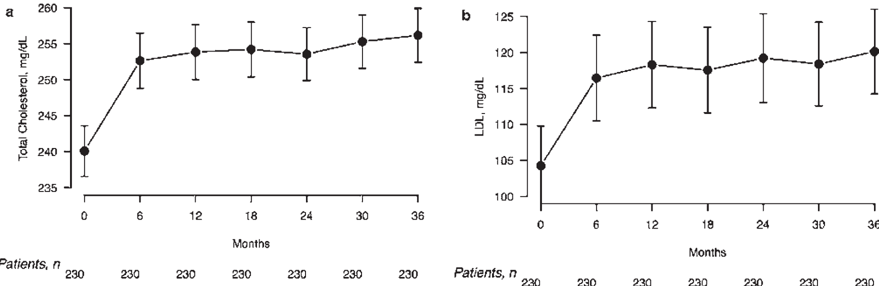

Fig. 6 Effects of long-term dutasteride therapy on total cholesterol (**a**) and LDL cholesterol (**b**) in men treated for BPH

We have recently reported on the effects of long-term dutasteride therapy in 230 men with BPH (Traish et al. 2017b). Dutasteride treatment for 36–42 months produced a progressive increase in fasting blood glucose and glycated hemoglobin (HbA1c) levels (Fig. 5a,b). These findings are consistent with those reported by Upreti et al. (2014), in which dutasteride treatment decreased glucose disposal during high-dose insulin infusion, most likely due to impaired insulin sensitivity in peripheral organs, including skeletal muscle and/or adipose tissue. Furthermore, Upreti et al. (2014) also noted that dutasteride reduced insulin-mediated suppression of nonesterified fatty acids (NEFAs), again suggesting impaired insulin sensitivity in adipose tissue. Joyce et al. (2017) reported that in older men who are free of CVD and diabetes, baseline levels of 5α-DHT were strongly associated with lower risk of diabetes and with less IR as assessed by HOMA-IR. These findings are also consistent with those reported by Hazlehurst et al. (2016) in which endogenous glucose production rate was significantly increased after dutasteride treatment and is consistent with increased hepatic IR. Dowman et al. (2013) also reported that 5α-reductase type 1-*deficient* mice developed IR and decreased insulin receptor expression.

Moreover, we note that TC and LDL cholesterol levels were also increased in men treated with dutasteride suggesting alteration in lipid metabolism (Fig. 6a,b).

Our findings are consistent with those of Hazlehurst et al. (2016) in which dutasteride had a significant effect on TC and LDL cholesterol, in the fasted state. Hazlehurst et al. (2016) demonstrated that intrahepatic lipid increased after dutasteride treatment and was associated with increased rates of de novo lipogenesis. Adipose tissue lipid mobilization was decreased by dutasteride treatment. These findings suggest that dutasteride treatment is associated with hepatic IR, hepatic lipid accumulation, and decreased adipose lipid mobilization without impacting peripheral insulin sensitivity (Hazlehurst et al. 2016). Furthermore, in the animal model, 5α-R type 1 deletion accelerated development of hepatic steatosis. Hepatic expression of genes involved in insulin signaling was reduced supporting a critical role of 5α-R type 1 in the evolution of NAFLD (Dowman et al. 2013). Thus, it is possible that long-term dutasteride therapy in men with BPH inhibits 5α-R type 1 function and contributes aforementioned adverse effects on metabolic processes.

The role of 5α-R type 1 activity was thought to be involved in regulating insulin sensitivity and lipid metabolism (Livingstone et al. 2015, 2017). In 5α-R type 1-KO mice, glucose intolerance was detected at 3 months of age, and a trend to hyperinsulinemia during glucose tolerance test (GTT) was detected at 5 months (Livingstone et al. 2015, 2017). 5α-R type 1-KO mice had increased susceptibility to hyperinsulinemia, fasting hyperglycemia, increased ratios of insulin to glucose, and liver fat accumulation (Livingstone et al. 2015, 2017). These animals exhibited suppression of liver lipolysis and induction of gene transcripts encoding enzymes involved in fatty acid β-oxidation and gluconeogenesis when animals fed a high-fat diet, while transcripts of genes favoring triglyceride esterification and cholesterol synthesis and excretion were disproportionately increased (Livingstone et al. 2015, 2017). These findings strongly suggest a critical role for 5α-R type 1 in glucose and lipid metabolism.

Zhang et al. (2013a) suggested that 5α-DHT is associated with decreased lipid accumulation and cholesterol synthesis in the liver by increasing expression of carnitine palmitoyltransferase 1 (CPT1) and phosphorylation of 3-hydroxy-3-methylglutaryl-CoA reductase (P-HMGCR) via an AR-mediated pathway (Fig. 4). Downregulation of cholesterol biosynthesis by increasing HMGCR phosphorylation was associated with androgen action. It was proposed that 5α-DHT modulates hepatic lipid metabolism and normalizes mitochondrial function. Since human liver expresses both 5α-Rs type 1 and type 2 enzymes and these isoforms play a significantly critical role in glucocorticoid metabolism and clearance as well as androgen metabolism, it is possible that inhibition of 5α-reductase type 1 activity by dutasteride increases endogenous glucocorticoid activities concomitant with reduction of androgenic activity producing marked alterations in glucose and lipid homeostasis and resulting in IR and lipid accumulation (Traish et al. 2015a, b; Livingstone et al. 2015; Hazlehurst et al. 2016; Livingstone et al. 2015, 2017; Dowman et al. 2013; Upreti et al. 2014).

Further, we observed increased liver activity of alanine and aspartate (ALA and AST) transaminases (Fig. 7a,b), suggesting that long-term treatment with dutasteride altered liver metabolic function (Livingstone et al. 2015; Dowman et al. 2013; Hazlehurst et al. 2016; Upreti et al. 2014; Traish et al. 2014c, 2015a, b, b). It is

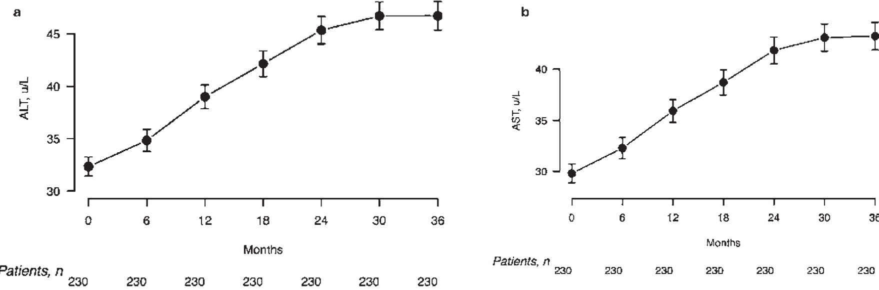

Fig. 7 Effects of long-term dutasteride therapy on alanine amino transferase (**a**) and aspartate amino transferase (**b**) in men treated for BPH

imperative to point out that 5α-DHT is a potent androgen and transformation of T to 5α-DHT may be critical to maintaining the functional metabolism in peripheral tissues (Zhang et al. 2013a; Livingstone et al. 2014, 2015, 2017; Upreti et al. 2014; Dowman et al. 2013; Hazlehurst et al. 2016). In summary, inhibition of the 5α-reductase family of enzymes (types 1, 2, and 3) by dutasteride contributes to a significant reduction (97%) in circulating 5α-DHT levels which are detrimental to metabolic function. Long-term dutasteride therapy may be associated with increased blood glucose, HbA1c, total cholesterol, and LDL cholesterol, potentially leading to increased onset of insulin resistance and NAFLD. In addition, dutasteride increased liver transaminase activity suggesting increased inflammation. These findings raise serious safety concerns regarding metabolic dysfunction of long-term dutasteride therapy.

We also noted that long-term dutasteride therapy resulted in a reduction in total circulating T levels (Fig. 8) consistent with previous observation with long-term therapy with finasteride (Traish et al. 2015a, b). Cross-sectional clinical studies have shown that low T concentrations are associated with increased hepatic steatosis in men (Kim et al. 2012; Völzke et al. 2010) and are consistent with findings in rodent models, suggesting that 5α-DHT treatment can decrease hepatic lipid accumulation (Zhang et al. 2013a; Nasiri et al. 2015). It is possible that reductions in T and/or 5α-DHT levels may contribute to altered glucose and lipid metabolism as well as liver dysfunction. Joyce et al. (2017) reported that baseline levels of 5α-DHT were strongly associated with lower risk of diabetes and with reduced IR, as assessed by HOMA-IR. In patients receiving ADT for treatment of PCa, Mohamedali et al. (2011) reported increased blood glucose and lipids in men undergoing 1 year of ADT. Similarly, Oka et al. (2016) reported that 1 month after ADT, the lipid profiles including TC, HDL-C, and LDL-C increased significantly. Finally, fnasteride and dutasteride treatment incrases teh aging male symptom score (AMS) suggesting increased metabolic and sexual dysfunction and reduced qualit of life (figure 8c) (Traish et al 2015a).

In view of our findings and those reported by others (Livingstone et al. 2014, 2015, 2017; Dowman et al. 2013; Hazlehurst et al. 2016; Upreti et al. 2014), it is important to consider the potential long-term consequences of dutasteride therapy

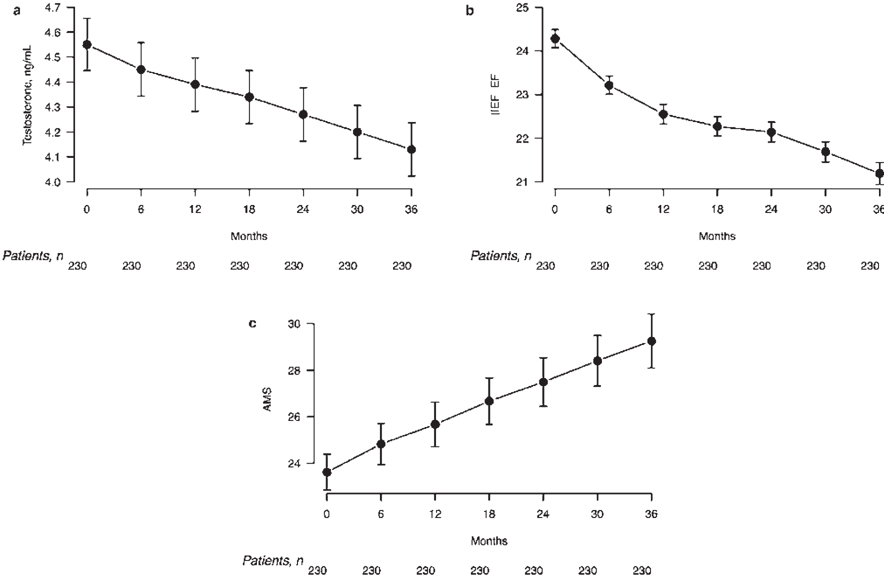

Fig. 8 Effects of long-term dutasteride therapy on testosterone levels (**a**) and erectile function (**b**) and aging male symptom (AMS) score (**c**) in men treated for BPH

on metabolic function, particularly IR and fat accumulation in liver. Because hepatic steatosis is a precursor to progression to more advanced stages of NAFLD including nonalcoholic steatohepatitis, it is likely that long-term therapy with dutasteride may contribute to onset of NAFLD.

Effects of 5αR Inhibitors Therapy on Sexual Function

Considerable contemporary literature exists relating to the importance of 5α-DHT to sexual function (for review cf. Traish et al. 2011c, Traish 2012, Traish et al. 2014b, c, 2015a, b, 2017b). Experimental animal studies have demonstrated that inhibition of 5αRs and blockade of T to 5α-DHT transformation resulted in sexual dysfunction (Bradshaw et al. 1981; Gray et al. 1980; Hart 1973, 1979; Mantzoros et al. 1995; Saksena et al. 1976; Baum et al. 1979; Lugg et al. 1995; Manzo et al. 1999; Seo et al. 1999; Zhang et al. 2013b; Pinsky et al. 2011; Oztekin et al. 2012; Traish et al. 1999). Clinical studies have also reported increased incidence of sexual dysfunction with agents that inhibit 5αRs activities (for comprehensive reviews cf. Traish et al. 2011c, 2014b, c, 2015a, b). The increased incidence of sexual dysfunction with agents such as finasteride and dutasteride is attributed to altering vascular and neural mechanisms involved in sexual activity.

In addition, we have reported that finasteride and dutasteride exert adverse effects on sexual function (Traish et al. 2011c, 2015a, b, 2017b) (Fig. 8b). These findings

are congruent with previous studies indicating that inhibition of T transformation to 5α-DHT may contribute to sexual dysfunction (Traish and Guay 2006; Traish et al. 2011c, 2014b, c). Our observations are consistent with previous study reporting that men with BPH who were treated with finasteride (Traish et al. 2015a, b) have increased aging male symptom (AMS) scores (Fig. 8c), suggesting that 5-αRI therapy induces functional androgen deficiency by reducing T levels (Fig. 8a) (Traish et al. 2017a, b) as well as 5α-DHT levels. This induced androgen deficiency increases TD-related symptoms concomitant with reduction in the quality of life, as reflected by the increased AMS score. A recent meta-analysis has confirmed the suspicion that inhibition of 5αR activities does contribute to erectile dysfunction (Corona et al. 2017). Therefore, we believe that inhibition of 5α-DHT formation is another form of induced androgen deficiency and its impact on health can no longer be ignored.

Discussion

In this chapter, we highlighted the significance of the adverse impact of TD on human health and quality of life. From the outset, we indicated that TD is a well-established medical condition that has a negative impact on men's health, and it merits treatment, irrespective of cause or etiology (Morgentaler et al. 2016). Several epidemiological studies have demonstrated prevalence of symptomatic TD in men with increasing age; these include the MMAS (Araujo et al. 2004), the EMAS (Wu et al. 2008, 2010), and the BACH study (Araujo et al. 2007). Thus, it is paramount that medical attention is re-focused on such vital medical condition that necessitates treatment. The negative impact of TD (idiopathic or acquired) on metabolic function is exemplified in alterations in carbohydrate metabolism as reflected by increased hyperglycemia, increased IR, and diabetes. Also, TD negatively impacts lipid metabolism and nitrogen retention. The negative consequence of TD on body composition, increased adipogenesis, and fat accumulation coupled with loss of lean body mass are no longer in dispute. TD is a risk factor for MetS and obesity (Laaksonen et al. 2004; Antonio et al. 2015). TD is associated with increased incidence of CVD and related mortality. Among the strong indications of TD are the reduced sexual desire and nocturnal penile erections (Wu et al. 2010). TD is also associated with diminished motivation, increased fatigue, decreased energy, reduced muscle mass and strength, depressed mood, irritability, and poor concentration (Wu et al. 2010; Araujo et al. 2004, 2007). Wu et al. (2008) suggested that age is associated with low free T and with elevated LH, indicating an impaired testicular function. TD is associated with overall sexual dysfunction encompassing loss of libido and reduced erectile function and increased orgasmic dysfunction. If remained untreated, TD has serious negative impact on men's health and quality of life.

We have previously postulated that TD plays a central role in the pathology of MetS, T2DM, and IR and contributes significantly to processes of adipogenesis and increased accumulation of visceral fat, resulting in obesity (Traish et al. 2009a, b, c, d).

Visceral fat serves as an endocrine organ, which produces pro-inflammatory cytokines affecting multiple tissues and organs and increasing the risk for IR, T2DM, MetS, and endothelial dysfunction. It is imperative that TD is recognized as a central factor in the development of various pathologies such MetS and obesity and contributing to vascular complications.

We suggest that there is a reasonable body of scientific and clinical evidence of proven benefits of T therapy in men with TD (for review, cf. Morgentaler et al. 2015a, b; Traish 2016a, b; Snyder et al. 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017). Although the safety concerns with regard to CVD risk are raised in some studies (Basaria et al. 2010; Xu et al. 2013; Vigen et al. 2013; Finkle et al. 2014) which are plagued by methodological, analytical, and interpretational flaws, a wealth of literature exists to support a host of benefits of T therapy without CVD risk (Maggi et al. 2016; Debruyne et al. 2017; Snyder et al. 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017; Traish et al. 2017a; Saad et al. 2016; Morgentaler et al. 2015a, b; Sharma et al. 2015, 2017; Wallis et al. 2016; Etminan et al. 2015; Baillargeon et al. 2014, 2015; Eisenberg et al. 2015; Tan et al. 2015; Cheetham et al. 2017; Hanske et al. 2017; Anderson et al. 2016). The findings from the most recent published placebo-controlled clinical trials on the benefits of T therapy in older men substantiate this argument (Snyder et al. 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017).

There is considerable evidence for proven benefits of T therapy in men with TD (Traish 2016a, b; Snyder et al. 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017). A more detailed discussion of these benefits can be found in the chapter of Dr. Saad in this current book. For this reason, no further discussion of T therapy will be presented here. In the following section, we wish to focus on the controversy of T therapy in men with nonclassical, idiopathic (functional) hypogonadism (TD) and the purported increased CVD risk. We wish also to address the FDA position on T therapy indications.

Although TD was recognized as medical condition since the 1940s and that such condition merits treatment, almost eight decades later, considerable controversies are raised regarding T therapy in men with TD due to its idiopathic nature. In 1981, the FDA approved T formulations for the treatment of “classical hypogonadism.” Since then, considerable advances in science and medicine have been achieved; nevertheless, the view from the FDA and others in the scientific and medical community still insists that only “classical hypogonadism” merits T therapy (Nguyen et al. 2015b). It was strongly proposed that T therapy is not indicated for “age-related,” i.e., idiopathic (functional) hypogonadism (Nguyen et al. 2015b). In our view, this argument has no scientific bases and should be dismissed. If age-related medical conditions do not warrant medical treatment, then we will not be treating age-related hypertension, age-related CVD, age-related thyroid deficiency, age-related cancer, etc. Nevertheless, all age-related medical conditions are treated, and therefore we find it absurd to suggest that age-related hypogonadism should be denied treatment.

The class label for T products to treat TD was introduced in 1981. At such time, there was limited information regarding the epidemiology, prevalence, and pathophysiological causes of TD. Since 1981, there were considerable scientific and clinical advances that contributed to a wealth of clinical knowledge regarding how important this medical condition is to men's health which were not known at the approval of the label in 1981. For instance, little was known in the 1980s about TD and other comorbidities, such as HIV infection, MetS, obesity, diabetes, renal failure, sleep apnea, and chronic opioid or glucocorticoid use. Given the evolving nature of clinical science, it is difficult to believe that the original causes of TD identified in the 1981 class label were intended to remain static and not subject to future revisions more than 30 years later, despite medical advances.

In April 2010 and in late 2013 and early 2014, four articles were published suggesting increased risk of cardiovascular disease with T therapy in men with TD (Basaria et al. 2010; Xu et al. 2013; Vigen et al. 2013; Finkle et al. 2014). The FDA reviewed all four studies together with other studies reported in the literature and concluded that there was no credible evidence to conclude that T therapy increases cardiovascular disease risk (cf. FDA response to the Public Citizen Petition to place a black box worning on T products; US Food and Drug Administration 2014). The European Medical Agency also arrived to a similar conclusion. Nevertheless, the FDA convened an advisory committee meeting in September 2014 to discuss the use of T for age-related hypogonadism and to address the recent controversies of potential cardiovascular risk. The FDA September 2014 advisory committee meeting addressed CV risk and the indicated populations for T therapy. The FDA concluded that the available evidence supports an indication for T therapy *only* in men with "classic hypogonadism" and emphasized that age-related hypogonadism is not an indication, as efficacy and safety of T products have not been established for this population. This conclusion limited the use of T therapy only to men with primary and secondary hypogonadism, which is limited to Klinefelter syndrome, undescended testicles, mumps orchitis, injury to the testicles, cancer treatment, Kallmann syndrome, pituitary disorders, inflammatory disease, HIV/AIDS, and medications. These are the only cases where FDA believes T therapy is indicated. The FDA panel concluded that "Given the widespread use of testosterone for age-related hypogonadism, the lack of substantial evidence to support such use, and the unknown effect of the label changes on prescribing patterns, the cardiovascular safety of testosterone products in older men remains an important public health concern" (Nguyen et al. 2015b). This statement is not supported by scientific evidence, and substantial evidence exists demonstrating significant benefits of T therapy in men without classical hypogonadism (Snyder et al. 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017). As to the argument of CVD safety, we are unaware of any evidence of CVD safety reported in men with "classical hypogonadism" albeit T is indicated for therapy in such group since 1981.

We should emphasize that benefits of T therapy in nonclassical hypogonadism were demonstrated in significant improvements on libido, erectile function, mood and depressive symptoms, body composition, bone mineral density, anemia, and

insulin sensitivity. The current literature provides *solid* evidence of benefits of T therapy in men with TD (Corona et al. 2011a, b, 2014; Isidori et al. 2005, 2014; Saad and Gooren 2011; Saad et al. 2011, 2012, 2013, 2016, 2017, Saad and Gooren 2009; Basurto et al. 2008; Snyder et al. 1999a, b, 2016, 2017; Kenny et al. 2010; Amory et al. 2004; Srinivas-Shankar et al. 2010; Wang et al. 2000, 2001, 2004; Cunningham et al. 2016; Hackett et al. 2013, 2014, 2016a, b; Jockenhövel et al. 2009; Amanatkar et al. 2014; Traish 2014a, 2016a, b, Traish et al. 2017a; Haider et al. 2014a, b). These include improvements in sexual function, such as libido and erectile function, as well as body composition (Corona et al. 2016) and bone mineral density. Also, there is good evidence of benefit in men with metabolic diseases (diabetes, obesity) of improvement with therapy with parameters of body fat, waist circumference, and insulin resistance. In addition, there is reasonable evidence of benefit with regard to mood and sense of well-being.

It should be noted that many of the studies on the benefit of T therapy in men with TD were performed in men aged 65 or older without "classical hypogonadism." For these reasons, we find that the distinction between "classical hypogonadism" vs "nonclassical hypogonadism" is neither clinically nor scientifically meaningful. Moreover, even if age did result in reduced T levels, why should the so-called age-related TD be denied benefits of treatment?

Recently, seven placebo-controlled clinical trials were reported on T treatment in older men (Snyder et al. 2016, 2017; Cunningham et al. 2016; Budoff et al. 2017; Resnick et al. 2017; Roy et al. 2017). The summary of such trials states that "These results, together with those of the other four trials (now completed), should inform decisions about T treatment for men 65 years of age or older whose levels are low for no apparent reason other than age." If such conclusion is made based on such trials, it is perplexing that the FDA and many others in the medical community remain opposed to T therapy in men with idiopathic TD. It is interesting that deficiencies of other hormones are treated based on the deficiency or absence of the hormone and not whether the cause is known or idiopathic. Obvious examples include diabetes and thyroid insufficiency. Physicians who opt to provide T therapy outside the FDA-approved labeling must educate themselves regarding the risks and benefits of T therapy and employ evidence-based medicine and maintain records of their decisions.

It is our view that the symptoms and signs of TD occur as a result of low T levels and many men with TD may benefit from T therapy, regardless of whether there is an identified underlying etiology or not. Also, we believe that there is no scientific basis for any age-specific recommendations against the use of T therapy in men. There is no evidence to suggest that the distinction between "classical hypogonadism" vs "nonclassical hypogonadism" is clinically or scientifically meaningful. In fact, even if age did result in low concentrations of T associated with symptoms of hypogonadism, why should the so-called age-related TD be denied benefits of treatment?

Most importantly a host of studies have demonstrated that T therapy in men with TD exerts a beneficial function on vascular health with no serious cardiovascular

events (Sharma et al. 2015, 2017; Wallis et al. 2016; Etminan et al. 2015; Baillargeon et al. 2014, 2015; Eisenberg et al. 2015; Tan et al. 2015; Cheetham et al. 2017; Hanske et al. 2017; Anderson et al. 2016).

Another critical issue that merits discussion in this chapter is the impact of inhibition of 5α-Rs on metabolic and sexual function. A body of growing evidence is accumulating regarding increased IR and lipid accumulation in the liver and predisposition to NAFLD in response to 5α-RI therapy (Upreti et al. 2014; Livingstone et al. 2014, 2015, 2017; Dowman et al. 2013; Hazlehurst et al. 2016). Bolduc et al. (2007) have investigated the effects 5α-DHT on several enzymes involved in glucose and lipid metabolism. α-DHT was shown to modulate biochemical pathways involved in energy metabolism and lipid mobilization. DHT increased the expression of carboxylesterase 3 expression, a lipase responsible for the hormone-sensitive lipase (HSL), independent lipolysis (Soni et al. 2004). In addition, the authors demonstrated that 5α-DHT upregulated carnitine acetyltransferase and enzymes involved in b-oxidation. However, 5α-DHT downregulates lipogenesis and synthesis of pyruvate through glycolysis. These observations are congruent with those proposed by Zhang et al. (2013a). Our recent findings (Traish et al. 2017b) and those discussed in this chapter (Livingstone et al. 2014, 2015, 2017; Dowman et al. 2013; Hazlehurst et al. 2016; Upreti et al. 2014) demonstrate that inhibition of 5α-Rs type 1 and 2 with dutasteride alters metabolic function with potential adverse metabolic ramifications. Simply, increased IR, lipid accumulation, and dysregulation of glucose metabolism and disposal are some of the observed effects of 5α-RIs therapy.

The negative impact of 5α-RIs on sexual function has been widely reported (reviewed in Traish 2012, Traish et al. 2015a, b, 2017b; Irwig & kolukula 2011, Irwig 2012, 2013, 2014). Considerable body of literature exists suggesting that a subset of patients treated with this therapeutic approach will experience adverse effects on libido, erectile, and orgasmic function (Traish et al. 2014a, b, c, 2015a, b, 2017b; Ali et al. 2015; Amory et al. 2008; Andriole et al. 2003, 2010; Basaria et al. 2016; Caruso et al. 2015; Cauci et al. 2017; Chi and Kim 2011; Chiriacò et al. 2016; Choi et al. 2016; Debruyne et al. 2004; Gupta et al. 2014; Mella et al. 2010; Kaplan et al. 2012; McConnell et al. 1992, 1998; Melcangi et al. 2013, 2017; Na et al. 2012; Roehrborn et al. 2002, 2008; Corona et al. 2017; Welk et al. 2017; Kiguradze et al. 2017; Guo et al. 2016; Liu et al. 2016). Although some investigators will argue that such drugs are considered safe (Skeldon et al. 2017; Wessells et al. 2003) and the number of afflicted individuals is relatively small, it is important to point out that almost three million men are treated with this therapeutic approach. If 9–10% of the treated men experience the serious adverse events on sexual function, this number may translate into several hundred thousand. In our view, this is not a small number of men who will suffer from loss of libido and erectile dysfunction. Given that a large number of older men treated with dutasteride for BPH, it is important that such adverse metabolic function be seriously taken into account prior to commencing such therapy (Corona et al. 2017). Furthermore, since this population is at greater risk for IR and diabetes, it is of great concern that treatment with this therapeutic

approach which may increase the onset of NAFLD, IR, and diabetes has not received much attention in the clinical setting. We strongly suggest that physicians discuss the potential adverse effects of finasteride and dutasteride therapy on metabolic (IR, lipid accumulation in liver, diabetes) and sexual function with their patients prior to initiating such therapy.

The fear that T and its metabolite 5α-DHT may increase the risk of prostate cancer has prompted the development of selective androgen receptor modulators (SARMs) which bind AR but exhibit tissue-selective activation of androgen-dependent genes (Bhasin et al. 2006; Narayanan et al. 2008). The major impetus for development SARMs has come from the unproven, potential, adverse effects of T supplementation on cardiovascular events, prostatic stimulation and enlargement, a possible increased risk of prostate cancer, and elevated hemoglobin levels. The key rationale for developing SARMs was that T serves as the AR ligand in vivo in many tissues but also serves as a substrate for 5α-DHT, a hormone which plays an important role in development and maintenance of secondary sexual characteristics and sexual function, germ cell development, and accessory sex organs. SARMs are thought to exhibit the potential to bind selectively on AR in various tissues and produce selective anabolic effects on muscle, without adverse effects on the prostate (Bhasin et al. 2005; Calof et al. 2005). It was believed that SARMs would be novel therapeutic agents with selective anabolic function in selective tissues, such as skeletal muscle and bone without the adverse effects associated with conversion of T to 5α-DHT or estradiol, and therefore will have no impact on prostate growth or related diseases (Negro-Vilar 1999; Edwards et al. 1998). Although some SARMs are in development, no SARM has been approved for clinical use by the US Food and Drug Administration. It should be pointed out that SARMs that may have properties of T but not 5α-DHT may in the long-term have unfavorable consequences on metabolic and sexual function. As we began to learn from the important role of 5a-DHT in liver and fat function and its critical role on maintaining sexual activity, it is paramount that any SARM developed and approved in human use should be tested for potential and significant adverse effects on metabolic and sexual function. It is my view that the exquisite and selective expression and function of 5a-Rs in various tissues are to serve critical and important physiological functions, in such tissues, including the central nervous tissue. The key question remains that any SARM that is resistant to transformation to 5α-DHT and or/aromatization to estradiol will have negative implication on a host of tissues physiology. Therefore, we believe that T is the best SARM. SARMs that may negate the effect of 5α-DHT or aromatization to estradiol will undoubtedly exert unexpected and unfavorable metabolic and sexual function in various tissues. Therefore, the rush to develop SARMs should be viewed with great deal of caution, and safety concerns need be taken seriously. Although SARMs have been in existence over the past 40 years, the question remains why no SARMs are currently in clinical use and why almost all SARMs in the pipelines of development by the pharmaceutical companies have been terminated.

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Testosterone Therapy and Glucose Homeostasis in Men with Testosterone Deficiency (Hypogonadism)

Farid Saad

Abstract Since the early 1990s, it has been recognized that testosterone (T) levels are lower in men with type 2 diabetes mellitus (T2DM) compared with nondiabetic men (controls). Hypogonadism has been reported in approximately 50% of men with T2DM with robust correlations with measures of obesity, such as waist circumference and body mass index (BMI). In longitudinal studies, hypogonadism has been identified as a predictor of incident T2DM. Experimental withdrawal of T led to acute decreased insulin sensitivity, which can be reversed by normalization of T concentrations. Androgen deprivation therapy, commonly used in men with advanced prostate cancer, increases the risk of incident T2DM significantly.

While short-term studies of T therapy in hypogonadal men with T2DM show only minor effects, long-term administration of T leads to meaningful and sustained improvements of glycemic control with parallel reductions in body weight and waist circumference. The more insulin-resistant and obese a patient is at the time of initiation of T therapy, the more improvements are noted. The observed effects are likely mediated by the increase in lean body mass invariably achieved by T therapy, as well as the improvement in energy and motivation, referred to as the psychotropic effects of T. As recommended by various guidelines, measuring T levels and, if indicated, restoring men's T levels into the normal physiological range can have a substantial impact on ameliorating T2DM in hypogonadal men.

Introduction

Testosterone (T) is a metabolic, vascular, and sexual hormone and exerts a diverse set of physiological functions in many tissues and organs (Traish 2014; Kelly and Jones 2013a). T modulates the function of muscle, adipose tissue, bone, epithelial, endothelial, and hematopoietic cells as well as regulates lipid, carbohydrate, and

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protein metabolism. More importantly, T is a key hormone in modulating vascular physiology and metabolism (Kelly and Jones 2013a, b, 2014; Rao et al. 2013; Traish 2014; Navarro et al. 2015).

T Deficiency (TD; Hypogonadism) and Its Implications on Overall Health

T deficiency (TD; also known as hypogonadism) is a well-recognized medical condition that negatively impacts sexual function, muscle mass, fat mass, body composition, bone density and strength, overall health, well-being, and quality of life (Morgentaler et al. 2016). Because T modulates muscle mass and function and also inhibits adipogenesis, it is postulated that TD may be associated with metabolic syndrome (MetS), obesity, and insulin resistance (IR) (Laaksonen et al. 2004; Antonio et al. 2015). Therefore, the interwoven relationship between TD and metabolic dysfunction leading to IR and type 2 diabetes mellitus (T2DM) has been a subject of intensive investigation.

TD has marked negative clinical manifestations on metabolic functions including reduced lean body mass (LBM) and increased fat accumulation (fat mass, FM), concomitant with increased IR (Kelly and Jones 2013a, b, 2014; Rao et al. 2013; Traish 2014; Morgentaler et al. 2015, 2016). TD is a risk factor for MetS, IR and T2DM, inflammation, dyslipidemia, hypertension, arterial stiffness, and atherosclerosis (Kelly and Jones 2013a, b, 2014; Rao et al. 2013; Traish 2014; Morgentaler et al. 2015, 2016; Snyder et al. 2016; Navarro et al. 2015). The complex relationship between TD and MetS components, such as hyperglycemia, IR, hypertension, dyslipidemia, and obesity, suggests an important role of T in metabolic function and homeostasis. One of the most obvious negative effects of TD is its relationship with accumulation of visceral fat and its deleterious endocrine function. It is well documented that TD promotes adipogenesis, which is associated with increased levels of circulating inflammatory cytokines (Kelly and Jones 2013a, b, 2014; Rao et al. 2013; Traish 2014; Morgentaler et al. 2015, 2016). The odds ratio for developing MetS is significantly greater in men with TD, irrespective of age, lifestyle changes, or comorbidities (Laaksonen et al. 2004; Antonio et al. 2015). Thus, the increased adiposity and inflammation as a pathophysiological response to TD promotes a vicious metabolic cycle and therefore contributes to metabolic dysfunction that leads to IR and diabetes (Kelly and Jones 2013a, b, 2014, 2015; Rao et al. 2013).

Relationship Between TD and Diabetes

Data from Cross-Sectional Studies

A large number of cross-sectional, longitudinal, and epidemiological studies have indicated that there are marked metabolic changes in patients with T2DM that contribute significantly to changes in circulating T levels. As early as 1990, it was recognized

that a positive association exists between visceral fat accumulation, insulin, glucose and C-peptide levels, and reduced T levels. These findings suggest that abdominal obesity in men is associated with reduced levels of sex hormone-binding globulin (SHBG) and total and free T levels (Seidell et al. 1990). Analyses of T concentrations in diabetic men showed that T and SHBG were lower in diabetic men, when compared with nondiabetic men. Furthermore, a strong negative correlation exists between plasma insulin and T levels. In men with TD, a marked increase in insulin and glucose levels and reduction in SHBG were recorded (Andersson et al. 1994). For example, a study of 103 consecutive men with T2DM showed that the proportion of men with TD is significantly larger than that of the general population. When diabetic patients were stratified by free T, total T, and bioavailable T concentrations, the percent of patients with TD were 33%, 44%, and 36%, respectively (Dhindsa et al. 2004). This observation led to the suggestion that TD diagnosed as hypogonadotropic hypogonadism (HH) occurs commonly in men with T2DM and increases significantly with age. Similar findings were reported by Kapoor et al., in which the proportion of patients with TD based on total T, bioavailable or free T, approached 50% (Kapoor et al. 2007).

The relationship between age, obesity, TD, and diabetes was also investigated in a number of studies. The prevalence of TD was particularly related to patients' body mass index (obesity) and to increased age, suggesting that the duration of androgen deficiency (i.e., the hypogonadal state) may contribute significantly to the increased prevalence of T2DM (Dhindsa et al. 2004; Kapoor et al. 2007). In studies by Dandona's group (Dhindsa et al. 2004, 2010), the proportion of men with TD and diabetes was highest in men with severe obesity approaching 58%. Similarly, when stratified by age, the proportion of men between 70 and 79 years of age with TD and diabetes approached 55%.

In a cohort of 1451 nondiabetic and 398 diabetic men, Dhindsa et al. determined free T levels and found that prevalence of reduced free T levels in lean, overweight, and obese nondiabetic men was 26%, 29%, and 40%, respectively. In contrast, in diabetic men, the prevalence of TD according to free T levels was 44%, 44%, and 50%, respectively. The mean free T level in diabetic men was significantly lower than that of nondiabetic men. Free T levels were negatively and significantly related to age, BMI, and SHBG. Based on these findings, it was suggested that the high prevalence of reduced free T levels may be attributed to obesity and the concomitant presence of diabetes, thus contributing to higher prevalence of TD (Dhindsa et al. 2010). Hackett reported increased glycated hemoglobin (HbA_{1c}) levels and reduced SHBG levels in men with reduced T levels (Hackett 2010). Furthermore, Brand et al. examined the relationship between endogenous T levels and SHBG and HbA_{1c} in 1136 men from the EPIC-Norfolk population. The authors noted that in nondiabetic men, HbA_{1c} levels were inversely associated with total T and calculated FT, independently of age, BMI, and smoking. When stratified by quartiles, HbA_{1c} levels were inversely related to total and free T levels, with lowest HbA_{1c} at the highest quartiles of total and free T. Interestingly, SHBG levels were also inversely associated with HbA_{1c} after multivariable adjustments (Brand et al. 2011). Together, these findings suggest that in middle-aged and older men, low endogenous T and SHBG levels are associated with glycemia, even below the threshold for diabetes. Therefore,

T and SHBG levels may be considered as markers of pathological processes resulting in elevated glucose levels, even among men without diabetes. Jones reviewed the epidemiological studies pertaining to T levels in men with T2DM and concluded that in diabetic men, reduced T levels, irrespective of what fraction of T was measured (total T, free T, or bioavailable T), were reported in all studies to date (Jones 2007). These epidemiological studies point out the negative impact of TD on glycemic control and metabolic function.

In a cross-sectional survey of 580 men with T2DM and 69 men with type 1 diabetes, Grossmann et al. investigated the prevalence of TD and the relationship between T and IR. Approximately 43% of men with T2DM had a reduced total T, and 57% had a reduced calculated free T. Interestingly, only 7% of men with type 1 diabetes had low total T. In contrast, 20.3% of men with type 1 diabetes had low calculated free T. Low T levels were independently associated with IR in men with type 1 diabetes as well as T2DM (Grossmann et al. 2008). In this latter study, age and BMI were major factors influencing both total T and calculated free T levels, consistent with previous studies (Kapoor et al. 2007; Selvin et al. 2007; Tsai et al. 2004). Data from experimental studies have also supported the observed decrease in total and free T and SHBG levels in T2DM in animals and humans (Maric 2009). In a recent study, Ho et al. examined 1306 men with normoglycemia (*n* = 577 (44.2%)), prediabetes (*n* = 543 (41.6%)), and diabetes (*n* = 186 (14.2%)), respectively. Total T, free and bioavailable T, and SHBG were determined in all men. The authors reported that prediabetes was associated with an increased risk of subnormal total T compared to normoglycemic patients. The authors further suggested that prediabetes is associated with an increased risk of TD, independent of obesity and MetS. After adjusting for MetS, the risk equals that of diabetes (Ho et al. 2013). Moreover, in another study of 196 prediabetics and 184 normoglycemic men, total T levels in prediabetes were markedly reduced compared to normoglycemic men (Rabijewski et al. 2015). TD was diagnosed in 30% of prediabetic men and in 13.6% normoglycemic men. In the prediabetic group, total T and calculated free T levels were lower in patients with impaired glucose tolerance than impaired fasting glucose.

Data from Longitudinal Studies

Data from longitudinal studies found that TD (hypogonadism) predicted incident diabetes in middle-aged men (Laaksonen et al. 2004). Levels of SHBG and total calculated free T and factors related to IR were determined at baseline in 702 middle-aged Finnish men participating in a population-based cohort study. These men had neither diabetes nor MetS at the start of the study. Men with TD (hypogonadal) who had no diabetes at baseline were more likely to be diagnosed with T2DM during 11 years of follow-up and after adjustment for age.

Epidemiological studies in healthy men also showed that low T predicts later onset of diabetes. In the Massachusetts Male Aging Study (MMAS) (1156 men followed up 7–10 years) (Stellato et al. 2000), the MRFIT study (528 men, 5 year

follow-up) (Haffner et al. 1996), the Rancho Bernardo study (294 men, 8 years follow-up) (Oh et al. 2002) and the "Gothenburg study" (659 men with 5 year follow-up) (Tibblin et al. 1996), total T, or free T were markedly reduced in men who progressed to become diabetic. In a prospective cohort study of 852 men free of diabetes and cardiovascular disease, Joyce et al. determined baseline levels of SHBG, T, and 5α-dihydrotestosterone (5α-DHT). IR estimated by homeostatic model assessment of insulin resistance (HOMA-IR) and insulin sensitivity estimated by the Gutt index in 1996 and incident diabetes (n = 112) were ascertained over a mean follow-up of 9.8 years. SHBG and 5α-DHT were inversely associated with IR after adjustments for demographics, alcohol consumption, current smoking, BMI, and other androgens. The authors suggested that, in older men, higher levels of 5α-DHT were inversely associated with IR and risk of diabetes over the ensuing 10 years, whereas levels of T were not (Joyce et al. 2017).

Experimentally Lowering Endogenous T Alters Glucose and Insulin Homeostasis

In the first decade of the new millennium, a group at Massachusetts General Hospital performed a number of elegant experimental studies. In a serious of experiments designed to investigate the potential mechanisms linking T and insulin sensitivity, Pitteloud et al. treated healthy volunteers with a GnRH antagonist which lowered their circulating T to castration levels. They then stimulated their endogenous T production by pulsatile GnRH and human chorionic gonadotropin (hCG). Patients underwent the hyperinsulinemic-euglycemic clamp procedure. The more T men produced under stimulation, the higher the increase in insulin sensitivity. This rapid change in insulin sensitivity with T stimulation occurred within 48 h and is therefore considered an immediate effect of T, which is not mediated by changes in body composition. These findings suggest that low T levels are associated with IR, resulting in part from an alteration in Leydig cell function (Pitteloud et al. 2005a). The studies by Pitteloud were further confirmed by Yialamas et al., in which acute sex steroid withdrawal reduced insulin sensitivity in young otherwise healthy men with TD due to idiopathic hypogonadotropic hypogonadism (IHH). In this study, 12 men with IHH on hormone therapy with normal T levels discontinued T therapy for 2 weeks. After discontinuation for 2 weeks, BMI remained unchanged. Serum T levels decreased and fasting insulin levels increased. HOMA-IR increased and insulin sensitivity index decreased (Yialamas et al. 2007). These findings suggest that acute T withdrawal increases IR in young IHH men confirming the conclusions by Pitteloud et al. that sex steroids modulate insulin sensitivity in the absence of apparent or detectable changes in body composition. These studies, however, were not designed to assess the effect of acute T withdrawal on beta-cell function. Further studies are needed to address this issue.

Androgen Deprivation Therapy (ADT) Alters Glucose and Insulin Homeostasis and Body Composition

Androgen deprivation therapy (ADT) by surgical or medical castration is considered standard treatment for men with advanced prostate cancer. This therapeutic approach offers a unique opportunity to study the effects of iatrogenically lowering men's T levels on metabolic and vascular function. Indeed, a large number of studies have investigated cardiometabolic outcomes of ADT. Significant changes in metabolic and body compositional parameters are noted in patients treated with ADT, even after only 1 or 3 months of therapy (Smith et al. 2001). In a prospective study of 32 men treated for 12 months with gonadotropin-releasing hormone (GnRH) agonists, patients experienced weight gain of 2.4%, increased body fat by 9.4%, and decreased lean body mass by 2.7% at 12 months. Changes in body composition with ADT were characterized by a reduction in lean body mass and an increase in fat mass. Furthermore, insulin concentrations rose despite unchanged plasma glucose, suggesting reduced insulin sensitivity and increased IR. The finding that ADT increases fat mass and insulin concentrations supports the concept that central abdominal adiposity is closely associated with disturbances in insulin and glucose metabolism in induced TD (Smith et al. 2002). Another prospective study of 25 patients without diabetes demonstrated that 12 weeks of ADT resulted in a 12.8% decrease in insulin sensitivity and a 25.9% increase in fasting plasma insulin. ADT also significantly increased HbA_{1c} levels and increased fat mass (Smith et al. 2006). Dockery et al. investigated the effects of complete ADT in 16 men with prostate cancer prior to and after 3 months of treatment for prostate cancer. The 16 men with prostate cancer underwent glucose tolerance and fasting lipids tests on both visits. After 3 months of T suppression, fasting insulin levels, total cholesterol, and high-density lipoprotein cholesterol increased in all treated men. These findings suggest that loss of androgens in men leads to increased serum insulin levels and may therefore adversely affect cardiovascular risk (Dockery et al. 2003). In addition, Basaria et al. reported on a cross-sectional study in which 53 men were evaluated, including 18 men with prostate cancer who received ADT for at least 12 months prior to study start (the ADT group), 17 age-matched men with nonmetastatic prostate cancer who had undergone prostatectomy and/or received radiotherapy but not ADT (the non-ADT group), and 18 age-matched controls (the control group). After adjustment for age and BMI, men in the ADT group had significantly higher fasting glucose, insulin, leptin levels, and HOMA index compared with men with prostate cancer but no ADT (n = 17) or healthy controls (n = 18). There was a significant negative correlation between total and free T levels with fasting glucose, insulin, leptin, and HOMA-IR. In this study, approximately 44% of men on ADT had fasting blood glucose greater than 126 mg/dL compared with only 11–12% in the other two comparison groups (Basaria et al. 2006). This latter study suggested that the adverse metabolic profile developed independent of age and BMI and appeared to be a direct result of androgen deprivation. In a subsequent review, Basaria suggested that long-term (12 months) ADT increases the prevalence of diabetes and MetS

compared with controls. In addition, men undergoing ADT also experience higher cardiovascular mortality (Basaria 2008).

In a retrospective analysis of 29 patients who had insulin-dependent diabetes mellitus prior to being diagnosed with metastatic prostate cancer and undergoing ADT, Haider et al. reported that glycemic control worsened substantially with concomitant increases of serum glucose and HbA_{1c} levels, suggesting impaired glycemic control and requiring increasing insulin dosages for treatment (Haider et al. 2007). In an observational study of a population-based cohort of 73,196 men, age 66 years or older who were diagnosed with locoregional prostate cancer, Keating et al. assessed the impact of GnRH agonists or orchiectomy on diabetes, coronary heart disease, myocardial infarction, and sudden cardiac death. It was reported that GnRH agonist use was associated with increased risk of incident diabetes, coronary heart disease, myocardial infarction, and sudden cardiac death. Men treated with orchiectomy were more likely to develop diabetes but not coronary heart disease, myocardial infarction, or sudden cardiac death (Keating et al. 2006).

It is well recognized that ADT decreases lean mass and increases fat mass and reduces insulin sensitivity. ADT increases low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides. ADT is thought to be associated with higher incidence of diabetes and cardiovascular disease (Keating et al. 2006; Saigal et al. 2007; Pilepich et al. 2005; Tsai et al. 2007). Additional studies by Saylor and Smith suggested that ADT is associated with increased risk and worsening of diabetes, coronary heart disease, myocardial infarction, and sudden death (Saylor and Smith 2009). Similarly, Hamilton et al. investigated changes in abdominal fat distribution and IR in 26 men with nonmetastatic prostate cancer during 1 year of ADT. After 12 months of ADT, visceral abdominal fat area increased by 22% and subcutaneous abdominal fat area by 13%. Fat mass increased by 14% and lean tissue mass decreased by 3.6%. IR (as assessed by HOMA-IR) increased by 12%. There was no change in fasting glucose or HbA_{1c} levels. The authors proposed that ADT induces TD with concomitant reduction of lean body mass and increased fat mass and visceral obesity. These pathophysiological changes lead to IR and T2DM in men treated with ADT for prostate cancer (Hamilton et al. 2011). In a recent study, Zitzmann et al. assessed the effects of androgen deprivation in 56 healthy men by progestins and subsequent replacement with T therapy. Total T levels were reduced, concomitant with increased IR (Quicki). IL-6 levels were increased and hemoglobin levels decreased. Subsequent therapy with T resulted in recovery of total T and significant improvement in HOMA index and recovery of hemoglobin levels with concomitant reduction in IL-6 (Zitzmann et al. 2017).

The underlying biochemical bases of testosterone deficiency and diabetes are supported by a body of evidence from clinical and preclinical studies; however, the exact molecular mechanisms of androgen deficiency and increased onset of diabetes, insulin resistance, and hyperglycemia remain poorly understood. Inaba et al. reported marked hyperglycemia and decreased β-cell function in prostate cancer patients treated with androgen deprivation therapy (Inaba et al. 2005), suggesting that severe androgen deficiency impairs β-cell function and leads to failure to compensate for insulin resistance (Mauvais-Jarvis 2016a, b). Recently, Navarro et al.

utilized a β-androgen receptor (AR) knock out mouse model (β-ARKO) and human and animal pancreatic β-islets in organ culture to investigate the potential biochemical mechanisms of androgen actions in β-cell function and the regulation of glucose-stimulated insulin secretion (GSIS). Based on findings from in vivo and in vitro experimentations, the authors postulated a novel non-genomic mechanism by which the extranuclear AR and its paracrine interactions with α-islets regulate the glucagon-like peptide-1(GLP-1) receptor which enhances β-cell function. Navarro et al. demonstrated that in adult male β-ARKO mice, GSIS was markedly decreased resulting in glucose intolerance and fasting and fed hypoinsulinemia and hyperglycemia when animals were fed high-fat diet (Navarro et al. 2016). Most importantly, T facilitated GSIS in cultured islets from animals and humans and was inhibited in β-ARKO islets and in human islets treated with flutamide, an androgen receptor blocker. In β cells, the AR stimulates GSIS by increasing intracellular cAMP biosynthesis, thus activating the cAMP-dependent protein kinase A (PKA). Furthermore, the insulinotropic effect of T was attributed to the activation of the GLP-1 receptor by islet-derived GLP-1 via paracrine mechanisms. These novel mechanisms shed new light on the potential role of androgen deficiency in increasing the risk of diabetes but also provide new therapeutic approaches that may help in prevention and/or treatment of diabetes. In additional experiments, Xu et al. found that AR-deficient is lets exhibited an altered expression of genes involved in insulin secretion, further underlining the importance of androgen action in regard to the devlopment of T2DM in men (Xu et al. 2017).

Effects of T Therapy on Lean Body Mass, Fat Mass, and Body Composition

As shown in Fig. 1, independent randomized, controlled trials have unequivocally resulted in a substantial increase in lean body mass in a magnitude between 3.4 and 4.8 kg within 1 year of T therapy in men with TD. One of these studies (Svartberg et al. 2008) was performed in community-dwelling men in Norway 60–80 years of age (mean: 69 years). Another study was carried out in Italy in men with MetS (mean age, 58 years) (Aversa et al. 2010). The lowest gain in muscle mass was achieved in Australian men with liver cirrhosis (mean age: 55 years) who gained "only" 3.4 kg. However, the placebo group lost muscle mass resulting in a mean adjusted difference between groups of 4.7 kg (Sinclair et al. 2016). In a fourth, short-term study (Hoyos et al. 2012) in obese men with obstructive sleep apnea in Australia, the T-treated group gained 1.2 kg lean body mass within as little as 18 weeks, while the placebo group lost 0.4 kg for a mean adjusted difference between groups of 1.6 kg. Another short-term study of only 24 weeks duration in men with T2DM between the ages of 30 and 65 years resulted in an increase in LBM by 2.6 kg in the T group, while the placebo group lost 0.8 kg with a mean

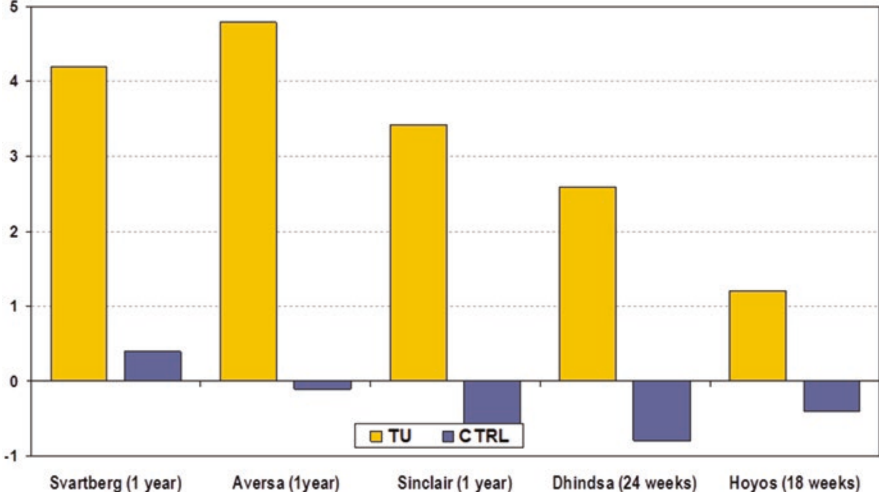

Fig. 1 Increase in lean body mass in five independent controlled studies using testosterone injections (Svartberg et al. 2008; Aversa et al. 2010; Sinclair 2016; Dhindsa et al. 2016; Hoyos et al. 2012)

between-group difference of 3.4 kg (Dhindsa et al. 2016). In all these studies, T was administered by injections.

In a long-term registry study in 411 obese men with TD receiving T therapy without lifestyle intervention, Saad et al. reported that weight loss was gradual but progressive and sustained. As shown in Fig. 2a, approximately 18 months of T treatment were required to achieve a mean weight loss of 5%. Similarly, it took a full year to achieve a reduction of HbA_{1c} by 0.5% (Fig. 2b). These findings strongly suggest that long-term T treatment is necessary to achieve sustained weight loss in obese men. The slow changes in weight in response to T therapy reflect a gradual return to a normal physiology and a reversal of a pathological condition such as obesity which takes many years to develop and progress (Saad et al. 2016a). The parallel reductions in weight and HbA_{1c} in patients with T2DM were recently confirmed in a Swedish systematic review of weight loss trials (Gummesson et al. 2017).

Ng Tang Fui et al. investigated 100 men who were randomized to either T or placebo. In one of the few randomized, placebo-controlled studies using T in exclusively obese men, patients underwent a very low energy diet (VLED) for 10 weeks followed by a maintenance diet for 46 weeks. They were randomized to receive either T undecanoate injections every 10 weeks following an initial 6-week interval or placebo. Loss of fat mass and lean mass was the same in both groups during the VLED phase. As shown in Fig. 3a–c, at the end of the 10-week VLED phase, both cases and controls lost the same body weight, with no difference in

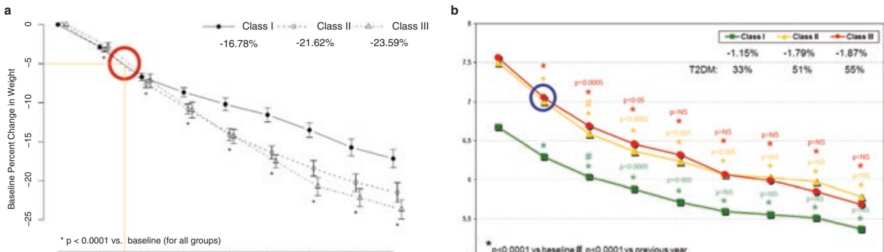

Fig. 2 (**a**) Weight loss (%) in 411 obese hypogonadal men receiving long-term testosterone treatment according to obesity class (Saad et al. 2016b). (**b**) HbA_{1c} (%) in 411 obese hypogonadal men on long-term testosterone treatment according to obesity class (Saad et al. 2016b)

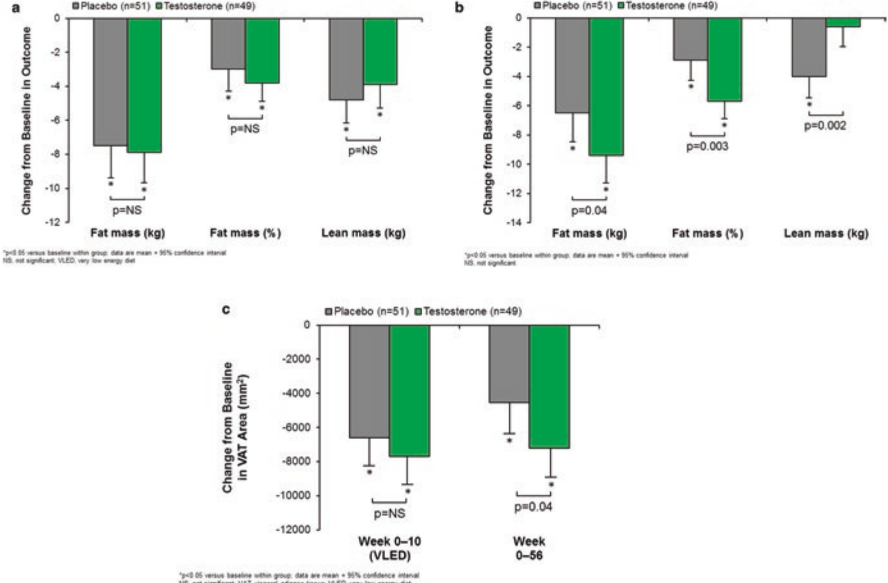

Fig. 3 (**a**) Loss of fat mass and lean body mass after 10 weeks of a VLED and treatment with testosterone or placebo. (**b**) Loss of fat mass and lean body mass after 56 weeks of treatment with testosterone or placebo. (**c**) Loss of visceral adipose tissue after 10 and 56 weeks of treatment with testosterone or placebo (Ng Tang Fui et al. 2016)

body composition (Fig. 3a). The reduction in fat mass from baseline was similar in both the placebo and the T-treated group. Also, the changes in lean body mass were similar in both groups during this brief period of time (10 weeks) (Fig. 3a). However, at the end of the study at 56 weeks, patients receiving T therapy had lost significantly more fat mass than the control group. More importantly, the T group

had completely regained their lean mass which was lost in the initial phase of the study, whereas the placebo group still had lost 4 kg of lean mass compared to baseline (Fig. 3b). When the loss of visceral adipose tissue was compared after 10 and 56 weeks of treatment with T or placebo, it was clear that the loss of fat mass was more significant in the T-treated group compared to placebo (Fig. 3c). The authors suggested that within the T group, there was a significant increase in physical activity and step count per day compared to baseline, measured by accelerometer. This was not observed in the placebo group although both groups underwent the same lifestyle intervention (Ng Tang Fui et al. 2016).

Effects of Testosterone Therapy in Men with TD (Hypogonadal) and IR or T2DM

In the early 1990s, Per Björntorp and his group in Göteborg were among the first to investigate the effects of varying doses of T administered to obese, middle-aged men. The authors administered T in the physiological range without resorting to very high doses. This therapeutic approach resulted in increased insulin sensitivity, which was most pronounced in men with low T at baseline. Mårin et al. reported that in 23 middle-aged abdominally obese men treated with T for 8 months or with placebo, T therapy resulted in decreased visceral fat mass, improved insulin sensitivity, and reduced blood glucose. Insulin sensitivity improved more in men with low baseline T values (Mårin et al. 1992a). Boyanov et al. investigated the effects of T therapy in men with TD on glucose homeostasis in middle-aged men with T2DM. After 3 months of therapy, the authors noted lower fasting, postprandial and mean daily blood glucose concentrations, and a decrease in HbA_{1c} (Boyanov et al. 2003). Similar findings were reported by others (Mårin et al. 1992a, b, c, 1993; Mårin and Arver 1998). T therapy for 8 months decreased visceral fat mass, IR, and fasting blood glucose. In 24 men with TD and T2DM treated with T or placebo for 3 months, Kapoor et al. reported that T therapy reduced the HOMA index suggesting improvement in fasting insulin sensitivity. Fasting blood glucose and HbA_{1c} were also significantly reduced. T therapy also produced a reduction in visceral adiposity as assessed by waist circumference (WC) (Kapoor et al. 2006). These findings suggest that T therapy in men with TD and diabetes improves insulin sensitivity and glucose homeostasis. Naharci et al. reported 24 patients with untreated TD (IHH) and 20 age-, sex-, and weight-matched eugonadal healthy control subjects. Patients with IHH, prior to T therapy, had higher fasting plasma glucose concentrations, higher fasting plasma insulin levels, a higher HOMA-IR score, and a lower QUICKI when compared with the control group. T therapy for 6 months in men with TD (IHH) improved HOMA-IR to levels comparable to those in the control group. Fat mass in men with IHH was reduced significantly with T therapy concomitant with significant increases in body mass index and lean body mass. Interestingly, a negative correlation was reported between T levels and HOMA

index in these men. HOMA index was higher in men with IHH prior to treatment and was significantly reduced after T therapy (Naharci et al. 2007).

In a prospective study, Wu et al. examined the effects of T therapy for 9 months in 26 men with HH and compared the findings to 26 healthy men. HOMA-IR and insulin areas under the curves (AUC) of 3-h oral glucose tolerance test were assessed in both groups. Prior to T therapy, HOMA-IR was significantly higher in the HH group than the healthy group. Similarly, insulin and fasting glucose levels were higher in the HH group than the healthy group. After 9 months of T therapy, significant reductions in HOMA-IR and insulin levels were recorded (Wu et al. 2009). In a randomized, double-blind, double-dummy, placebo-controlled, parallel group, single-center study (Aversa et al. 2010), 50 patients were randomized 4:1 to receive T therapy or placebo for 24 months. After 1 year, T therapy produced significant improvement in insulin sensitivity which was maintained for 2 years during therapy. Patients who were on placebo were shifted to T therapy and demonstrated marked improvements in HOMA-IR with reductions in fasting glucose and fasting insulin. The changes in HbA_{1c} after 12 and 24 months of therapy were significant, and switching from placebo to T therapy during the second year produced marked reductions in HbA_{1c} with treatment. The changes in HOMA-IR were also confirmed by the Quicki index in that T therapy produced marked reduction in IR (Aversa et al. 2010). The changes in HbA_{1c} observed in this and other studies were confirmed by Zitzmann et al. in a subgroup of hypogonadal men from the IPASS cohort with elevated HbA_{1c} at baseline. In the group of patients whose baseline level of HbA_{1c} was elevated (>6.1%) and with impaired glucose metabolism/T2DM, T therapy produced a decline of 1.1% points within the treatment period, suggesting the benefit of T therapy in regulating hyperglycemia (Zitzmann et al. 2013). Cornoldi et al. compared HOMA-IR changes in 87 elderly men (mean age: 74 years) with diabetes and proven coronary artery disease (CAD) after 12 weeks of T therapy or placebo. The authors reported improvement in the HOMA-IR in men who received T therapy (Cornoldi et al. 2010).

In a multicenter, prospective, randomized, double-blind, placebo-controlled study, Jones et al. assessed the efficacy and safety of T therapy for 12 months in 220 hypogonadal men with T2DM and/or MetS. The authors reported that T therapy reduced HOMA-IR in the overall population by 15.2% at 6 months and 16.4% at 12 months. In T2DM patients, glycemic control was significantly better in the T therapy group when compared with the placebo group at 9 months. The authors concluded that T therapy was associated with beneficial effects on IR in men with TD (hypogonadal), with T2DM, and/or MetS (Jones et al. 2011). The DIMALITE study reported on findings from 32 men aged 35–70 years who were newly diagnosed with T2DM with HbA_{1c} >6.5% but <9.0%. These patients had not received any oral antidiabetic medications and no insulin treatment. All men received supervised diet and exercise (D&E). One group (16 men) received T therapy and the other group remained untreated and served as control. No glucose-lowering agents were administered prior to or during the study period. After 52 weeks of T therapy, significant improvements were noted in glucose and insulin levels in the T group compared with D&E alone. The group on D&E plus T therapy had significantly lower

HbA_{1c} levels and reduced HOMA-IR than the control group (D&E only), suggesting improvements in insulin sensitivity. T therapy together with supervised D&E resulted in greater therapeutic improvements of glycemic control after 52 weeks of treatment in hypogonadal patients with the MetS and newly diagnosed T2D (Heufelder et al. 2009).

Contrary to the findings of the studies discussed above, Gianatti et al. reported that in a randomized, double-blind, placebo-controlled trial in 88 men with T2DM, aged 35–70 years with an HbA_{1c} <8.5% (69 mmol/mol), and a total T level of <12.0 nmol/L (346 ng/dL), T therapy or matching placebo was given over a 40-week period. T therapy did not improve glucose metabolism or visceral adiposity in obese men with moderately controlled T2DM and subnormal T levels typical for men with T2DM (Gianatti et al. 2014). T therapy did not improve insulin sensitivity and did not reduce IR, as assessed by HOMA-IR or glycemic control compared with placebo despite a decrease in fat mass and an increase in lean mass. However, this study was criticized because mean baseline HOMA-IR was very low to begin with at 2.11 and baseline HbA_{1c} 6.8% in the T-treated group, suggesting that these patients had almost no IR at the beginning of the study and their diabetes and glycemia were very well controlled (Jones 2014).

TD is associated with impaired diabetes control, greater BMI, increased WC, and increased severity of ED in men with T2DM. T therapy in men with T levels below 8 nmol/L for 30 weeks was associated with significant improvement in sexual function, including desire and morning erections (Hackett et al. 2013). The Aging Males' Symptoms (AMS) scale, a quality of life instrument, improved markedly but not metabolic parameters apart from WC. T therapy resulted in reductions in weight, BMI, HbA_{1c}, total cholesterol, and Hospital Anxiety and Depression Scale (HADS). The full benefits of treatment took at least 12–18 months and required the achievement of therapeutic trough levels of T at 14–15 nmol/L. Significant metabolic improvements were seen only in the *mild* group by 30 weeks and only in the *severe* group at 12–18 months when adequate levels were achieved (Hackett et al. 2013).

The effects of T therapy in 156 obese, diabetic men with TD were examined in a prospective, observational study (Haider et al. 2014). All 156 subjects had BMI ≥30 kg/m^2 and WC ≥94 cm with several comorbidities, including T2DM and dyslipidemia. Among these, 153 men had hypertension, 37 men had a history of CAD, and 19 men had previously had a myocardial infarction. T therapy of obese diabetic men with TD produced reductions in weight and WC, which were statistically significant at the end of each year compared to the previous year over the full observation time of 6 years. T therapy improved blood glucose levels (Fig. 4a) and HbA_{1c} levels (Fig. 4b). The decrease in HbA_{1c} was statistically significant at the end of each year compared to the previous year over the first 5 years. Also, T therapy improved systolic and diastolic blood pressure in this population, as well as lipid profiles, demonstrated by an increase in high-density lipoprotein cholesterol (HDL-C) and significant reductions in total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG). In addition, T therapy reduced the activities of liver aspartate transaminase (AST) and alanine transaminase (ALT)

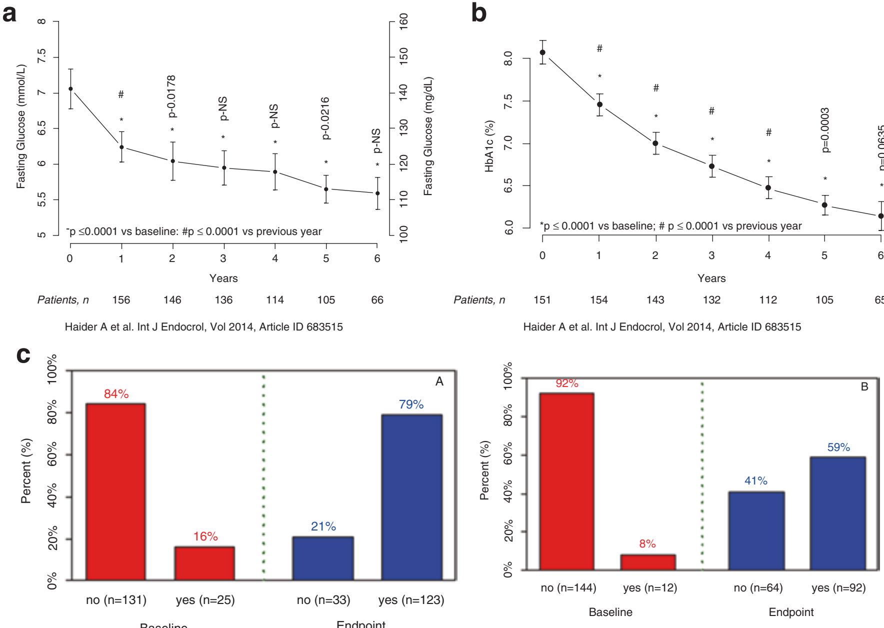

Fig. 4 (**a**) Fasting glucose (mg/dL or mmol/L) in 156 obese hypogonadal men with type 2 diabetes mellitus treated with testosterone undecanoate injections for up to 6 years. (**b**) HbA_{1c} (%) in 156 obese hypogonadal men with type 2 diabetes mellitus treated with testosterone undecanoate injections for up to 6 years. (**c**) Patients reaching HbA_{1c} target of ≤7% (**a**) and ≤6.5% (**b**) at baseline and end of observation time after treatment with testosterone undecanoate injections for up to 6 years (Haider et al. 2014)

suggesting a reduction in liver fat content, a reduced inflammatory response, and improvement in liver function (Haider et al. 2014). These findings were confirmed by the same investigators reporting even longer follow-up data (Traish et al. 2014; Saad et al. 2016a; Traish et al. 2017).

In Haider's "diabesity study" (Haider et al. 2014) in patients on standard diabetes treatment, before initiation of T therapy, only 16% and 8% of patients had achieved target HbA_{1c} targets of 7% and 6.5%, respectively (Fig. 4c). However, subsequent to T therapy, these targets were achieved by 79% and 59% of patients, respectively, at the last observation (Fig. 4c). These proportions further increased under continued T treatment for up to 8 years, as shown by the same group of authors (Saad et al. 2016b) and even up to 12 years (Saad et al. 2017).

As shown in Fig. 2b, 1 year of T therapy was necessary to observe a reduction in HbA_{1c} by 0.5% in obese men with TD. In contrast to other diabetes interventions, the gradual reduction in HbA_{1c} in response to T therapy was progressive and sustained throughout the follow-up period. In class I obesity, the prevalence of T2DM was 32.7%, in class II obesity 51.3%, and in class III obesity 55.3%. The mean baseline HbA_{1c} of approximately 7.5% in class II and class III obesity shows that it

is a major challenge to achieve HbA_{1c} targets recommended in guidelines for the treatment of diabetes by standard therapy (Saad et al. 2016a).

Dhindsa et al. investigated the effects of T therapy on IR in men with TD (hypogonadal) and T2DM. A total of 94 men were recruited, 50 men had normal T levels, and 44 men had TD. Men with hypogonadism were randomized to receive T therapy or placebo for 24 weeks. As shown in Fig. 5a**,** HOMA-IR decreased significantly in the T group, from 4.1 ± 0.9 to 2.7 ± 0.5 (p = 0.03), while there was a statistically nonsignificant increase in the placebo group. The difference between groups was −1.72 which remained statistically significant (p = 0.03). As shown in Fig. 5b, insulin sensitivity was determined by the hyperinsulinemic-euglycemic clamp methodology. T treatment at 24 weeks increased the glucose infusion rate (GIR) from 6.66 ± 4.36 to 8.73 ± 4.27 by 32% (p = 0.004), while there was no change in the placebo group (Fig. 5b). This was statistically significant between groups even after adjustment for confounding factors (p = 0.03). It should be noted that in this study, the total lean body mass increased in the T group from 70.6 ± 9.2 to 73.2 ± 10.7 kg (p = 0.001) and decreased in the placebo group from 69.1 ± 13.4 to 68.3 ± 13.0 (p = 0.41) with a mean difference between groups of 3.4 kg (p = 0.003) (Fig. 1) (Dhindsa et al. 2016).

This study also demonstrated that after 24 weeks of T treatment mRNA expression as well as protein expression levels of insulin signaling mediators (insulin receptor beta (IR-b), insulin receptor substrate-1 (IRS-1), glucose transporter type 4 (GLUT4), and serine/threonine kinase containing src homology domain 2 (AKT-2) were significantly altered in adipose tissue (Dhindsa et al. 2016).

The reported improvements in insulin sensitivity were also confirmed by the findings of a meta-analysis in which T therapy was shown to reduce IR, as assessed by reductions in fasting glucose and HOMA-IR (Corona et al. 2011).

Once T therapy is discontinued, improvements achieved during therapy are no longer maintained. This was shown to be the case for muscle mass and strength, in

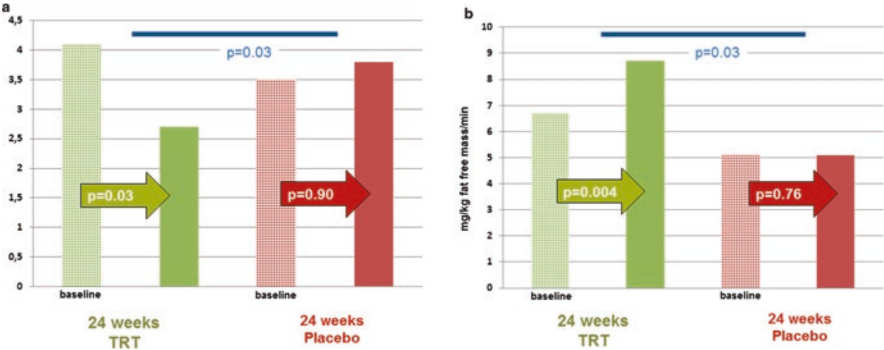

Fig. 5 (**a**) Insulin resistance assessed by HOMA-IR in hypogonadal men with type 2 diabetes receiving testosterone treatment vs placebo. (**b**) Insulin sensitivity assessed by glucose infusion rate (GIR) (Dhindsa et al. 2016)

a geriatric population of frail elderly men, where muscle mass and strength increased during 6 months of T therapy, but these effects were lost after cessation of treatment during the following 6 months (O'Connell et al. 2011). Similar results were reported by Ng Tang Fui et al. where beneficial effects on body composition and weight were lost after discontinuation of treatment at the end of the study (Ng Tang Fui et al. 2017). These findings are consistent with results from a long-term prospective registry study in which, after 5.5 years of treatment, T therapy was interrupted in a subset of patients for a mean duration of 14.5 months due to reimbursement issues. During this intermission phase, weight regain and a steep increase in HbA_{1c} occurred. After recommencing treatment, all measured parameters began to improve again. The authors concluded that hypogonadism may require lifelong T therapy (Yassin et al. 2016a, b).

Until today, no clinical study has assessed β-cell function by either HOMA-beta or hyperglycemic clamp. This represents a knowledge gap which is, however, being assessed in ongoing studies whose results will be published in the near future.

Testosterone Deficiency, T Therapy, and the Progression of Prediabetes

A considerable body of evidence exists demonstrating the link between reduced T levels and diabetes (Kapoor et al. 2007; Dhindsa et al. 2004; Grossmann et al. 2008; Haffner et al. 1996; Oh et al. 2002; Stellato et al. 2000; Lakshman et al. 2010; Ho et al. 2013). Limited studies are available on the relationship between TD and prediabetes, a state which is considered the preceding stage of overt diabetes. Ho et al. suggested that, after adjustment for MetS, men with prediabetes were at the same risk of subnormal total T as men with diabetes (Ho et al. 2013). Colangelo et al. suggested that sex hormones are associated with T2DM independently of BMI and WC (Colangelo et al. 2009). Tsai et al. also reported that T levels were inversely associated with fasting blood glucose (FBG) level and IR, and the association was independent of total body fat or abdominal fat (Tsai et al. 2004). The Rancho Bernardo study suggested that men with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) as defined by The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997) had lower total T than those with normal glucose tolerance (Goodman-Gruen & Barrett-Connor 2000). It should be noted that the relationship between T levels and diabetes is confounded by abdominal fat and central obesity (Grossmann 2011; Tsai et al. 2004). T therapy in men with TD and T2DM and/or MetS improves insulin sensitivity, and it is possible that T therapy in androgen-deficient men with prediabetes may prevent progression to overt diabetes (Kapoor et al. 2006; Kalinchenko et al. 2010; Jones et al. 2011; Heufelder et al. 2009). The relationship between prediabetes and T remains controversial (Vermeulen et al. 1993; Pitteloud et al. 2005b; Yeap et al. 2009; Grossmann 2011). Recently, it has been reported that prediabetic men had lower total T levels than men

without prediabetes (Arthur et al. 2017; Selvin et al. 2007; Colangelo et al. 2009; Grossmann 2011; Shin et al. 2012; Kim and Halter 2014; Rabijewski et al. 2014). Potentially, the increased synthesis of SHBG due to hyperinsulinemia in men with prediabetes may be responsible for the masked relationship between T concentrations and prediabetes (Pasquali 2006). Hyperinsulinemia may contribute to TD by impairing Leydig cell function or downregulation of testicular steroidogenesis (Pitteloud et al. 2005a; Giovannucci et al. 2010). It has been suggested that men with low T are at increased risk for developing diabetes and IR (Buysschaert et al. 2015; Stellato et al. 2000; Grossmann et al. 2008) and T and SHBG levels may predict new cases of diabetes (Stellato et al. 2000). Obese men with TD treated with T therapy demonstrated improved insulin sensitivity (Mårin 1998). Goodman-Gruen & Barrett-Connor reported that men with IFG or IGT had significantly lower total T levels compared with those with normal glucose tolerance, even after adjustment for age and BMI (Goodman-Gruen & Barrett-Connor 2000). Corona et al. reported that men with IFG had ED, reduced penile blood flow, and biochemical evidence of TD compared to their euglycemic counterparts (Corona et al. 2012). In men with prediabetes, Ho et al. reported that the OR for decreased total T levels compared to individuals with normoglycemia was 1.87 (95% CI 1.38–2.54) and 2.38 (95% CI 1.57–3.6) in those with T2DM. After adjustment for the metabolic syndrome, the OR in men with prediabetes was equal to that of diabetic men (1.49 vs. 1.50). The authors also reported that HbA_{1c} appeared to be a stronger predictor of low T (Ho et al. 2013). T therapy in men with TD improves insulin sensitivity and reduces IR (Mårin 1998), HbA_{1c}, WC, cholesterol, and fasting glucose levels (Kapoor et al. 2006; Naharci et al. 2007; Jones et al. 2011; Saad et al. 2013, 2015; Haider et al. 2014; Traish et al. 2014, 2017). There is an urgent need for studies to evaluate the impact of T therapy in men with TD on halting the progression from prediabetes to diabetes. Preliminary data presented at a congress of the European Association for the Study of Diabetes (EASD) suggest that T therapy in men with TD completely prevented progression to T2DM in an 8-year observational study (Saad et al. 2015). These findings were confirmed by the same group in comparison to untreated men with TD (hypogonadal control group): while no patient receiving T therapy progressed to T2DM during an observation time of up to 12 years, 37% of the untreated controls did during an observation time of up to 8 years (Haider et al. 2017). This is of importance since the incidence and prevalence of diabetes is a public health concern.

Discussion

T is a steroid hormone with pleotropic functions and is involved in fuel metabolism. The key biochemical and physiological effects of T are exerted on a variety of tissues, influencing many metabolic functions and altering body composition (Navarro et al. 2015). TD, irrespective of its etiology, is associated with increased fat mass, reduced lean body mass, and perturbations in several metabolic processes (Traish et al. 2014).

The changes in body composition accompanying TD are thought to contribute to reduced insulin sensitivity and poor glucose disposal, resulting in hyperglycemia and IR. TD is also associated with increased triglycerides and cholesterol and low HDL-cholesterol. In addition, TD negatively contributes to a host of comorbidities such as MetS, obesity, sexual dysfunction, fatigue, lethargy, depressed mood, and reduced quality of life.

Data from population studies strongly suggest that reduced T levels are an independent risk factor for diabetes and MetS (Laaksonen et al. 2004; Antonio et al. 2015). Men with IR or T2DM are often diagnosed with TD (Tomar et al. 2006; Haffner et al. 1996; Muller et al. 2005; Basaria 2008). Pitteloud et al. inferred that reduced T levels are related to the adverse metabolic condition, altering mitochondrial function and insulin sensitivity, and therefore promoting MetS (Pitteloud et al. 2005b). Along these lines, Yialamas et al. concluded that acute T withdrawal impairs insulin sensitivity in young healthy men with TD (due to IHH). One key finding was that the rapid changes in T levels concomitant with absence of changes in BMI suggest that T modulates insulin sensitivity in the absence of apparent or detectable changes in body composition during the short duration of the androgen withdrawal (Yialamas et al. 2007). This should not come as a surprise, since T is a metabolic and vascular hormone with an important role in muscle function and inhibition of differentiation of pre-adipocytes to adipocytes. Thus, TD may be a significant contributor to obesity, poor glucose homeostasis, and lipid metabolism (Xia et al. 2015; Kelly and Jones 2013a, 2014, 2015).

Recent studies have shown that T therapy ameliorates MetS components and reduces body weight, WC, BMI, and IR (Traish 2014; Saad et al. 2013, 2016a, b, Tsujimura 2013). Based on such findings, one would expect that T therapy would reduce IR and improve insulin sensitivity, together with increased muscle mass and reduced fat mass concomitant with improved glucose homeostasis. What is not yet fully appreciated is the impact of TD on IR and T2DM. The fact that T therapy reduces IR and ameliorates MetS could provide a novel therapeutic strategy for men with TD (hypogonadal) and T2DM. The fact that HOMA-IR, insulin AUC, and fasting glucose level in young men with IHH were significantly higher than in healthy control men suggests that TD is a risk factor for IR. Since T therapy significantly improves insulin sensitivity, this therapeutic approach may be useful to halt and prevent men with TD from developing T2DM (Wu et al. 2009). Interestingly, Ho et al. proposed that prediabetes is associated with an increased risk of TD, independent of obesity and MetS even after adjusting for MetS, the risk equaling that of diabetes (Ho et al. 2013).

Considerable evidence exists linking low T levels and T2DM (Hackett 2010). Reduced circulating T levels were thought to predict increased risk of developing T2DM over 5 years in men (Atlantis et al. 2016), and T therapy improves glucose control in men with, or at risk of, low T levels (Boyanov et al. 2003; Caminiti et al. 2009; Cornoldi et al. 2010; Emmelot-Vonk et al. 2008; Gopal et al. 2010; Heufelder et al. 2009; Kapoor et al. 2006; Malkin et al. 2006; Mårin et al. 1992a, 1993; Nair et al. 2006; Schroeder et al. 2004; Svartberg et al. 2008) and IR, assessed by HOMA-IR (Caminiti et al. 2009; Cornoldi et al. 2010; Emmelot-Vonk et al. 2008;

Gopal et al. 2010; Heufelder et al. 2009; Kapoor et al. 2006; Malkin et al. 2006; Schroeder et al. 2004; Svartberg et al. 2008) over short and medium terms as well as improved IR in men with T2D and/or MetS (Grossmann 2014). In addition, evidence suggests that benefits of T therapy for glucose control may be greatest when combined with lifestyle interventions (Heufelder et al. 2009). Holmboe et al. reported that reduced levels of T and SHBG were associated with a significantly increased risk of subsequent T2DM. The authors suggested that primary hypogonadism per se is not associated with an increased risk of T2DM and came to the conclusion that reduced T is a risk marker, but not a risk factor for T2DM (Holmboe et al. 2016). Several studies have reported that TD is associated with increased risk of T2DM (Vikan et al. 2010; Salminen et al. 2015; Schipf et al. 2011).

TD is associated with increased visceral adiposity, BMI, and HbA_{1c} levels, IR and other clinical symptoms, particularly ED (Kapoor et al. 2007). The postulated biochemical and physiological mechanisms for linking TD with T2DM encompasses increased expression and activity of aromatase and increased biosynthesis of estradiol in the peripheral tissues, including visceral adipose tissue. Reduced T levels will increase differentiation of pre-adipocytes into adipocytes with concomitant increased accumulation of visceral fat and activation of lipoprotein lipases leading to IR (Hackett et al. 2009; Jones 2010). The increase in abdominal obesity with TD brings about increased expression and activity of aromatase, with concomitant reduction in T levels, increased lipoprotein lipase enzyme activity and triglyceride uptake leading to increased visceral obesity and risk of IR. Pro-inflammatory adipocytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) may also produce further inhibition of T biosynthesis due to negative feedback on the pituitary-gonadal axis (Jones 2007). Also, visceral fat produces leptin, which by feedback on the hypothalamic-pituitary axis reduces LH and hence further lowers testosterone. There is now strong evidence that low T levels precede the onset of visceral obesity and are not simply a consequence.

TD is common in men with diabetes, regardless of the type (Grossmann et al. 2008), and approximately 43% of men with T2DM had reduced total T levels, and 57% had reduced calculated free T. Reduced T levels were also independently associated with IR in men with diabetes. Grossmann et al. reported a meta-analysis to assess the effects of T therapy on glucose metabolism in men with T2DM and/or MetS and low to low-normal T levels. While T therapy results in improved glucose-insulin profiles as assessed in the fasting state using HOMA-IR model 1, which is consistent with a beneficial effect to ameliorate IR, this improvement was not established when IR was assessed by HOMA-IR model 2. The authors stated that T therapy had no significant effect on glycemic control when assessed by HbA_{1c} levels. Furthermore, it was postulated that the reduction in IR reported with T therapy in the various studies were predominantly reported in men with MetS but without established T2DM, and for this reason, it is possible that T therapy may be more effective in improving glycemic outcomes in men with MetS compared to men with established T2DM. Because all but one of only seven selected studies had a duration of less than 1 year, the authors stated that longer treatment duration may have more marked effect on glucose metabolism (Grossmann et al. 2015). Magnussen et al.

reported that values for HOMA-IR and HbA_{1c} levels were unchanged after T therapy. T therapy in aging men with low bio-T levels and T2DM controlled on metformin monotherapy improved body composition; however, glycemic control, peripheral insulin sensitivity, EGP, and substrate metabolism were unchanged. However, this study had a short duration of only 24 weeks (Magnussen et al. 2016).

Gummesson et al. recently reported on the effects of weight reduction and HbA_{1c} in T2DM patients. The authors concluded that for each 1 kg or percent of weight loss, the mean HbA_{1c} is reduced by 0.1% units in the average T2DM population. Interestingly, the authors noted that HbA_{1c} reduction was more effective in populations with poor glycemic control than in well-controlled populations even at the same degree of weight loss (Gummesson et al. 2017).

It has been reported that bariatric surgery results in complete resolution of diabetes in a large proportion of obese T2DM patients (Buchwald and Oien 2009; Li et al. 2012), and HbA_{1c} levels progressed toward the normal range following significant weight loss in all surgery groups.

The role of androgens and the androgen receptor (AR) in regulating muscle and adipose tissues which play a role in insulin sensitivity has been well recognized. However, the role of the AR in regulating insulin secretion from pancreatic islets remains to be investigated (Navarro et al. 2015; Mauvais-Jarvis 2016a). The group of Mauvais-Jarvis investigated the effects of T on insulin secretion in mice with β cell-selective AR deficiency (βARKO). The authors reported that adult male βARKO mice exhibit decreased glucose-stimulated insulin secretion (GSIS) leading to glucose intolerance (Navarro et al. 2016). Mice fed western diet developed fasting hyperglycemia. T enhanced GSIS in cultured human and mouse islets, an effect that was blocked in βARKO islets and in human islets treated with the AR antagonist flutamide.

IR is thought to contribute to skeletal muscle loss. Recently it was reported that the risk of developing low relative appendicular skeletal muscle mass (ASM) at 4.6-year follow-up was 2.9 times higher among subjects with HOMA-IR levels more than 2.3, and after adjusting for age, the risk increased to ≥3.9 times (Alemán-Mateo et al. 2014). Since muscle is the principle tissue of glucose metabolism and disposal, it is possible that loss of muscle mass contributes to hyperglycemia and IR (López Teros et al. 2015). Hyperinsulinemia as an early marker of IR was associated with the loss of ASM in a cohort study of community-dwelling older men and women without other chronic health conditions. The use of fasting insulin levels >8.4 mU/mL may help clinicians identify individuals in the geriatric population who are at high risk of loss of ASM. Srikanthan and Karlamangla suggested that skeletal muscle mass relative to body weight is inversely associated with IR and the risk of prediabetes (Srikanthan and Karlamangla 2011). It is known that the pathophysiology of T2DM causes atrophy of muscles, due to declines in the activity of anabolic hormones (e.g., IGF-I, T, ghrelin), increased inflammation, increased expression of acrogens that increase protein degradation, and the detrimental effects of T2DM on blood supply to muscle. It should be noted that T2DM is also characterized by decreased functional β-cell mass and inadequate insulin secretion. Thus, increased glycemia in T2DM is the result not only of increased IR (which is related

to decline in muscle mass) but also of decreased β-cell function. Fornari et al. reported that in obese patients, higher lean mass is directly linked to a lower inflammatory profile and to better insulin sensitivity and better metabolic profile (Fornari et al. 2015).

The increased lean body mass with T therapy is a critical physiological component of improving glucose utilization and disposal, as well as ameliorating hyperglycemia and improving insulin sensitivity. A large number of studies demonstrated unequivocally that T therapy increases lean body mass and reduces fat mass thus improving body composition, amelioration of MetS components, concomitant with weight loss, reduction in WC, BMI, improvement in glucose, HbA_{1c}, lipid profiles, and blood pressure (Traish 2014; Traish 2016). Insulin-mediated glucose disposal is carried out primarily in muscle tissue. Therefore, the negative impact of low muscle mass on IR and diabetes is well recognized (Srikanthan et al. 2010; Dominguez and Barbagallo 2007; Atlantis et al. 2009). Higher muscle mass improves insulin sensitivity due to efficient glucose metabolism and disposal and reduces the incidence of diabetes. To this end, we wish to point out that it is highly possible that the changes in body composition invariably resulting from T therapy may be the key unique effect which cannot be achieved by any other diabetes medication.

Srikanthan and Karlamangla examined data from 13,644 subjects and assessed HOMA-IR, HbA_{1c}, prevalence of transitional/pre- or overt diabetes, and prevalence of overt diabetes mellitus. The authors reported that with every 10% increase in skeletal muscle index, an 11% relative reduction in HOMA-IR and 12% relative reductions in prediabetes prevalence were observed, even after adjusting for age, ethnicity, sex, and generalized and central obesity (Srikanthan and Karlamangla 2011). These findings clearly suggest that skeletal muscle mass relative to body weight is inversely associated with IR and the risk of prediabetes. In addition, the incidence of low relative ASM is significantly associated with IR in men aged 60–72 years age. Also, men who developed low relative ASM at 4.6-year follow-up had significantly higher HOMA-IR values at baseline than normal men. These findings suggest that the risk of developing low relative ASM is increased by 3.9 times when the HOMA-IR values were greater than 2.3, after adjusting for age (Alemán-Mateo et al. 2014). In a follow-up study, the authors examined the relationship between hyperinsulinemia, as an early predictor of IR, and loss of ASM. An elevated baseline insulin concentration is associated with increases of ASM, while at 4.6-year follow-up hyperinsulinemia increased risk of ASM loss in both older men and women. This relationship remained significant even after adjusting for age, gender, smoking, alcohol use, fat mass, heart disease, hypertension, physical activity, and use of medications. These findings suggest that hyperinsulinemia is a significant risk marker for the loss of ASM in an apparently healthy sample of older nondiabetic men with no pronounced loss of ASM (López Teros et al. 2015). These findings are congruent with the studies reported by Fornari et al. who showed that higher lean body mass was associated with a better insulin sensitivity and a lower inflammation status in an obese population (Fornari et al. 2015).

Men with TD experience fatigue and lethargy and reduced physical activity. Thus, one important aspect of T therapy in men with TD is the improvement in mood, energy, and increased physical activity, in turn promoting a healthier lifestyle. The increased energy and improvement in mood and physical activity is expected to contribute to improved muscle mass and improved insulin sensitivity. In addition to improvements in body composition, T therapy has significant additional health benefits such as psychotropic effects that contribute to increased physical activity, increased motivation, and an overall positive attitude and mood (Zitzmann et al. 2013). In a placebo-controlled study in obese men, there was a significant increase in physical activity and step count per day compared to baseline, measured by accelerometer within the T group but not in the placebo group although both groups underwent the same lifestyle intervention (Ng Tang Fui et al. 2016). Jockenhövel et al. reported that T therapy significantly reduced depression and fatigue scores within 6 weeks concomitant with improvement in scores of anxieties and concentration (Jockenhövel et al. 2009). A modest, yet significant, increase in mood and self-confidence was also reported (Wang et al. 2004; Snyder et al. 2016). Zitzmann et al. noted a marked gradual increase in symptoms and reduced vigor with reduced T levels and suggested that patients who are on long-term injection T therapy notice when they are becoming androgen deficient by lacking vigor and libido (Zitzmann et al. 2006). A similar observation regarding the threshold for TD symptoms is reported in men with TD (Kelleher et al. 2004). Men with TD treated with T therapy reach distinctively individual trigger TD symptoms and seek retreatment.

All-cause mortality is greater in diabetic men with low T compared with normal total T levels (Muraleedharan et al. 2013; Hackett et al. 2017). Studies in men with T2DM further suggest that T therapy reduces all-cause mortality (Muraleedharan et al. 2013; Shores et al. 2012; Hackett et al. 2016, 2017).

Figure 6 shows the absolute risk reduction (ARR) and the relative risk reduction (RRR) and lifetime risk reduction expected by treating men with T at 55 and 65 years of age. Lifetime risk reduction is significantly greater when T therapy is initiated at 55 years of age. The RRR associated with T therapy is similar at both ages selected (Ramachandran et al. 2017).

T therapy was shown to improve the quality of life, as assessed by changes in the quality of life (QoL) SF-12 questionnaire (Tong et al. 2012). Among the most significant improvement in the SF-12 scores was vitality, followed by general health, social functioning, physical role functioning, and emotional role functioning. The physical and mental health composite scores were significantly improved in the active treatment group. There was a significant difference in the physical and mental health composite scores between the active treatment and placebo groups. The effectiveness of T therapy in improving the mental health component of QoL in men with TD was also reported by Tong et al. While the improvement in SF-12 composite scores was apparent within 30 weeks of treatment, it is important to note that the physical composite scores continued to improve at 48 weeks of treatment. Therefore, T therapy may be indicated in men who have a poor QoL due to TD. Zitzmann et al. investigated scores of mental and psychosexual functions (libido, vigor, overall

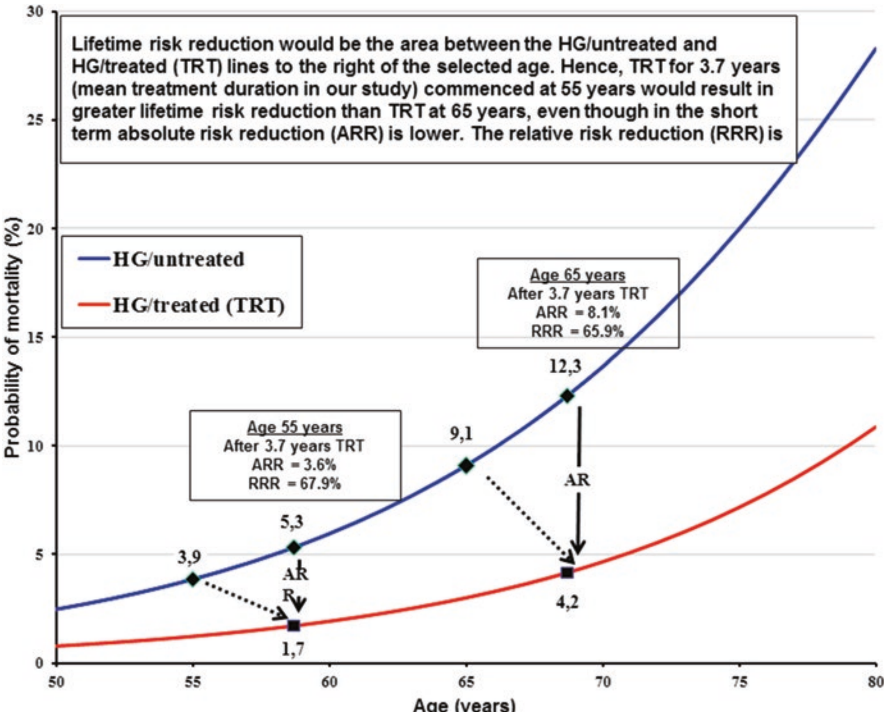

Fig. 6 Comparison of absolute risk reduction, relative risk reduction, and lifetime risk reduction in man with hypogonadism (sexual symptoms and total testosterone <12 nmol/l (348.1 ng/dl) and T2DM, treated and untreated with testosterone (Ramachandran et al. 2017)

mood, and ability to concentrate) in 1493 hypogonadal men treated with T therapy. The results indicate that the proportions of patients who reported low levels of well-being declined during T therapy. Significant improvements were recorded in the overall level of vigor/vitality and improved mood and increased ability to concentrate (Zitzmann et al. 2013).

In conclusion, long-term T therapy in hypogonadal men with T2DM brings about meaningful and sustained improvements of glycemic control with parallel reductions in body weight and waist circumference. These effects are likely mediated by the increase in lean body mass, which is always achieved by T therapy, as well as improvements in energy and motivation which may contribute to adopting a healthier lifestyle. As recommended by various guidelines, testosterone should be measured in men with T2DM. If hypogonadism is diagnosed, adequate long-term testosterone therapy can have a substantial positive impact on ameliorating T2DM.

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Sex Differences in Androgen Regulation of Metabolism in Nonhuman Primates

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Abstract The in-depth characterization of sex differences relevant to human physiology requires the judicious use of a variety of animal models and human clinical data. Nonhuman primates (NHPs) represent an important experimental system that bridges rodent studies and clinical investigations. NHP studies have been especially useful in understanding the role of sex hormones in development and metabolism and also allow the elucidation of the effects of pertinent dietary influences on physiology pertinent to disease states such as obesity and diabetes. This chapter summarizes the current state of our understanding of androgen effects on male and female NHP metabolism relevant to hypogonadism in human males and polycystic ovary syndrome in human females. This review will also focus on the interaction between altered androgen levels and dietary restriction and excess, in particular the Western-style diet that underlies significant human pathophysiology.

Introduction

There is a growing appreciation of the role of sex differences in biology that has been inadequately addressed in previous biomedical research as well as in clinical trials. This has led to a renewed emphasis on evaluation of the role of sex in important aspects of physiology and pathology, largely driven by the realization that sex differences may affect the efficacy or adverse effects of established and emerging therapies for human disease. The importance of sex differences was illustrated by the recent demonstration that the majority of mammalian phenotypic traits are influenced by sex (Karp et al. 2017). A major aspect of sex differences is their effect on metabolism (Varlamov et al. 2014; Mauvais-Jarvis et al. 2017; Mauvais-Jarvis

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2015) which is particularly relevant in light of the worldwide increase in obesity and its complications such as cardiovascular disease (Spence and Pilote 2015). While the most obvious (and most studied) factor in determining sex differences is the role of sex steroids such as estrogens and androgens, it is important to note that many examples of sex differences are more directly due to the presence and copy number of sex chromosomes that can be independent of sex steroid effects per se. These studies have employed powerful rodent models such as the "four-core genotypes" and XY* systems, in which sex chromosome vs sex steroid effects can be distinguished (De Vries et al. 2002; Arnold and Chen 2009; Chen et al. 2012; Mauvais-Jarvis et al. 2017; Arnold 2014). Connections between sex chromosome complement and sex steroid effects can obviously occur as well, exemplified by the effect of sex on adrenal androgens (Rehman and Carr 2004).

A comprehensive and translatable understanding of the effects of sex on major physiological processes such as metabolism requires the study of appropriate experimental systems. Clinical studies provide directly relevant information, while rodent systems allow the use of elegant genetic models. Clinical studies have obvious logistical and ethical limitations, while rodent metabolic control mechanisms do not always extrapolate to humans (Kowalski and Bruce 2014; Bunner et al. 2014; Mauvais-Jarvis et al. 2017). Nonhuman primates (NHP), particularly macaque species such as rhesus and cynomolgus, represent important preclinical models that combine greater similarity to human physiology than rodents with greater possibilities for experimental intervention than human studies. NHPs exhibit sex differences in basic biology as well as in complex physiology (Resko and Roselli 1997; Key and Ross 1999; Reinius et al. 2008; Simpson et al. 2016).

With respect to the control of metabolism in particular, there are two crucial tissues that illustrate the similarities between NHPs and humans and differences from rodents that make the NHPs especially important experimental systems. The first is the pancreatic islet that regulates glucose metabolism through insulin and glucagon. Basic islet architecture in primate (macaque and human) islets involves a lower proportion of insulin-producing β-cells and extensive intermingling of cell types, which results in primarily heterotypic interactions (Brissova et al. 2005; Cabrera et al. 2006; Bosco et al. 2010; Dolensek et al. 2015; Arrojo e Drigo et al. 2015). In contrast, rodent islets are composed of a central core comprised of β-cells surrounded by a thin layer of non-β (α, δ, and PP)-cells. Thus, interactions between insulin- and glucagon-producing cells are much more pronounced in primate islets. Other structural features that distinguish primate from rodent islets include aspects of vascularization (Brissova et al. 2015; Cohrs et al. 2017) and basement membrane composition (Otonkoski et al. 2008; Virtanen et al. 2008; Kharouta et al. 2009), innervation (Rodriguez-Diaz et al. 2011), and gene expression patterns (Macdonald et al. 2011; Dai et al. 2012; Amisten et al. 2017). These differences underlie important differences in function such as proliferative capacity and hormone secretion (Butler et al. 2010; Genevay et al. 2010; Conrad et al. 2016).

Another important metabolic tissue is the set of adipose depots that play the major role in lipid metabolism as well as important roles in glucose metabolism. Humans and NHPs have a variety of specialized adipose depots (Hudson et al. 1996;

Colman et al. 1999; Varlamov et al. 2010), including several white adipose tissue (WAT) depots and brown adipose tissue. Rodents and primates, however, differ in the anatomy and exact types of WAT (Isler 2014; Chusyd et al. 2016). Additionally, rodent and primate adipose tissue differ in gene expression and regulation as well as lipolysis control (Mynatt and Stephens 2001; Lindroos et al. 2013; Guller et al. 2015; Zuriaga et al. 2017; Sengenes et al. 2002).

These species differences in major organs that regulate metabolism and their conservation between NHPs and humans make NHPs invaluable tools for the elucidation of important aspects of sex-specific control of metabolism. While the role of estrogens in metabolic control is crucial (Newell-Fugate 2017; Clegg et al. 2017), the role of androgens is also important in the metabolic state of both males and females (Schiffer et al. 2017). In this chapter, we review the effects of androgens on various aspects of metabolism in NHP models of altered androgen levels as well as their interactions with diet.

Effects of Androgens on Male NHP Metabolism

Our previous NHP studies demonstrated that androgen deprivation under conditions of a low-fat chow diet did not result in the development of obesity or insulin resistance (Varlamov et al. 2012), suggesting that other diet-related factors and/or increased caloric intake may increase the vulnerability of hypogonadal males to metabolic disturbance. This study also showed that androgen deprivation for 1 year achieved via surgical orchiectomy induced abnormal cellular morphology in retroperitoneal WAT (Varlamov et al. 2012). Morphological alterations induced by androgen deprivation included a multilocular phenotype (the presence of multiple lipid droplets) and an increase in a percentage of smaller adipocytes (Fig. 1). Furthermore, adipocyte insulin signaling via the Akt pathway and adipogenic gene expression were decreased in androgen-deprived males. In contrast, orchidectomized males receiving a physiological dose of testosterone (T) during the last 6 months of the study displayed a normal unilocular WAT phenotype (a single central lipid droplet), an increased percentage of larger adipocytes, an improved adipocyte insulin sensitivity, and an increased expression of adipogenic genes.

Effects of Androgens on Female NHP Metabolism

Effects of Prenatal Hyperandrogenism

When early- to mid-gestation prenatal hyperandrogenism is derived from transplacental delivery of experimentally induced maternal hyperandrogenism (Resko et al. 1987; Abbott et al. 2008), exposed female NHPs exhibit a variety of polycystic ovary syndrome (PCOS)-like neuroendocrine, ovarian, endocrine, and metabolic

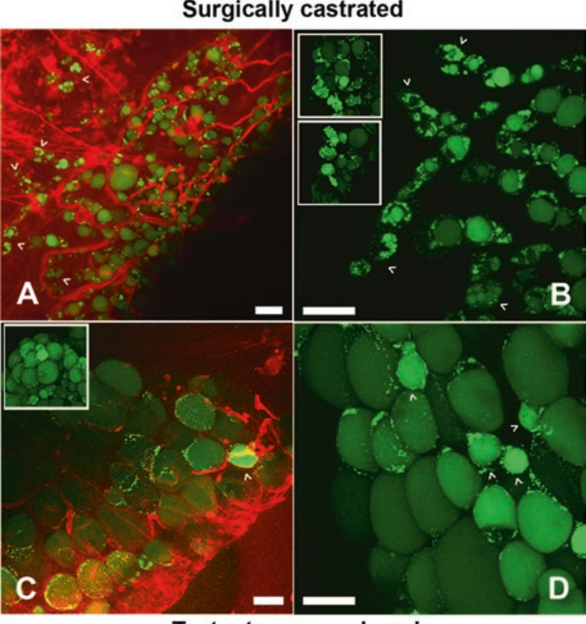

Fig. 1 Testosterone deficiency induces a multilocular phenotype in male WAT. Insulin-stimulated WAT explants from castrated (**a** and **b**), and testosterone-replaced (**c** and **d**), adult male macaques were labeled with green fluorescent fatty acid BODIPY-C12 (lipid droplets) and red fluorescent wheat germ agglutinin (blood vessels) and analyzed by confocal microscopy. *Arrowheads* indicate small multilocular adipocytes in castrated (**a** and **b**), and small unilocular adipocytes in testosterone-replaced (**c** and **d**) animals. *Scale bar*, 50 μm (Adapted from Varlamov et al. 2012)

traits, including type 2 diabetes mellitus (T2DM) (Abbott et al. 2016). With specific regard to metabolic dysfunction, it must be noted that all these prenatal hyperandrogenic manipulations were performed on NHPs fed a non-obesogenic low-fat, high-fiber diet.

Visceral adipocytes in female NHPs exposed to early-gestation hyperandrogenism demonstrate differential methylation in ~100–300 gene promoter sites when they reach infancy or adulthood (Xu et al. 2011). Bioinformatic analysis of this epigenetic reprogramming identified altered TGF-β signaling as the most functionally defective pathway, implicating alterations in TGF-β receptor-mediated bone morphogenetic protein and anti-mullerian hormone regulation of adipogenesis (Xu et al. 2011). One phenotypic consequence was the preferential accumulation of abdominal fat in visceral compared to subcutaneous (SC) depots as BMI increases, in contrast to preferential lipid accumulation in SC depots in controls (Eisner et al. 2003; Bruns et al. 2007). Such differential adipose accumulation is consistent with impaired maturation of SC adipocytes, accompanied by accelerated commitment of adipocyte stem cells to preadipocytes in androgen-exposed female NHPs, as evidenced by disproportionally increased numbers of small SC adipocytes together with a reciprocal decrease in C/EBPα and an increase in Zfp423 gene expression, respectively (Keller et al. 2014). C/EBPα is an androgen-regulated transcription

factor enabling adipocyte maturation, while Zfp423 is an insulin-regulated transcription factor committing adipose stem cells to a preadipocyte phenotype (Keller et al. 2014). Such a phenomenon may diminish SC abdominal storage of lipid and therefore explain why visceral fat, as an alternative fat depot, increases in amount with BMI in androgen-exposed, but not control, female NHPs (Bruns et al. 2007). Interestingly, an increase in the number of small SC adipocytes is linked with metabolic dysfunction in humans (Mclaughlin et al. 2014), as is an alternative increase in SC adipocyte size in some PCOS women (Manneras-Holm et al. 2011), implying that a change in either direction away from the optimal size of SC adipocytes might have metabolic consequences.

Together, these NHP adipogenic findings suggest that when energy intake exceeds the capacity of normal SC adipose to safely store fat, excess free fatty acid (FFA) becomes deposited in abnormal locations, such as the muscle, liver, and pancreas, where FFAs induce oxidative/endoplasmic reticulum stress tightly linked with insulin resistance and inflammation (Manneras-Holm et al. 2011; De Zegher et al. 2009; Virtue and Vidal-Puig 2010; Sorensen et al. 2010; Lee and Pratley 2005). This sequence of events is important because metabolic dysfunction in humans likely results from ectopic lipid accumulation in nonadipose cells (Sorensen et al. 2010). Such a notion is consistent with abdominal fat or visceral fat correlating negatively with insulin sensitivity in androgen-exposed female NHPs, alone (Bruns et al. 2007), and the progressive appearance of excess FFA, pancreatic beta cell decompensation, insulin resistance, and higher incidence of type 2 diabetes mellitus in these same female NHPs (Eisner et al. 2000; Abbott et al. 2005; Zhou et al. 2007).

Interestingly, pancreatic dysfunction appears as early as the newborn infant in female NHPs exposed to hyperandrogenism during early gestation. Transient newborn hypoglycemia, excessive numbers of β-cells, and small pancreatic islets, together with inappropriate β-cell compensation and increased body weight, suggest that transient gestational hyperglycemia unexpectedly induced by maternal hyperandrogenism may contribute to fetal reprogramming of metabolic function in androgen-exposed female NHPs (Abbott et al. 2010; Nicol et al. 2014).

Exposure of female NHPs to maternal androgen excess during late gestation, in contrast, while inducing greater total body adiposity in adulthood (Eisner et al. 2003) does not induce metabolic dysfunction, including an absence of T2DM (Abbott et al. 2005; Bruns et al. 2007).

Effects of Postnatal Hyperandrogenism

The models of prenatal hyperandrogenemia described above have examined how early exposure to androgens can program changes in adult metabolism. These findings have clear implications for congenital adrenal hyperplasia, wherein elevated androgens produced by the fetal adrenal gland can result in masculinization of female offspring at birth and lifelong metabolic and reproductive complications (Merke and Bornstein 2005). This model also offers insight into PCOS, since many reproductive and metabolic phenotypes of PCOS are recreated in models of prenatal

androgen exposure. However, whether gestational hyperandrogenemia happens in women who go on to develop PCOS is unclear. Clinical studies have identified elevated androgens around the time of puberty in a population of girls that appear at increased risk for the development of PCOS, indicating that this later postnatal developmental time window may also have profound effects to program adult metabolism (McCartney et al. 2007; Apter et al. 1994). Adolescence is also a critical time for the programming of obesity, lending further support to the hypothesis that androgen elevation during this time may have long-lasting effects on metabolism (Dietz 1994).

An NHP model of postnatal androgen treatment has been developed to investigate how androgen exposure during puberty may alter adult metabolism. Importantly, this model uses a fourfold excess of T to imitate the similar increase in androgens observed in peripubertal girls at risk for the development of PCOS in adulthood (McCartney et al. 2007), and treatments were initiated between 1 and 2.5 years of age. Initial pilot studies in prepubertal 1-year-old animals did not reveal significant differences in weight or insulin resistance between control and androgen (T)-treated animals (T group) consuming a chow diet (Mcgee et al. 2014). However, challenge with a western-style diet (WSD) later in development did reveal increased weight gain in androgen-treated females, indicating a worsened adaptive response to this caloric challenge. Hyperandrogenemia also reduced basal lipolytic activity and the expression of hormone-sensitive lipase in visceral omental (OM)-WAT (Varlamov et al. 2013). Surprisingly, these effects were only observed during the luteal phase, when the levels of estrogens and progesterone are higher compared to menses. This same study revealed increased FFA uptake and insulin signaling in OM-WAT ex vivo. The effects of androgens on FFA uptake were observed at menses, under conditions of low estrogen and progesterone levels, but not during the luteal phase.

More recent studies have examined the effects of androgen treatment beginning at the initiation of puberty (roughly 2.5 years of age) and demonstrated that this treatment also appeared to cause negative metabolic outcomes. In particular, increased weight and fat mass gain as well as increased abdominal circumference were observed in androgen-treated females (True et al. 2017). However, this study included androgen-treated females on a control diet and a WSD, and many effects that were statistically associated with androgen exposure appeared to be driven largely by the combination of WSD and androgens (see following section). Hyperandrogenemia was also associated with an altered pattern of physical activity, with androgen-treated females showing a delay in the usual puberty-associated decline in activity, followed by a more rapid decline later in the study, corresponding to the period when weight gain was observed. Similar to the findings in hyperandrogenized prepubertal animals described above, peripubertal treatment with androgens resulted in impaired lipolysis ex vivo (Varlamov et al. 2017). Basal lipolytic activity was significantly reduced in OM and SC-WAT, while the β-adrenergic lipolytic response was significantly decreased only in SC-WAT (Fig. 2). These findings of postnatal androgen effects on whole-body metabolism and adipocyte expansion are largely consistent with findings in rodents (Alexanderson et al. 2007; Nilsson et al. 1998; Kauffman et al. 2015).

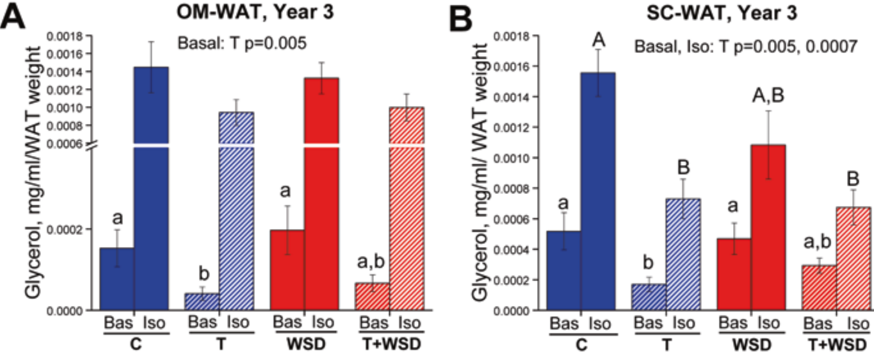

Fig. 2 The suppression of lipolysis by androgens in female WAT. Female rhesus macaques were randomly assigned at 2.5 years of age (near menarche) to receive either cholesterol (C; $n = 20$)- or testosterone (T; $n = 20$)-containing silastic implants to elevate T levels fivefold above baseline. Half of each of these groups was then fed either a low-fat monkey chow diet or a WSD, resulting in four treatment groups (C, control diet; T alone; WSD alone; T + WSD; $n = 10$/group) that were maintained until the current analyses were performed at 5.5 years of age (3 years of treatment, young adults). OM (panel **a**) and SC-WAT (panel **b**) biopsies were collected and analyzed longitudinally for changes in basal (Bas) and isoproterenol (Iso)-stimulated lipolysis. In year 3 of treatment, basal lipolysis was blunted in the T and T + WSD groups in both WAT depots, while isoproterenol-stimulated lipolysis was significantly blunted in the T and T + WSD groups only in SC-WAT (Adapted from Varlamov et al. (2017))

In addition to the long-term effects of androgen on whole-body metabolism in females, androgens exert acute effects on adipocyte physiology. In contrast to the effects of long-term postnatal androgen exposure, acute ex vivo treatment of female retroperitoneal WAT explants with the nonaromatizable androgen dihydrotestosterone increased basal but reduced insulin-stimulated FFA uptake (Varlamov et al. 2012). Additionally, androgen-stimulated basal FFA uptake was greater in WAT of ovariectomized females compared to WAT of intact females and ovariectomized females replaced with estrogen and progesterone in vivo (Varlamov et al. 2012).

Interactions Between Androgens and Diet

Androgen Deprivation in NHP Males

Our earlier studies in males (Varlamov et al. 2012) did not address the effect of androgen deprivation on SC-WAT and OM-WAT and was limited to a non-obesogenic low-fat control chow diet. In a follow-up study, we compared the effect of androgen deprivation in intact vs orchidectomized middle-aged male rhesus macaques, using three types of diet (Cameron et al. 2016). Both groups of animals were maintained for 2 months on a chow diet and then shifted to a WSD. Following 6 months on a WSD, the individual caloric intake was reduced by 30% (on chow

diet). This experimental design allowed us to collect and longitudinally study WAT biopsies derived from the same anatomical sites. Androgen deprivation did not have a significant effect on the WSD-induced increase in the average size of OM and SC adipocytes, while both groups developed insulin resistance. However, orchidectomized animals exhibited less reduction in the size of OM and SC adipocytes after caloric restriction, which was associated with persistent insulin resistance.

Hyperandrogenemia in NHP Females

We have also examined the interaction of peripubertal hyperandrogenemia and WSD on female metabolism. Strikingly, animals receiving androgens and WSD (T+WSD group) beginning at puberty did worse metabolically than animals receiving either treatment alone (True et al. 2017). This was observed for measures of body weight, fat gained over the experiment, and measures of insulin resistance. Adipocytes also appeared to be affected by the combined treatment, with larger visceral adipocytes and increased FFA uptake in visceral WAT in the T+WSD group compared to all other groups. Female rhesus macaques exposed to hyperandrogenemia and WSD exhibited an increase in the average size of visceral adipocytes that correlated with greater insulin resistance compared to animals treated with T or WSD alone (True et al. 2017; Varlamov et al. 2017) (Fig. 3). As noted above, certain metabolic effects were often statistically identified as being driven by either diet or androgen in isolation; however, the group receiving both androgens and WSD was often significantly different from all other groups, indicating a more extreme metabolic phenotype when both conditions were present. In addition, the T+WSD group showed metabolic heterogeneity, with some animals maintaining metabolic parameters similar to controls and some developing a worsened metabolic phenotype (increased weight, increased fasting insulin, etc.). This variability resembles the clinical picture of PCOS, where hyperandrogenemia is associated with obesity and insulin resistance in the majority of, but not all, patients (Legro et al. 2001; Ovalle and Azziz 2002).

Conclusions

NHP Males

These NHP studies suggest that T, at least in the presence of low-fat diet, is essential for the maintenance of normal WAT morphology (Fig. 1), adipogenic gene expression, and adipocyte insulin sensitivity (Fig. 4). Furthermore, T is involved in adipocyte hypotrophy following the transition from WSD to caloric restriction (Fig. 4). Although the mechanism of male obesity associated with low T and

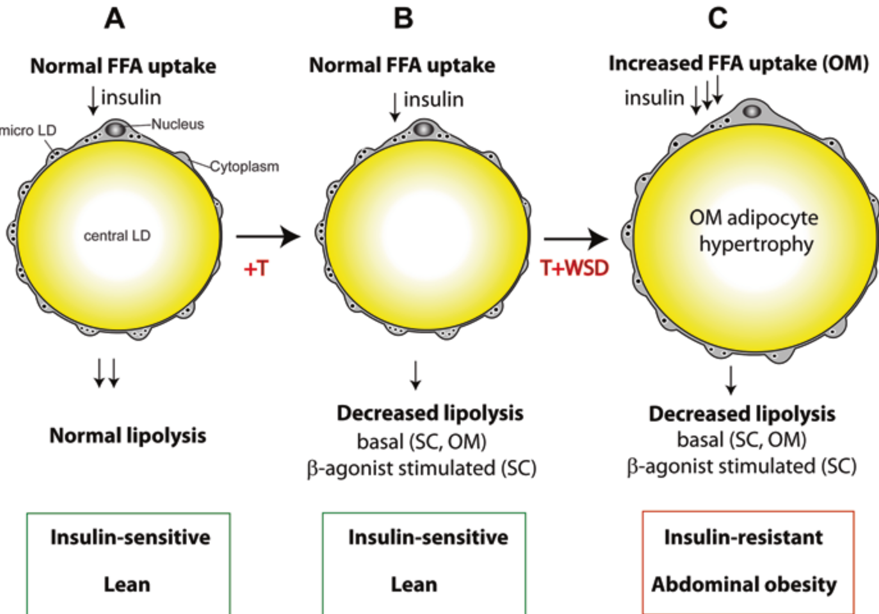

Fig. 3 The effect of hyperandrogenemia on female adipocytes. (**a**) Insulin-stimulated free fatty acid (FFA) uptake in adipocytes is coupled to unidirectional FFA esterification, followed by the packaging of triglyceride into the central lipid droplet (LD). Micro-LDs are strategically located at the interface of the cytoplasm, endoplasmic reticulum, and the central LD, being responsible for and/or associated with insulin-stimulated triglyceride synthesis and packaging in unilocular adipocytes (Chu et al. 2014). The opposite process of triglyceride degradation, termed lipolysis, is potentiated by β−adrenergic stimuli provided by local sympathetic innervation. (**b**) Hyperandrogenemia (T excess) inhibits basal lipolysis both in subcutaneous (SC) and visceral omental (OM) WAT depots while significantly suppressing β-agonist-stimulated lipolysis in SC-WAT. In animals fed a control low-fat chow diet, the T-induced lipolytic defect does not evoke adipocyte hypertrophy and insulin resistance. (**c**) T-induced suppression of lipolysis persists in animals fed a WSD with the same depot specificity. Additionally, insulin-stimulated FFA uptake in OM adipocytes is significantly elevated in response to a combined exposure to hyperandrogenemia and WSD. In combination, elevated FFA uptake and suppressed lipolysis are associated with OM adipocyte hypertrophy, abdominal obesity, and systemic insulin resistance

therapeutic androgen deprivation is currently unknown, it is possible that it is related to the development of sarcopenia (muscle loss) observed in T-deficient males (Fig. 4). Because skeletal muscle is responsible for the majority of glucose disposal and fatty acid β-oxidation, reduced muscle mass may result in insufficient substrate utilization and increased fat storage in WAT. Furthermore, androgen deprivation and muscle loss in males may also evoke secondary effects through reduced overall physical activity.

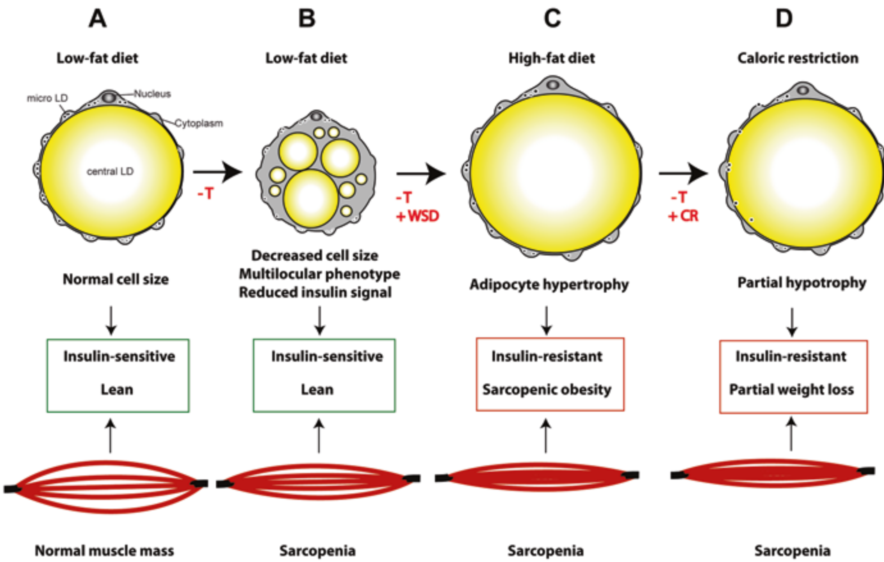

Fig. 4 The effect of T deficiency on male adipocytes and the skeletal muscle. (**a**) The cytoarchitecture of a unilocular adipocyte (described in Fig. 3a). (**b**) In control diet-fed animals, T deficiency induces a multilocular phenotype, the appearance of a population of smaller adipocytes, impairs insulin signaling in WAT, and triggers rapid muscle loss (sarcopenia). (**c**) WSD stimulates visceral (OM) and SC-WAT hypertrophy, resulting in sarcopenic obesity and insulin resistance. (**d**) The reversal of WSD with caloric restriction causes partial normalization of adipocyte size and reduces fat mass but does not eliminate systemic insulin resistance and the progression of sarcopenia

NHP Females

Our research has indicated that both prenatal and postnatal androgen exposure in females can cause metabolic dysfunction. Early- to mid-, but not late, gestational exposure to androgens produces postnatal defects in insulin secretion and action, as well as increased body weight accumulation, in female offspring as early as infancy, well preceding pathological defects in adulthood leading to increased incidence of type 2 diabetes. Adipogenic constraint may amplify accelerated weight (and likely lipid) accumulation into lipotoxicity and its adult sequelae. Postnatal exposure to androgens before puberty is associated with increased insulin resistance, similar to the human condition of PCOS. Our studies indicate that peripubertal hyperandrogenemia inhibits lipolytic responsiveness (Figs. 2 and 3) and accelerates FFA uptake in female WAT (Fig. 3), while estrogen and/or progesterone can protect female WAT from androgen-induced lipid overload. These studies suggest that an increase in visceral WAT mass is associated with ectopic lipid deposition in visceral organs and skeletal muscle, being principally responsible for the development of peripheral insulin resistance. In contrast, SC-WAT can play a protective role against

systemic lipotoxicity and insulin resistance. Our studies show that hyperandrogenemia alters the functional properties of visceral adipocytes, which leads to visceral obesity and metabolic disturbances.

The various NHP studies described above support the notion that androgens play important roles in organ-specific (adipose) as well as systemic aspects of metabolism, including a requirement for androgens in male adipose function and metabolic control and the adverse effects of androgen deficiency, and the similar effects of androgen excess in females. The specific molecular mechanisms that result in sex-specific effects on metabolism of the same ligand acting through the same receptor remain to be elucidated. Also requiring more understanding are the interactions between androgens, developmental windows, and diet that are just beginning to be appreciated in conditions such as PCOS and that are at play in males as well.

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Prenatal Testosterone Programming of Insulin Resistance in the Female Sheep

Muraly Puttabyatappa and Vasantha Padmanabhan

Abstract Insulin resistance, a common feature of metabolic disorders such as obesity, nonalcoholic fatty liver disease, metabolic syndrome, and polycystic ovary syndrome, is a risk factor for development of diabetes. Because sex hormones orchestrate the establishment of sex-specific behavioral, reproductive, and metabolic differences, a role for them in the developmental origin of insulin resistance is also to be expected. Female sheep exposed to male levels of testosterone during fetal life serve as an excellent translational model for delineating programming of insulin resistance. This chapter summarizes the ontogeny of insulin resistance, the tissue-specific changes in insulin sensitivity, and the various factors that are involved in the programming and maintenance of the insulin resistance in adult female sheep that were developmentally exposed to fetal male levels of testosterone during the sexual-differentiation window.

Introduction

"Programming," a term coined by Lucas (1991), describes the process whereby a developmental insult, at a critical period in development, has lifelong consequences. Developmental programming is a primitive mechanism, as evident from the effect of temperature on sex determination in reptiles. The eggs of the American alligator when incubated at 30 °C develop into females and at 33 °C into males (Deeming and Ferguson 1989). In this animal, the fundamental sex is female, and it appears that conversion to a male developmental pathway requires a transcription factor that is under environmental control. These findings in an egg-laying species have major implications in the developmental origin of diseases in higher animals including the human. Strong epidemiological evidence indicates that the hormonal, nutritional, and metabolic environment to which the fetus is exposed during gestation permanently alters many aspects of fetal development and subsequent expression of

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physiology and behavior during adulthood (Barker 2004; Nijland et al. 2008; Hanson and Gluckman 2011; Padmanabhan et al. 2016).

Fundamentally, the concept of developmental programming is embedded in how males and females differentiate. In mammals, the default phenotype is female, and the internal genitalia (oviducts, uterus, and cervix) and external genitalia (clitoris, labia majora and minora) develop from the Mullerian system in the absence of gonadal hormones. On the other hand, the genetic male produces a local hormone/substance (Mullerian-inhibiting substance/anti-Mullerian hormone) to repress the formation of the female internal genitalia and a systemic hormone (testosterone [T]) to stimulate its own internal genitalia (epididymis, vas deferens, seminal vesicles) from the Wolffian duct system. This gonadal sexual differentiation also extends to the brain leading to masculinization of the brain. How and when this occurs is best typified by the studies of sexual differentiation of the surge mode of gonadotropin secretion. Whether androgens were present or not during a sensitive perinatal period was found to program the hypothalamus in such a way that the pattern of gonadotropin secretion of the offspring could be produced to facilitate the pattern of gamete release (male, continuous; female, discontinuous) (Jost 1983). This fascinating story in developmental biology has many practical implications in medicine and disease in the context of programming of reproductive, behavioral, stress, and metabolic phenotypes.

Because steroids orchestrate the dialogue between the uterine environment and the developing fetus, inappropriate exposure to sex steroids during critical windows of differentiation could reprogram the developmental trajectory of several organ systems leading to maladaptive changes during adulthood. This is exemplified by the fact that female rats are masculinized in utero by hormones emanating from male littermates sharing the same uterine horn (Meisel and Ward 1981; Vom Saal 2016). Such females are less attractive to males, reproductively compromised, and more aggressive. Similarly, exposure to excess androgens in utero leads to sterility and metabolic dysfunctions in monkeys and sheep (Abbott et al. 2013; Padmanabhan and Veiga-Lopez 2013). Experimental manipulation of the prenatal sex steroid environment provides a powerful experimental tool for understanding mechanisms that underlie prenatal programming of the metabolic axis. Relative to this, precocious large animal models such as sheep are excellent translational models to study sex differences in phenotypic outcomes and understand the role played by the sex hormones in developmental programming. Added to the similarity to human in fetal and adult body size, adiposity, physiology, and timing of organ differentiation, the benefits of sheep come from the large body of well-characterized physiologic data and unique investigative approaches that are already available. These relate to (i) detailed profiles of reproductive hormone secretion; (ii) information relative to behavioral sex differences; (iii) uniqueness of hypophyseal portal sampling approach that allows procurement of hypothalamic samples for assessing neurosecretory patterns (Clarke 1992); (iv) ability to cannulate and procure serial samples from fetuses for profiling fetal hormone secretion (Barry and Anthony 2008); (v) the extended developmental timeline (months) allowing targeting of discrete critical windows of differentiation; (vi) the short period of time (months) to puberty allowing integrative, sequential studies to be performed on same animals from timing of early develop-

mental insults to adulthood when pathology is manifested; (vii) ability to perform studies in natural setting keeping social interactions intact and avoiding artificial housing conditions; (viii) avoidance of litter effects due to the singleton or twin pregnancy; and more importantly (ix) the similarity in precocial timing of organ differentiation that parallels human (Padmanabhan and Veiga-Lopez 2014).

Using this translational model of human relevance, this chapter addresses the impact of exposure to male pattern fetal T exposure on development of insulin resistance in female sheep.

Insulin Resistance

Insulin, a major anabolic hormone secreted by the body, stimulates glucose uptake by the muscle and adipose tissue, glycogen and protein synthesis in the muscle and liver, and lipid synthesis and storage in the liver and adipose tissue. It also inhibits catabolic processes such as fatty-acid oxidation, glycogenolysis, and gluconeogenesis. Failure of metabolic tissues to respond to insulin stimulation and reduce buildup of blood glucose underlies development of insulin resistance. While pancreatic islet cells compensate initially by increasing insulin secretion, failure of this adaptive mechanism to overcome the inability of target tissues to respond to insulin culminates in insulin resistance and compensatory hyperinsulinemia.

From a disease perspective, insulin resistance is associated with several metabolic diseases such as type 2 diabetes, obesity, nonalcoholic fatty liver diseases (NAFLD), polycystic ovary syndrome (PCOS), and metabolic syndrome. The prevalence rate of type 2 diabetes mellitus (T2DM) in the United States, which accounts for 90–95% of all diabetic patients, is 9.1% with prediabetes tallying at 38.0% (Menke et al. 2015). The obesity prevalence rate in the United States is about 37.9% (Flegal et al. 2016). The prevalence estimate of NAFLD, which results from accumulation of fat in the liver, in the United States is ~18–24% (Sayiner et al. 2016). The prevalence of PCOS varies from 7% to 14% with up to 75% manifesting insulin resistance (Moran et al. 2015). The National Health and Nutrition Examination Survey (NHANES) estimate from 2003 to 2012 places the prevalence of metabolic syndrome at ~35% (Aguilar et al. 2015). These prevalence estimates of the major noncommunicable diseases stress the importance of understanding the origin of insulin resistance.

Sex Differences in Insulin Sensitivity and Role of Sex Hormones

It is widely accepted that there are differences in metabolic variables between the sexes although the mechanisms underlying these differences are unclear. The sexual dimorphism in metabolic mediators appears to relate to differences in muscle mass,

adiposity, prenatal and adult hormonal milieu, and developmental differences in growth trajectory. While low lean body mass and high subcutaneous fat deposits characterize women, men have higher lean mass and higher visceral fat deposits (Rochlani et al. 2015). Although women are 41% more insulin sensitive compared to men (Nuutila et al. 1995), the higher circulating concentrations of free fatty acids and myocellular lipid content predispose them for insulin resistance (Mauvais-Jarvis 2015). The increased insulin sensitivity in women appears to relate to increased capacity of skeletal muscles for glucose uptake (Nuutila et al. 1995). The loss of this increased sensitivity as women age is postulated to be a function of loss of estrogen's protective action following onset of menopause (Pradhan 2014).

A wealth of information is available to document that sex hormones at physiological concentrations positively influence tissue insulin sensitivity. Estrogens promote insulin sensitivity in the liver and muscle and pancreatic beta cell insulin secretion thus facilitating maintenance of glucose homeostasis (Ropero et al. 2008). Studies with estrogen receptor alpha knockout mice and aromatase gene mutations in humans have established the specificity of estrogen's role in regulating insulin sensitivity (Ohlsson et al. 2000; Morishima et al. 1995). Similarly, T enhances insulin action and glucose uptake in target tissues thus helping maintain insulin sensitivity (Rao et al. 2013). Low levels of estradiol in females and T in males or high levels of T as in women with PCOS or estrogen and progesterone during pregnancy have been shown to increase the risk for development of insulin resistance (Rao et al. 2013; Boonyaratanakornkit and Pateetin 2015; Livingstone and Collison 2002). Likewise, expression of sex hormone-binding globulins that bind sex steroids and regulate their availability can also influence insulin sensitivity (Wallace et al. 2013). In addition to the role played by sex steroids in maintaining insulin homeostasis, it is becoming increasingly apparent that sex hormones play a key role in the developmental ontogeny of insulin resistance.

Prenatal T-Treated Sheep, a Model for Studying Developmental Programming of Metabolic Defects

Days 30–90 of fetal development in sheep encompasses the period of sexual differentiation and susceptibility window relative to the organization of reproductive and metabolic organs (Padmanabhan and Veiga-Lopez 2014). The timing of this developmental window in the sheep parallels a similar developmental timeline in the humans (Padmanabhan and Veiga-Lopez 2013). Generation of female sheep that manifest metabolic features of PCOS including insulin resistance involves treatment of pregnant sheep with twice-weekly intramuscular injections of T propionate at levels seen in intact males from days 30–90 of gestation. This mode of treatment produces T levels in the female fetuses that are comparable to that seen in control male fetuses and encompasses the sexual differentiation window (Veiga-Lopez et al. 2011). Treatment paradigms involving T-treatment from gestational days

60–90 or 62–102 also produce comparable metabolic phenotypes as the 30–90 treatment window suggesting that the critical period for metabolic programming resides between days 60 and 90 of gestation (Padmanabhan and Veiga-Lopez 2013; Ramaswamy et al. 2016).

Phenotype

The phenotypic characteristics of sheep prenatally exposed to male patterns of T mimic reproductive and metabolic features of women with PCOS. On the reproductive side, these include oligo-anovulation, increased sensitivity to GnRH, LH excess, polyfollicular ovarian morphology – the result of increased recruitment and follicular persistence – and functional hyperandrogenism (Padmanabhan and Veiga-Lopez 2013). On the cardiometabolic side, the defects include insulin resistance and hypertension (Cardoso et al. 2015). The oligo-/anovulatory, functional hyperandrogenism, and multifollicular/polycystic features evidenced in prenatal T-treated sheep meet the varying criteria put forth for PCOS by NIH, Rotterdam, as well as AE-PCOS society. The classic NIH criteria require chronic anovulation and clinical and/or biochemical signs of hyperandrogenism (Zawadzki and Dunaif 1992). The Rotterdam criteria require meeting two of the three diagnostic features, namely, oligo- or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries (Rotterdam 2004). The AE-PCOS society criteria define PCOS as condition with presence of clinical/biochemical signs of hyperandrogenism and oligomenorrhea or polycystic ovaries (Azziz et al. 2006).

Since diagnosis of PCOS occurs after puberty, no information on fetal and neonatal phenotypes is available in human although there is some evidence that offspring with low birth weight develop PCOS phenotype (Melo et al. 2010). Studies in Spanish cohort support increased incidence of low birth weight babies and early perturbations in female offspring of PCOS women (Sir-Petermann et al. 2005, 2012). Prenatal T-treated sheep, a resource to address developmental origins of metabolic phenotype in PCOS, manifest intrauterine growth restriction (IUGR), low birth weight, and postnatal catch-up growth (Manikkam et al. 2004; Steckler et al. 2005), features also linked to adult metabolic perturbations including insulin resistance (Morrison et al. 2010). Importantly, these females manifest hyperinsulinemia early in life (Cardoso et al. 2016). Mechanistic studies during early life found the manifestation of IUGR and low birth weight to be associated with reduced bioavailability of insulin-like growth factors (IGF) during the fetal life but increased IGF bioavailability during the period of subsequent catch-up growth (Crespi et al. 2006; Manikkam et al. 2004).

Peripheral Insulin Resistance

Assessment of presence or absence of insulin resistance in animal models and human disease states has been mostly obtained from cross-sectional single time point studies. Longitudinal studies are required to determine developmental ontogeny of perturbations so early interventions can be developed to prevent progression and severity of diseases. Longitudinal studies carried out only in female sheep exposed to male pattern T levels during their fetal life manifest age-specific changes in peripheral insulin sensitivity (Cardoso et al. 2016). This is reflected as reduced insulin sensitivity during infantile (Recabarren et al. 2005; Cardoso et al. 2016) and early juvenile life (Padmanabhan et al. 2010), improvement in insulin sensitivity during postpubertal period (Veiga-Lopez et al. 2013; Cardoso et al. 2016) (possibly a compensatory process to overcome pathology), and reemergence of insulin resistance during adulthood (Padmanabhan et al. 2010), indicating lack of sustainability of such compensatory mechanisms (Fig. 1). These findings emphasize the need for longitudinal assessment of insulin resistance and careful interpretation of differing outcomes in cross-sectional studies in the context of when during the life span such studies are being carried out.

Relative to the role sex hormones play in the developmental programming of insulin resistance, the findings that insulin sensitivity indices of prenatal T-treated female sheep are comparable to that of control male sheep (Recabarren et al. 2005) emphasize the role male pattern exposure to T during fetal life may play in establishing gender-specific differences in insulin sensitivity.

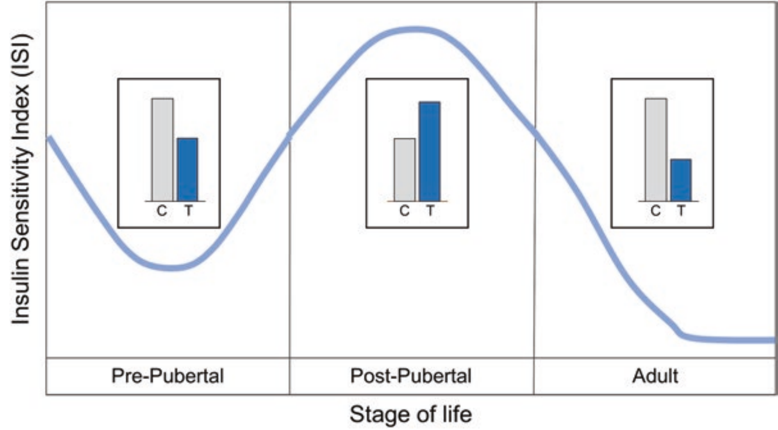

Fig. 1 Schematic showing the developmental progression of changes in insulin sensitivity index (ISI) in female sheep prenatally exposed to fetal male levels of T

Tissue-Specific Changes in Insulin Resistance

Insulin elicits its function in metabolic tissues by inducing a series of signaling cascades starting with insulin binding to its receptor (IR). A major target protein activated through phosphorylation is Akt/protein kinase B, while mitogen-activated protein kinase extracellular signal-regulated kinase (ERK) and mammalian target of rapamycin (mTOR) could also be similarly activated (Saltiel and Kahn 2001). Phosphorylation of Akt promotes translocation of the glucose transporter (GLUT) 4 from intracellular vesicles to the cell membrane and inactivates glycogen synthase kinase (GSK), which permits the activation of glycogen synthase. These changes promote glucose uptake in the muscle and fat cells and glycogenesis in the liver. Impairment at any level of the insulin signaling pathway could result in hyperglycemia (Rask-Madsen and Kahn 2012). Target-specific changes in the insulin signaling pathway (Table 1) induced in female sheep by male pattern exposure to T during fetal life are discussed below.

Liver

The liver contributes to the maintenance of blood glucose homeostasis through glycogenesis and gluconeogenesis. Insulin activates AKT and mTOR members of its signaling cascade, promotes glycogenesis, and reduces gluconeogenesis in the liver mainly through inactivation of GSK that permits the activation of glycogen synthase and reduction of gluconeogenic enzyme expression through phosphorylation of the transcription factor, Forkhead box protein O1 (FOXO1) (Soumaya 2012). Early effects of prenatal T excess evident in 90-day fetuses include higher mTOR protein and phosphorylation states of ribosomal protein S6 kinase beta-1 (p70S6K) and GSK3β thus favoring increased glycogen synthesis and promoting insulin sensitive state (Lu et al. 2016). Intervention studies utilizing androgen antagonist and insulin sensitizer co-treatments indicate involvement of androgenic but not insulin-dependent pathway in the gestational T-induced activation of mTOR (Lu et al. 2016). In contrast to the insulin-sensitive status of 90-day fetuses, during adulthood, prenatal T-treatment induces transcriptional changes that are consistent with the liver being insulin resistant (Nada et al. 2010). These transcriptional changes included downregulation of insulin receptor (IR) 2, a predominant form in the liver, insulin receptor substrate 2, Akt, and mTOR. While such changes were not evident at the protein level of Akt, ERK, and mTOR under basal state, Akt phosphorylation was blocked under conditions of insulin stimulation (Lu et al. 2016). Transcriptional changes consistent with insulin-resistant state were also observed in gestational day 62–102 T-treated female sheep with increased expression of mitogen-activated protein kinase kinase 4 (MAP2K4) that increases stress and pro-inflammatory gene expression and UDP-glucose ceramide glucosyltransferase (UGCG) that catalyzes the initial glycosylation during the synthesis of glycosphingolipids and found to be elevated in fatty livers (Hogg et al. 2011). Intervention studies involving treatment

Table 1 Tissue-specific changes in the members of insulin signaling pathway induced in female sheep by prenatal exposure to fetal male levels of T

	Adipose tissue		Liver		Muscle	
Insulin signaling member	Transcriptional changes (Adult)[a]	Protein changes (Fetal/Adult)[b]	Transcriptional changes (Adult)[a]	Protein changes (Fetal/Adult)[b]	Transcriptional changes (Adult)[a]	Protein changes (Fetal/Adult)[b]
Insulin receptor (IR)	No change	Not examined	↑IR2	Not examined	↑IR1/IR2 ratio	Not examined
Insulin receptor substrate (IRS)	↑IRS2	Not examined	↓IRS2	Not examined	↑IRS1	Not examined
Protein kinase B/AKT	↑	*Fetal* No change *Adult* Total protein: no change Phosphoprotein: *Basal* = No change *Insulin stimulated*: ↑	↓	*Fetal* No change *Adult* Total protein: no change Phosphoprotein: *Basal* = No change *Insulin stimulated*: ↓	No change	*Fetal* No change *Adult* Total protein: no change Phosphoprotein: *Basal* = No change *Insulin stimulated*: ↓
Mitogen-activated protein kinase – ERK	No change	*Fetal* No change *Adult* Total protein: no change Phosphoprotein: *Basal* = No change *Insulin stimulated*: not examined	No change	*Fetal* No change *Adult* Total protein: no change Phosphoprotein: *Basal* = No change *Insulin stimulated*: not examined	↓ERK1	*Fetal* No change *Adult* Total protein: no change Phosphoprotein: *Basal* = No change *Insulin stimulated*: not examined
Mammalian target of rapamycin complex (mTORC)	↓mTOR	*Fetal* No change *Adult* Not examined	↑Rictor	*Fetal* ↑mTOR *Adult* Not examined	↑Rictor	*Fetal* No change *Adult* Not examined

Insulin signaling member	Adipose tissue		Liver		Muscle	
	Transcriptional changes (Adult)[a]	Protein changes (Fetal/Adult)[b]	Transcriptional changes (Adult)[a]	Protein changes (Fetal/Adult)[b]	Transcriptional changes (Adult)[a]	Protein changes (Fetal/Adult)[b]
Ribosomal protein S6 kinase beta-1 (p70S6K)	Not examined	*Fetal* No change *Adult* Not examined	Not examined	*Fetal* ↑	Not examined	*Fetal* No change *Adult* Not examined
Glycogen synthase kinase (GSK)	No change	*Fetal* No change *Adult* No change	No change	*Fetal* ↑GSK3β phosphorylation *Adult* No change	↑GSK 3α and 3β	*Fetal* ↓GSK3β phosphorylation *Adult* No change
Glucose-6-phosphate	No change	Not examined	↑	Not examined	No change	Not examined
Phosphoenolpyruvate carboxykinase (PEPCK)	No change	Not examined	No change	Not examined	No change	Not examined

↑ = increase; ↓ = decrease
[a]From Nada et al. (2010)
[b]From Lu et al. (2016)

with androgen antagonist and insulin sensitizer revealed neither androgenic nor metabolic pathways are involved in the maintenance of this defect, suggestive of a role for estrogen in this programming. Together, the findings during fetal and adult life indicate that the hepatic insulin-resistant state involves impairment at both transcriptional and functional levels that are likely programmed during fetal life via androgenic pathway.

Muscle

The muscle is the major organ of postprandial blood glucose disposal through glycogen synthesis. Insulin promotes this by stimulating glucose uptake by increasing the phosphorylation of Akt that causes translocation of GLUT to the cell membrane (Dimitriadis et al. 2011). Gestational exposure of female fetuses to male pattern T exposure decreased GLUT4 protein and phosphorylation of GSK3β in the fetal muscle indicative of a reduced insulin-sensitive state in the fetal muscle (Lu et al. 2016). Such changes appear to involve both androgenic and metabolic pathways. In adult females, prenatal T-treatment increased IR1, insulin receptor substrate (IRS) 1, mTOR complex subunit rictor, and GSK-3α and GSK-3β mRNAs (Nada et al. 2010). These changes correlate with insulin-resistant states as IR1 is internalized at a higher rate reducing effectiveness of insulin signaling and increasing GSK expression, which is inhibitory for glycogen synthesis (Savkur et al. 2001; Nikoulina et al. 2000). This insulin-resistant state was also evident at the functional level, as insulin stimulation failed to increase phosphorylation of Akt in prenatal T-treated animals (Lu et al. 2016). Thus similar to the liver, insulin-resistant state of the muscle is conferred by defects at both transcriptional and functional levels in the insulin-signaling pathway.

Adipose Tissue

Glucose uptake in the adipose tissue is mainly dependent on insulin and is regulated in similar fashion as the skeletal muscle (Dimitriadis et al. 2011). Since adipose tissue glucose uptake accounts for only 5–10% of whole body glucose uptake, it does not appear to have a role in regulation of postprandial glucose homeostasis. However, adipose tissue has a major role in conferring insulin resistance as evident from several animal models and disease states (Kahn and Flier 2000). During the fetal stage, prenatal T-treatment did not alter the phosphorylation states of Akt, mTOR, ERK, GSK3β, and p70S6K in the visceral adipose tissue (VAT) indicative of lack of effect on insulin sensitivity at this time point (Lu et al. 2016). In the adult female, however, male pattern fetal exposure to T increased mRNA expression of IR2, mTOR, Akt, phosphatidylinositol-3-kinases (PI3K), and peroxisome proliferator-activated receptor gamma (PPARG) in the VAT (not studied in subcutaneous adipose tissue [SAT]), a gene expression profile consistent with promotion of insulin signaling (Nada et al. 2010). The absence of effect of prenatal T-excess on insulin-stimulated

increase in phospho-Akt in the VAT and SAT is also consistent with the insulin-sensitive status of adipose tissue of the prenatal T-treated animals (Lu et al. 2016). Overall the transcriptional and protein expression profile in the VAT (and SAT) of prenatal T-treated animals indicates they do not manifest insulin resistance both during fetal life and adult life in stark contrast to the insulin-resistant phenotypes of the muscle and liver.

Cardiac Tissue

Cardiac tissue can also utilize glucose during fetal life and periods of stress although free fatty acids are its primary fuel (Abel 2004). Insulin influences glucose uptake and storage in cardiac tissue in a manner similar to that occurring in skeletal muscle. While data is not available in the fetal ages, cardiac tissue from adult prenatal T-treated sheep manifests elevated expression of mTORC1 and increased phosphorylation of PI3K, Akt, and mTOR (Vyas et al. 2016). These changes carried out under basal states indicate cardiac tissue is not insulin resistant in the prenatal T-treated female sheep. Whether the transcriptional responses to insulin stimulation differ from basal state is not known. Similarly, to what extent the increased expression of these signaling molecules leads to a maladaptive state reflective of the cardiac hypertrophic and hypertensive phenotype (Vyas et al. 2016) and development of hypertension (King et al. 2007) remains to be determined.

Pancreatic Islet Cells

Beta cells in the pancreatic islet of Langerhans are the site of insulin secretion in response to increase in blood glucose. Insulin-resistant state is associated with compensatory increase in insulin secretion and, if the insulin sensitivity is not restored, leads to development of hyperinsulinemia. While the hyperinsulinemia of prenatal T-treated sheep can reflect compensation to overcome the insulin-resistant state, a primary pancreatic defect could also be a contributory factor. To this end, increase in pancreatic β cell number has been observed in female fetuses exposed to T from days 62 to 102 of gestation (Rae et al. 2013). In vitro studies have found the pancreas from these animals have elevated basal but not glucose-stimulated insulin secretion indicative of functional defects. Direct fetal administration of T at gestational days 62 and 82 also resulted in increased β cell numbers at both fetal and adolescent ages and elevated basal insulin secretion (Ramaswamy et al. 2016). The presence of this phenotype only in animals that received gestational treatment with T and not diethylstilbestrol or dexamethasone suggests that these changes are likely mediated through the androgenic pathway (Ramaswamy et al. 2016). Therefore, findings thus far – while limited – indicate the pancreas is also subject to developmental reprogramming by male pattern T exposure during fetal life.

Mechanisms Underlying Tissue-Specific Changes in Insulin Resistance

Tissue insulin sensitivity can be influenced by various factors such as those that promote hormonal action (positive mediators) to those that inhibit or reduce the efficiency of the hormones (negative mediators). The positive mediators that promote insulin signaling are adiponectin and antioxidants, which act by enhancing hormone action and/or reducing the inhibitory factors. The negative mediators of insulin action include inflammation, oxidative stress, and free fatty acids, and these act by reducing the expression of the members of the insulin signaling pathway, preventing receptor binding, deactivating proteins through phosphorylation or dephosphorylation, or damaging the cellular and molecular structures involved. Thus, the net insulin sensitivity of the tissue is governed by the predominance of either the positive or negative mediators that influence insulin action. Tissue-specific changes in the mediators of insulin sensitivity and their role in the development of insulin-resistant state in female sheep prenatally exposed to male pattern T exposure are discussed below.

Adiponectin

Adiponectin is an adipokine mainly secreted by the adipose tissue but also by other cell types such as skeletal and cardiac muscle and endothelial cells (Caselli 2014). It exerts its effects mainly through two forms of adiponectin receptors ADIPOR1 and ADIPOR2 (Kadowaki and Yamauchi 2005). Adiponectin activates various downstream signaling molecules of which the main one is AMP-activated protein kinase (AMPK) (Fu 2014). Adiponectin exists in the circulation in varying molecular weight forms that are produced by multimerization. Of these the high molecular weight (HMW) form of the adiponectin has the most insulin-sensitizing effect in metabolic tissues (Achari and Jain 2017). Adiponectin promotes antidiabetic, anti-inflammatory, and anti-atherogenic actions, thus functioning as an insulin sensitizer (Achari and Jain 2017; Caselli 2014). In the muscle, adiponectin facilitates insulin sensitivity by enhancing translocation of GLUT4, promoting glucose uptake and lipid oxidation, and reducing myocellular lipid accumulation (Liu and Sweeney 2014). In the liver, adiponectin lowers blood glucose by primarily suppressing gluconeogenesis and glycogenolysis (Ruan and Dong 2016). In adipose tissues, adiponectin ameliorates inflammation by decreasing pro-inflammatory cytokines and preventing macrophage infiltration (Achari and Jain 2017; Caselli 2014). The multiple metabolic site-specific actions of adiponectin help promote insulin action and maintain insulin sensitivity in the metabolic tissues. Consistent with this, serum concentrations of adiponectin (especially its HMW form) have been found to be lower in disease states such as obesity and PCOS that are associated with insulin resistance (Caselli 2014; Villa and Pratley 2011).

Paradoxically, fetal exposure to male pattern T-induced changes in circulating adiponectin levels and tissue AMPK levels in adult female sheep that are inconsistent with their insulin-sensitizing role (Puttabyatappa et al. 2017). This was reflected as lack of changes in adiponectin mRNA expression in adipose tissue and muscle, increase in circulating concentrations of HMW adiponectin, and increases in phospho-AMPK levels in muscle and liver (Puttabyatappa et al. 2017). These changes in HMW adiponectin and phospho-AMPK appear to involve both androgenic and metabolic reprogramming. This paradoxical finding in the face of insulin resistance might reflect a compensatory response to overcome the insulin-resistant state or alternatively a function of the time point being studied relative to the progression of insulin resistance.

Oxidative Stress

Reactive species (RS) such as reactive oxygen and nitrogen species (ROS and RNS) are byproducts of aerobic metabolism. Buildup of reactive species in cells causes damage to DNA, RNA, and proteins and may cause cell death. Antioxidants maintain RS homeostasis by neutralizing reactive oxygen and nitrogen species thus protecting from cellular damage. Various factors including hormonal or growth factors, pro-inflammatory cytokines, immune cell infiltration, radiation, lipids, endoplasmic reticulum stress, xenobiotics, and environmental endocrine-disrupting chemicals influence the formation of RS (Bashan et al. 2009). RS have been implicated in regulating insulin action with low concentrations of RS promoting insulin signaling through reversible protein modifications, activation of mitogen-activated protein kinase (MAPK), or modulation of expression of genes such as adiponectin and GLUTs (Bashan et al. 2009). In contrast, under conditions that lead to oxidative stress, RS negatively affects tissue insulin sensitivity through irreversible modification of proteins, activation of stress-related signaling, and DNA and lipid oxidation (Bashan et al. 2009). Oxidative stress is implicated in obesity-associated insulin resistance (Rani et al. 2016; Verdile et al. 2015). Elevated markers of oxidative stress have also been reported in other metabolic conditions such as nonalcoholic steatohepatitis and PCOS (Macut et al. 2013; Liu et al. 2016).

Consistent with their expected role, an oxidative stress phenotype was observed in female sheep exposed during fetal life to levels of T found in control male fetuses (Puttabyatappa et al. 2017). This was manifested as elevated circulating concentrations of nitrotyrosine, an outcome consistent with the peripheral insulin-resistant status of the female sheep. Similarly, an elevation in nitrotyrosine was also evident in the liver in line with their insulin-resistant state. Paradoxically, adipose tissue, which was insulin sensitive, also had elevated markers of oxidative stress. However, the parallel increase in antioxidant expression in the adipose tissue suggests that the negative effects of oxidative stress are counterbalanced by the antioxidants. While these changes appear to be programmed during the fetal life independent of androgenic or metabolic pathways, both pathways are implicated in the maintenance of the adult phenotype as postnatal treatment with androgen antagonist or insulin sen-

sitizer overcomes the prenatal T-treatment induced increases in liver and adipose tissue nitrotyrosine content (Puttabyatappa et al. 2017). As opposed to findings in the liver where antioxidant mechanisms appear not to overcome the negative effects of nitrotyrosine, in the adipose tissue, the increase in antioxidants likely helps protect from oxidative stress, thus enabling the maintenance of the insulin-sensitive state.

Inflammation

Inflammation in tissues is characterized by increased expression of pro-inflammatory cytokines and infiltration of immune cells which sets in motion a vicious cycle of production of more pro-inflammatory cytokines and infiltration of immune cells (Romeo et al. 2012). Insulin resistance in various animal models and human disease states such as obesity is associated with adipose tissue inflammation so much so that inflammatory state of adipose tissue state is now thought to influence the insulin sensitivity of the whole body (Esser et al. 2014). The pro-inflammatory cytokines negatively affect insulin signaling by activating stress-activated protein kinases and deactivating members of the insulin signaling, while infiltration of immune cells especially macrophages promotes lipolysis and increases circulating lipid concentrations (Romeo et al. 2012).

Not consistent with the insulin-sensitive phenotype of the adipose tissue and insulin-resistant state of the muscle and liver, prenatal T-treated animals had higher mRNA expression of pro-inflammatory cytokines (interleukin 1β, interleukin 6, tumor necrosis factor alpha, and chemokine CC ligand 2) and macrophage marker (CD68) in the VAT but not the SAT, liver, or muscle (Puttabyatappa et al. 2017). Changes in protein levels of these markers are not known. These changes at the mRNA level appear to be mediated both during the period of programming (fetal life) and maintenance (adult life) via androgenic and metabolic pathways. Whether the pro-inflammatory phenotype of VAT is counterbalanced by activation of anti-inflammatory markers and the extent to which the increase in pro-inflammatory markers in VAT contributes to peripheral, liver, and muscle insulin resistance is unknown.

Lipotoxicity

Lipids such as free fatty acids, sterols, fatty acid esters, and phospholipids are required for maintaining cellular homeostasis, cell signaling, immune function, and energy metabolism. However, improper deposition of lipids at ectopic sites such as the liver and skeletal and cardiac muscle can lead to lipotoxicity and compromise cell and tissue function (Ertunc and Hotamisligil 2016). Excess accumulation of lipids has been shown to activate protein kinase C and ceramide, which in turn causes inflammation and RS generation, aspects that can negatively affect insulin signaling (Ertunc and Hotamisligil 2016). Lipid accumulation in the liver leads to

steatohepatitis, a form of nonalcoholic fatty liver disease, which can culminate in hepatic fibrosis, cirrhosis, and loss of hepatic function (Liu et al. 2016). Increased circulating lipids and ectopic lipid accumulation in the liver and skeletal muscle is evident in conditions that manifest insulin-resistant states such as animals fed high-fat diet or obesity in human (Liu et al. 2016; Brons and Grunnet 2017). Dyslipidemia and increased incidences of nonalcoholic steatohepatitis have also been reported in women with PCOS (Macut et al. 2013; Jones et al. 2012), the attributes of whom the prenatal T-treated sheep manifest.

Relative to the model being discussed and consistent with their insulin-resistant state, elevated plasma lipid concentrations were present in female sheep that were exposed to fetal male levels of T (Veiga-Lopez et al. 2013; Puttabyatappa et al. 2017). Androgenic or metabolic pathways appear not to be involved in either organizing or maintaining the dyslipidemic state (Puttabyatappa et al. 2017). The increase in oil red O staining and triglyceride content in the liver and elevated triglyceride content in muscle (Puttabyatappa et al. 2017) are also consistent with their insulin-resistant status. Both androgenic and metabolic pathways appear to be involved in the programming and maintenance of the tissue phenotype. Lipotoxicity associated with elevated lipids in the liver and muscle may therefore underlie the pathogenesis of peripheral, liver, and muscle insulin resistance in these female sheep exposed prenatally to fetal male levels of T.

Integrating Changes in Negative and Positive Mediators of Insulin Sensitivity

Considering the changes in various positive and negative mediators of insulin sensitivity, it appears that the homeostatic mechanisms involved in maintenance of insulin sensitivity are disrupted in the liver and muscle from prenatal T-treated sheep with the balance shifted toward increase in negative mediators of insulin sensitivity thus promoting insulin-resistant state (Fig. 2). This regulation appears to be also tissue specific with both oxidative stress and lipotoxicity contributing to the development of insulin resistance in the liver, while only lipotoxicity underlies muscle insulin resistance. In contrast, the insulin-sensitive status of the adipose tissue appears to be maintained by the balance of oxidants and antioxidants (Fig. 2). As insulin induces lipogenesis in adipose tissue, the hyperinsulinemic status of the prenatal T-treated females (Padmanabhan et al. 2010) may enhance insulin signaling in adipocytes leading to excess lipogenesis. Because these females have a reduced adipose tissue lipid storage capability stemming from reduced adipocyte size in the absence of changes in adiposity (Veiga-Lopez et al. 2013; Cardoso et al. 2016), the excess lipid produced likely leads to ectopic lipid buildup in the circulation, liver, muscle, and potentially other organs thereby negatively affecting insulin signaling and contributing to the insulin-resistant state of these animals.

Tissue	Positive Mediators of Insulin Sensitivity	Negative Mediators of Insulin Sensitivity	State of Insulin Sensitivity
Adipose Tissue	Antioxidants	Inflammation Oxidative Stress	*Sensitive*
Liver	AMPK Activation	Lipid Accumulation Oxidative Stress	*Resistant*
Muscle	AMPK Activation	Lipid Accumulation	*Resistant*
Systemic	High Molecular Weight Adiponectin	Dyslipidemia Oxidative Stress	*Resistant*

Fig. 2 Schematic showing changes in the positive and negative mediators of insulin sensitivity in the metabolic tissues and peripheral circulation relative to the insulin-sensitive phenotype of adult female sheep prenatally exposed to fetal male levels of T

Conclusions

Although female fetuses are protected from T by the placental aromatization, about 40% of human female fetuses are exposed to T at levels seen in male fetuses during midpregnancy (Beck-Peccoz et al. 1991). Because this period encompasses the sex differentiation window, where the male pattern exposure to T establishes sex-specific genital, behavioral, reproductive, and metabolic outcomes, inappropriate exposure of female fetuses to male levels of testosterone during development places them at risk of reprogramming that culminates in adult pathologies. This concept as it relates to developmental origin of insulin resistance has been discussed in this chapter using a prenatal T-treated sheep model. Because loss of insulin sensitivity characterizes many metabolic disorders in the humans, the findings discussed in this chapter using this model are of translational significance especially considering that the developmental progression of organ differentiation in sheep parallels that of human and gets completed in utero. Since steroids including T can induce epigenetic changes in stem/progenitor cells (Bramble et al. 2016) leading to developmental reprogramming (Tang and Ho 2007), the epigenetic mechanisms contributing to the development of insulin resistance in female offspring exposed to male pattern T during fetal life and the potential for these reprogrammed changes to be passed on to subsequent generations either via multigenerational (repetitive establishment of same phenotype, which requires careful phenotyping at each generation – an aspect not incorporated in many studies) or epigenetic transgenerational inheritance

pattern are fruitful avenues to pursue in future research. The findings from this precocial model are also of relevance in addressing the impact of developmental exposure to environmental steroid mimics and assessing if T can serve as a biomarker during early pregnancy for identifying offspring at risk of developing metabolic pathologies.

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The Role of Androgen Excess in Metabolic Dysfunction in Women

Androgen Excess and Female Metabolic Dysfunction

Héctor F. Escobar-Morreale

Abstract Polycystic ovary syndrome (PCOS) is characterized by the association of androgen excess with chronic oligoovulation and/or polycystic ovarian morphology, yet metabolic disorders and classic and nonclassic cardiovascular risk factors cluster in these women from very early in life. This chapter focuses on the mechanisms underlying the association of PCOS with metabolic dysfunction, focusing on the role of androgen excess on the development of visceral adiposity and adipose tissue dysfunction.

Introduction

Polycystic ovary syndrome (PCOS), the most common endocrine disorder in premenopausal women (Asuncion et al. 2000; Sanchon et al. 2012), is characterized by the association of androgen excess with chronic oligoovulation and/or polycystic ovarian morphology, provided that other disorders such as hyperprolactinemia, nonclassic congenital hyperplasia, and androgen-secreting tumors have been excluded (Azziz et al. 2006, 2009).

Metabolic disorders and classic and nonclassic cardiovascular risk factors cluster in women with PCOS from very early in life (Wild et al. 2010). Therefore, metabolic prevention in women with PCOS should start as earlier as possible, usually meaning at diagnosis. The present chapter focuses on the mechanisms underlying the association of PCOS with metabolic dysfunction, focusing on the role of androgen excess on the development of visceral adiposity and adipose tissue dysfunction.

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Androgen Excess as the Primary Defect in PCOS

More than a decade ago, McAllister and collaborators (Wickenheisser et al. 2006) demonstrated that exaggerated androgen secretion by ovarian theca cells is a primary feature of PCOS. Theca cells from women with PCOS secreted increased amounts of androgens and their steroid precursors compared with cells obtained from women without androgen excess disorders even after several passes in primary culture. This was accompanied by increased expression of all the enzymes involved in androgen synthesis in these cells (Wickenheisser et al. 2006). Hence, these cells maintained an increased capacity for androgen synthesis when isolated from the endocrine and metabolic milieu characteristic of women with PCOS, strongly suggesting that androgen excess is a primary ovarian defect in this syndrome (Wickenheisser et al. 2006). Possibly such a predisposition toward increased androgen synthesis and secretion is also shared by the adrenal glands of many women with PCOS because adrenal hyperandrogenism is quite prevalent in this disorder (Luque-Ramirez and Escobar-Morreale 2016), yet a definite proof is still lacking since the adrenals are essential for life and adrenal tissue cannot be obtained safely from these patients.

Androgen Excess from an Evolutionary Perspective

Survival advantage contributes to explain the present epidemics of obesity, insulin resistance, diabetes, and cardiovascular disease (Fernandez-Real and Ricart 1999). Thrifty genotypes and phenotypes in which insulin resistance and weight gain favored survival may have been selected during ages of prolonged famine and environmental stress (Fernandez-Real and Ricart 1999). However, with the sudden improvement in environmental conditions that occurred in the past century, in which food is not restricted for most of the human population and severe trauma and infection are relatively rare, such thriftiness is no longer favorable leading to obesity, insulin resistance, and associated disorders (Fernandez-Real and Ricart 1999).

Because pregnancy and delivery were the major cause of death in women until Semmelweis introduced hand disinfection standards in obstetrical clinics in 1847 (Semmelweis 1861), androgen excess might have favored survival of affected women and their progeny in these earlier ages (Escobar-Morreale et al. 2005b): aside from favoring assertive behavior, androgen excess may associate on the one hand an earlier maturation of the reproductive axis extending the reproductive age of affected girls; on the other hand, the subfertility derived from oligoovulation would reduce the fertility rate, decreasing the certain possibility of death from parturition of affected women and improving the care of their progeny, thereby increasing the chances of both maternal and infant survival (Escobar-Morreale et al. 2005b).

Androgen Excess May Influence Metabolism from Early Human Life Stages in PCOS

Albeit the clinical onset of PCOS is usually peripubertal, the systemic consequences of androgen excess might start earlier in life, even during fetal life (Abbott et al. 2007). The fetal ovary expresses the steroidogenic enzymes needed to secrete androgens and estrogens from mid-gestation driven mostly by placental human chorionic gonadotropin (Polin et al. 2016). Hence, possibility exists that women predisposed to PCOS may suffer from increased androgen concentrations during fetal life. This hypothesis, however, is still in need of confirmation by the finding of increased testosterone concentrations in umbilical artery blood in girls born from PCOS mothers (Barry et al. 2011), from whom they may have inherited the predisposition toward androgen excess.

Nevertheless, several animal models indicate that fetal exposure to androgen excess predisposes the offspring not only toward the development of PCOS-like symptoms in females during adult life but also toward visceral adiposity, insulin resistance, and associated metabolic derangements both in female and male animals (Abbott et al. 2007), similar to what has been described in daughters and sons of women with PCOS (Sir-Petermann et al. 2009; Recabarren et al. 2008). The origin in humans of such prenatal exposure to androgen excess might be maternal, since women with PCOS maintain increased androgen levels throughout pregnancy (Sir-Petermann et al. 2002), but considering the efficiency of placental aromatase in converting maternal androgens into estrogens, inheritance by the female fetus of the same adrenal and/or ovarian mechanisms that led to androgen excess in their PCOS mothers is a more likely event (Abbott et al. 2002).

Androgen Excess Masculinizes Body Fat Distribution and Adipocyte Function

We have recently hypothesized that women with PCOS suffer from a vicious circle whereby androgen excess favoring the abdominal deposition of fat further facilitates androgen secretion by the ovaries and adrenals in PCOS patients (Fig. 1) (Escobar-Morreale and San Millan 2007). Visceral fat accumulation and adipose tissue dysfunction may lead to insulin resistance and compensatory hyperinsulinism, facilitating androgen secretion because insulin acts as a co-gonadotropin at the ovary (Sam and Dunaif 2003).

The possibility that androgens influence body fat distribution and visceral adipose tissue dysfunction is supported by recent studies. Women with PCOS present with increased thickness of intraperitoneal and mesenteric fat depots and, when considered together with non-hyperandrogenic women and with men, the thickness of visceral adipose tissue depots correlated positively with serum androgen concentrations and negatively with serum estradiol levels (Borruel et al. 2013). Of note, the

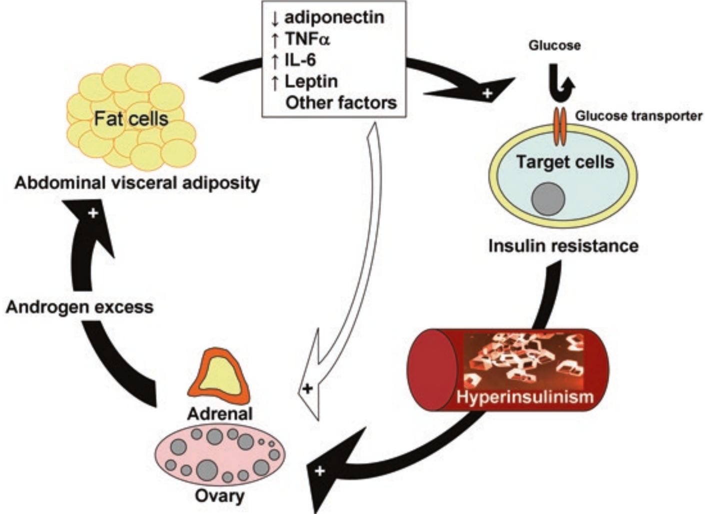

Fig. 1 Unifying hypothesis explaining the interplay between the polycystic ovary syndrome and abdominal adiposity as the result of a vicious circle represented by the black arrows: androgen excess favors the abdominal deposition of body fat, and visceral fat facilitates androgen excess of ovarian and/or adrenal origin by the direct effects (white arrow) of several autocrine, paracrine, and endocrine mediators or indirectly by the induction of insulin resistance and hyperinsulinism (Reproduced from Escobar-Morreale and San Millán (2007), with permission. Copyright Elsevier, 2007)

increase in visceral adiposity occurs even in lean women with PCOS (Borruel et al. 2013).

Intervention studies in humans also support a role of androgens in the development of visceral adiposity in women. Testosterone administration in female-to-male transsexuals increases visceral adipose tissue and decreases subcutaneous fat depots (Elbers et al. 1997, 2003). Also, the pure nonsteroidal antiandrogen flutamide decreases markedly visceral fat in women with PCOS after 12 months of treatment (Gambineri et al. 2006).

Moreover, both genomic and proteomic nontargeted studies of visceral adipose tissue indicated substantial differences in the gene expression profiles and in the proteomes of severely obese patients with PCOS compared with control women (Corton et al. 2007, 2008), in conceptual agreement with a putative role of androgens in the sexual dimorphism of adipose tissue distribution and function (Wells 2007; Insenser et al. 2012). Of note, the promoter regions of several of the genes dysregulated in visceral adipose tissue of women with PCOS contain putative androgen response elements, suggesting that androgen excess might influence adipose tissue dysfunction in these patients (Corton et al. 2007). To this regard, reduction of androgen excess by means of laparoscopic ovarian electrocautery in infertile patients with PCOS was followed by an improvement in insulin resistance, glucose tolerance, and adipokine secretion (Seow et al. 2007a, b), in conceptual

agreement with the role that androgens may play on the development of metabolic dysfunction in these women.

Obesity Plays a Major Impact on the Development of Metabolic Disorders in PCOS

The development of exogenous obesity aggravates the vicious circle of androgen excess, abdominal adiposity and adipose tissue dysfunction, insulin resistance, and further androgen excess in women with PCOS, increasing markedly the metabolic and cardiovascular risk of affected women. In fact, our recent metabolomic data indicate that insulin resistance is not universal in PCOS and that obesity is the major culprit of this association (Escobar-Morreale et al. 2012).

Even though patients with PCOS as a group were hyperinsulinemic and insulin resistant compared with the controls, nonobese patients with PCOS showed a metabolic profile consisting of suppression of lipolysis and increased glucose utilization in peripheral tissues, and PCOS patients as a whole showed decreased 2-ketoisocaproic and alanine concentrations, suggesting utilization of branched-chain amino acids for protein synthesis and not for gluconeogenesis (Escobar-Morreale et al. 2012). These metabolic processes required effective insulin signaling; hence, insulin resistance was not present in all tissues of these women, and different mechanisms such as a decrease in insulin clearance possibly contributed to their hyperinsulinemia (Ciampelli et al. 1997).

On the contrary, in obese women with PCOS, the increase in plasma long-chain fatty acids, such as linoleic and oleic acid, and glycerol suggests increased lipolysis, possibly secondary to impaired insulin action at adipose tissue (Escobar-Morreale et al. 2012). Therefore, obesity appears to be the major determinant of metabolic heterogeneity in PCOS.

Although abdominal adiposity, metabolic dysfunction, and markers of subclinical atherosclerosis may also be present in nonobese women with PCOS (Yildirim et al. 2003; Carmina et al. 2006; Luque-Ramirez et al. 2007c), obesity is clearly related to the development of metabolic disorders in PCOS (Gambineri and Pasquali 2006), explaining why not every patient is at increased risk of metabolic and cardiovascular disease (Wild et al. 2010). Accordingly, the frequency of dyslipidemia and disorders of glucose tolerance in women with PCOS increases markedly with obesity (Ehrmann et al. 1999; Legro et al. 1999, 2001). In Spaniards, abdominal adiposity is an intrinsic characteristic of PCOS, yet obesity increases markedly the amount of fat in the visceral adipose tissue depots of these women (Borruel et al. 2013), and, in fact, it is obesity the actual responsible of the association of PCOS with the metabolic syndrome (Alvarez-Blasco et al. 2006), hypertension (Luque-Ramirez et al. 2007b), hyperuricemia (Luque-Ramirez et al. 2008), and decreased health-related quality of life (Alvarez-Blasco et al. 2010).

Therefore, obesity is a major player in the association of PCOS with metabolic dysfunction, and its prevention and management must be a priority when designing strategies for the long-term management of PCOS (Salley et al. 2007; Wild et al. 2010).

PCOS as a Heterogeneous Disorder in Terms of Metabolic Dysfunction

The metabolic heterogeneity of PCOS may be explained by the existence of a continuum in the relative contribution of androgen excess, on the one hand, and of abdominal adiposity and insulin resistance, on the other (Escobar-Morreale and San Millan 2007). In one extreme of this spectrum, women presenting with severe androgen excess may develop PCOS without the participation of any other pathophysiological mechanism (Fig. 2). In the other extreme of the spectrum,

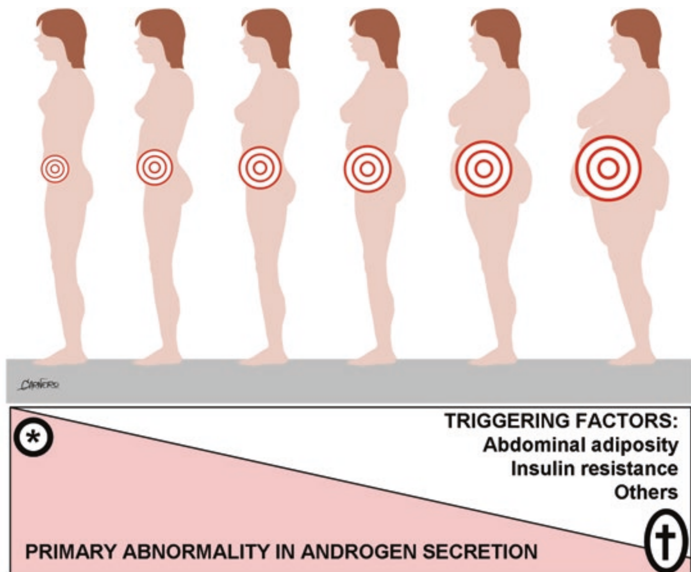

Fig. 2 The polycystic ovary syndrome as the result of the interaction of a primary abnormality in androgen synthesis, manifesting as androgen excess, with environmental factors such as abdominal adiposity, obesity, and insulin resistance. In one extreme (*), in some patients the disorder is severe enough to result in PCOS even in the absence of triggering environmental factors. In the other extreme (†), a very mild defect in androgen secretion is amplified by the coexistence of abdominal adiposity, obesity, and/or insulin resistance. Between the two extremes, there is a spectrum in the severity of the primary defect in androgen secretion, explaining the heterogeneity of PCOS patients with regard to the presence of obesity and metabolic comorbidities. Yet all patients share a primary defect in androgen secretion (Reproduced from Escobar-Morreale and San Millán (2007), with permission. Copyright Elsevier, 2007)

women with mild androgen excess only develop PCOS when another pathophysiological mechanism, such as abdominal adiposity, obesity, insulin resistance, and/or hyperinsulinemia, acts as a trigger (Fig. 2). The latter may explain the increasing prevalence of PCOS in Spaniards with increasing grades of obesity, from 6.5% in lean women (Asuncion et al. 2000) to 28% in overweight and obese females (Alvarez-Blasco et al. 2006) and up to 47% in women presenting with morbid obesity (Escobar-Morreale et al. 2005a). Obviously, the most severe phenotypes are observed in patients presenting with both severe androgen excess and severe obesity. But in order to develop PCOS, a primary defect in androgen secretion, from mild to severe, is needed. In the absence of such a defect, PCOS cannot develop, explaining how half of morbidly obese women do not suffer the syndrome even in the presence of massive abdominal adiposity and severe insulin resistance (Escobar-Morreale et al. 2005a, 2017). And because there is a continuum between both extremes of the spectrum, the relative contributions of androgen excess and of visceral adiposity and obesity to the clinical picture of the individual patient with PCOS must be established accurately in order to apply the most adequate prevention and therapeutic strategies for each particular case.

Clinical Management of Metabolic Dysfunction in PCOS and the Role of Amelioration of Androgen Excess

Current recommendations from the Androgen Excess and PCOS Society (AE-PCOS) provide guidance on how to screen women presenting with hyperandrogenic phenotypes of PCOS for metabolic dysfunction and suggest different approaches for their long-term management (Wild et al. 2010). Of note, non-hyperandrogenic PCOS phenotypes rarely associate metabolic dysfunction (Moghetti et al. 2013), and, therefore, the following recommendation do not apply to women presenting with ovulatory dysfunction and polycystic ovarian morphology in the absence of clinical and biochemical hyperandrogenism.

According to AE-PCOS guidelines, women with PCOS should be screened for metabolic dysfunction at diagnosis and every visit by measuring body mass index, waist circumference, and office blood pressure. Dyslipidemia should be screened every 2 years by obtaining a complete lipid profile, and glucose tolerance should be screened every 2 years by performing a standard 75 g oral glucose tolerance test in women with obesity and in nonobese women over 40 years old, personal history of gestational diabetes, or family history of type 2 diabetes (Wild et al. 2010).

The cornerstone for management of metabolic dysfunction in PCOS is lifestyle modification, especially diet-induced weight loss (Salley et al. 2007; Wild et al. 2010). There is evidence nowadays that lifestyle intervention improves body composition, hyperandrogenism, and insulin resistance in women with PCOS (Moran et al. 2011). On the contrary, there is not enough evidence of effect for lifestyle intervention on improving glucose tolerance or lipid profiles and no

literature assessing clinical reproductive outcomes, quality of life, and treatment satisfaction (Moran et al. 2011). However, the long-term efficacy of lifestyle intervention in patients with PCOS has still to be proven. In general, the magnitude of the weight loss usually attained after caloric restriction combined with increased physical activity is usually moderate, in the range of 5–10% of the initial body weight, and is frequently not maintained for long periods of time (Norman et al. 2002; Yanovski and Yanovski 2002).

Pharmacological treatment for metabolic dysfunction in patients with PCOS should be aggressive because such a dysfunction may start much earlier in these women compared with those in the general population (Wild et al. 2010). To this regard, current evidence supports the use of metformin for glucose intolerance and diabetes and lipid lowering drugs in dyslipidemic patients (Wild et al. 2010; Duleba 2012). Moreover, in this context surgical management of obesity appears as an alternative therapeutic approach for metabolic dysfunction in PCOS when lifestyle intervention and drug treatment fail. It must be highlighted that there is no enough evidence at present to universally recommend bariatric surgery for patients with PCOS and metabolic dysfunction, as the studies addressing this issue are scarce. But a recent meta-analysis indicates that PCOS is present in as many as 36% (95CI 22–50) of women submitted to bariatric surgery for severe obesity and that PCOS resolves in a striking 96% (95CI 89–100) of them following surgically induced weight loss (Escobar-Morreale et al. 2017). This makes of bariatric surgery the most effective mean, by far, of treating PCOS in obese women, because both clinical and biochemical androgen excess and ovulatory dysfunction improve in parallel to the improvement in insulin resistance and metabolic disorders (Escobar-Morreale et al. 2017).

A more controversial issue is that of the possible metabolic consequences of treating androgen excess in women with PCOS (Diamanti-Kandarakis et al. 2003). Considering that patients with this disorder present frequently with insulin resistance and its associated metabolic comorbidities as stated earlier, and because combined oral contraceptives may worsen insulin resistance and glucose tolerance in the general population, reputed authors in the field suggested these drugs should be replaced by metabolically safe and effective insulin sensitizer drugs (Diamanti-Kandarakis et al. 2003). However, a 2007 Cochrane Review of four randomized clinical trials comparing metformin with COC did not find a higher metabolic risks with the latter (Costello et al. 2007), a result confirmed by later trials (Luque-Ramirez et al. 2007a). Moreover, treatment of patients with PCOS with the nonsteroidal androgen receptor blocker flutamide may reduce visceral adiposity more markedly than metformin (Gambineri et al. 2004, 2006), strongly suggesting that androgen excess is actually a contributor to abdominal adiposity and metabolic dysfunction in these women and not only a consequence of insulin resistance and hyperinsulinemia (Escobar-Morreale and San Millan 2007).

Conclusions

We propose that PCOS and its associated metabolic comorbidities could be explained by the existence of a vicious circle whereby a chronic androgen excess of ovarian and/or adrenal origin starting early in life, or even prenatally, results in abdominal visceral adiposity in affected women. Abdominal adiposity favors further hyperandrogenism indirectly as a result of insulin resistance and hyperinsulinism or directly by the effects of several mediators secreted by adipose tissue. Accordingly, obesity aggravates all the manifestations, risks, and metabolic comorbidities of PCOS. Therefore, screening for metabolic dysfunction should be performed routinely in hyperandrogenic women with PCOS, and preventive strategies, focused on weight excess avoidance and/or management, should be started as soon as the disorder is diagnosed. Therapeutic approaches should include amelioration of androgen excess, weight loss, and early treatment of metabolic dysfunction with appropriate drugs.

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Part IV
Transgender Biology and Metabolism

Sex, Gender, and Transgender: Metabolic Impact of Cross Hormone Therapy

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Abstract Most preclinical and clinical, animal, and human research has been biased with respect to sex and even more so with respect to gender. In fact, little is known about the impact of sex and even less about the influence of gender on overall metabolic processes. The National Institutes of Health has recognized this gap in scientific knowledge and now mandates that studies be conducted in both sexes and to include gender as variables influencing physiological processes such as metabolism. It is therefore critical to understand and appreciate how to incorporate sex and gender in preclinical and clinical research in order to enhance our understanding of the mechanisms by which metabolic processes differ by sex and gender. In this chapter, we define sex and gender and discuss when sex and gender are not aligned, such as that which occurs in transgender individuals, and how this impacts metabolic processes. We discuss the importance of understanding the influence and

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interactions between sex hormones and sex chromosomes rather than focusing on their relative contributions to metabolism in isolation. This knowledge will optimize therapies specific for individuals which need to encompass sex and gender.

NIH Mandates Including Sex and Gender as Biological Variables in Metabolic Research

The National Institutes of Health (NIH) emphasized the inclusion of both sexes in biomedical research in 1993 with the NIH Revitalization Act (1993). This act mandated that all clinical trials funded by the NIH are needed to include women and minorities (unless the research question was specific for one sex, such as prostate cancer). By 2015 the percentage of women involved in clinical studies had increased; however, women were still underrepresented in investigations of major health issues, including cardiovascular disease and cancer (Mazure and Jones 2015). Moreover, despite the fact that more investigators include both sexes in their study designs, very few report or analyze data according to sex (Mazure and Jones 2015; Nieuwenhoven and Klinge 2010). Therefore, the NIH recently enhanced the stringency with which it mandated the inclusion of both sexes and the influence of gender in biomedical research (Clayton and Collins 2014).

Sex and Gender Influence Metabolism

With the NIH mandate to incorporate sex as a biological variable, it is important to define and use the terms "sex" and "gender" appropriately. *Sex* "refers to a set of *biological attributes* in humans and animals." Sex is primarily associated with physical and physiological features including sex chromosomes, gene expression, hormone levels, and reproductive/sexual anatomy. In contrast, *gender* refers to how people perceive themselves and others, as well as how they act and interact.

Gender is a continuum and refers to social behaviors, expectations, expressions, and identities of girls, women, boys, men, and gender-diverse people. Where there has been an increasing amount of knowledge gained as to the influence of sex on biological processes, little is known as to the influence of gender. Importantly, assessments of health and disease risk need to take both *sex* and *gender* into account (Phillips 2005). Below are considerations pertaining to how *sex* and *gender* should be applied to preclinical and clinical research.

The role of *sex* in scientific discovery, disease detection, diagnosis, and treatment is often underappreciated and, even more specifically as previously mentioned, is infrequently factored as a biological variable. This becomes important when one considers that nearly every cell has a sex – for males, this is represented by the sex chromosomes X,Y, and for females, this is represented by the sex chromosomes

X,X. Importantly, the sex of the cell influences its function, and this could be independent of sex hormones (Mauvais-Jarvis et al. 2017). For while many intrinsic properties of cells can appear hormone-independent, cells may also exhibit differential variations upon exposure to sex hormones. An example is that female and male cells respond differently to chemical and microbial stressors. What is striking is that where it is appreciated that male and female cells differ, basic science research using cell lines rarely factors sex into the biology studied. Importantly, the hormonal milieu for which the cells are studied contains estrogenic compounds and sex hormones, which are rarely taken into consideration and factored into the overall analysis of the results.

Sex Chromosome Impact on Metabolic Processes

As previously mentioned, sex chromosomes, independent of sex hormones, are also linked to sex differences in disease risk (Wijchers et al. 2010). The X and Y chromosomes evolved over the past 160 million years from a pair of autosomes. Although they were both initially about the same size, the Y chromosome has gradually lost its ability to exchange genetic information with the X chromosome and therefore began to evolve independently. Subsequently, the Y chromosome contains only 3% of the genes that it once shared with the X chromosome.

The Y chromosome is present exclusively in males and contains the sex-determining region (*Sry*), the primary determinant of testicular development, spermatogenesis, and masculinization. It was recently discovered that the genes conserved on the Y chromosome are expressed in cells and tissues throughout the body and are involved in decoding and interpreting the entirety of the genome (Bellott et al. 2014). Despite this, the Y chromosome has mostly been excluded from the larger genome-wide association studies (GWAS), due to the belief that the Y chromosome is a "genetic wasteland" (Marshall Graves 2002). This concept has been challenged recently as many Y chromosomal genes were found to be haploinsufficient regulatory genes (Bellott et al. 2014). Additionally, certain single-nucleotide polymorphisms (SNPs) on the Y chromosome (Charchar et al. 2012) are correlated with risk factors associated with CVD, and this is independent of sex hormones.

Females on the other hand, have two X chromosomes and therefore two copies of every X-linked gene. To compensate for the fact that men have only one X chromosome, one copy of the female X chromosome is randomly inactivated or turned off. Inactivation of the X chromosome allows for adjustments in gene expression between sexes, individuals, and tissues (Deng et al. 2014). When X-inactivation occurs, it utilizes cellular properties that are found only in females, making females more vulnerable than males to genetic or environmental perturbations during embryonic development (Chen et al. 2008). In fact, Wu et al. found that cells silence X chromosomes in different patterns providing a mechanism by which individual differences may be derived (Wu et al. 2014). Despite this, the X chromosome has

also been basically "ignored" in the analysis of GWAS data, with only 33% of the reported studies from 2010 to 2011 factoring in the X chromosome (Wise et al. 2013).

Divergence from the normal number of X and Y chromosomes, called sex chromosome aneuploidy (SCA), accounts for approximately half of all chromosomal anomalies in humans, with a total frequency of 1:400 (Passarge 1995). Aneuploidy occurs in at least 5% of all pregnancies, which is a relatively high frequency, and is the most commonly recognized chromosome abnormality in humans (Hassold and Hunt 2001). Examples include:

Turner syndrome: Also referred to as monosomy X (45X) which occurs only in girls and women when the X chromosome is totally or partially missing. Affected individuals experience abnormal growth patterns, heart defects, and certain learning disabilities, and they are short in stature, generally lack prominent female secondary sexual characteristics, and are sterile (The Mayo Clinic 2016b).

XXX females: Referred to as triple X syndrome, or trisomy X, females experience normal development of sexual traits and are fertile. Affected individuals are usually taller than average and have slender builds and no severe phenotype (U.S. National Library of Medicine 2016; The Mayo Clinic 2016a).

Klinefelter syndrome: Klinefelter syndrome (47XXY or XY/XXY mosaic) is the most pervasive sex chromosomal anomaly (Klinefelter et al. 1942), and males with Klinefelter syndrome carry two or more X chromosomes resulting in abnormal development of the testis, leading to hypogonadism and infertility (Bojesen et al. 2004).

XYY males: Men inheriting an additional Y chromosome have higher than average levels of T and are usually taller than average, and affected males are typically fertile, and many are unaware that they have a chromosomal abnormality.

Relatively little information is available on how sex chromosomes interact and/or impact the phenotypes induced by gonadal/sex hormones in humans. Because sex differences result from both endocrine/hormone and cell autonomous/sex chromosome effects, future investigations on the role of sexual dimorphisms and their impact on metabolic function will require incorporation of classical endocrinology with respect to hormone synthesis and action, modern molecular genetic methods to alter hormone action in a cell type-specific manner, and sophisticated methods to manipulate the copy number and expression of X and Y genes that underlie constitutive genetic differences in XX and XY cells (Wintermantel et al. 2006; Monks et al. 2007).

Consistently, sex differences often derive from interactions of the hormonal milieu with gene expression on sex chromosomes and autosomes. It is also important to note that there are data to suggest that cells retain their memory of the sex and environment of the donor from which they are derived. Therefore, it is pertinent to differentiate between biological functions determined by the action of sex chromosomes alone and the associations brought about by sex steroids interacting with hormone-responsive genes either at the time of sexual differentiation, at the time of puberty, or during adulthood.

Organizational Versus Activational Effects of Sex Hormones

To begin to understand the impact of sex on physiological processes such as metabolism, it is important to appreciate that the interaction between sex chromosomes and sex hormones is influenced by the *organizational* (long-lasting or permanent) vs *activational* (reversible) exposure of sex hormones, referring to the timing and duration of the effects. The o*rganizational* or *activational* concept of the biological actions of sex hormones emerged almost 60 years ago in a publication by Phoenix et al. (1959), who stated “developmental exposure to gonadal/sex steroids acts on the brain to organize the neural substrate, which is then selectively activated in the adult to induce expression of sex specific behavior.” The basic tenant, therefore, is that gonadal/sex hormones cause permanent sex changes during tissue differentiation and growth. This has contributed to both clarity and confusion on the role of sex steroids in biology, because the timing at which the hormone levels or activity at their receptors is explored becomes important. If investigators are manipulating hormone levels in adult animals or humans, the tissues have already been primed by the sex hormone during development. On the other hand, experimental manipulation of sex hormones in fetal or neonatal animals will provide differing results. The “timing” of hormonal manipulation in humans and animal models is critical with respect to data interpretation (Mauvais-Jarvis et al. 2017; Mauvais-Jarvis 2014), and this becomes even more critical when factoring in the influence of sex hormones in transgender individuals (discussed below) who are utilizing cross hormone therapy to obtain a desired gender.

The Activational Influence of Sex Hormones on Disease Risk: Estrogens

When sex is factored into disease risk such as cardiovascular disease (CVD), it is well established that premenopausal women are relatively protected from CVD when compared to body weight and age-matched men (Skafar et al. 1997; Yanes and Reckelhoff 2011; Ren and Kelley 2009; Collins et al. 2002). This “sex advantage” disappears after menopause, leading to the generally accepted conclusion that it is the activational influence of sex hormones, and in particular estrogens (E2), which provided the protection against the metabolic syndrome. There are data suggesting there is a “U”-shaped dose-response curve with respect to estrogens such that low levels of estrogens are associated with increased CVD risk, with several lines of evidence linking hypoestrogenemia (HypoE) in young women to increased CVD. The timing of this association is important, in that early menopause (≤45 years) is associated with accelerated atherosclerosis and a 2.6-fold increase in the risk of CVD (95% CI 2.05–3.35) (Kannel and Wilson 1995), as well as increased CVD mortality (Jacobsen et al. 1997; Cooper and Sandler 1998), compared to women experiencing later menopause**.** Conditions resulting in severe HypoE,

including Turner syndrome (TS) and primary ovarian insufficiency (POI), are also associated with elevated rates of CVD in young women (Swerdlow et al. 2001).

The activational influence of estrogens is important in men as well, because testosterone (T) can be aromatized to estrogens with more than 80% of circulating E in men being derived from aromatization of T (Carani et al. 1997). Finkelstein et al. found that blocking the aromatization of T results in increased adiposity and reduced sexual function in men (Finkelstein et al. 2013), further supporting the concept that E deficiency is largely responsible for some of the key metabolic and endocrine consequences in men. As serum levels of T decline with aging, there is a concomitant decline in serum levels of E. In a related report, Jankowska et al. demonstrated that men with the lowest quintile of estradiol (E2) (lowest 20%, <12.90 pg/mL) were found to have the highest death rates from congestive heart failure over a 3-year period, while men with E2 in the range of 20–30 pg/mL had the lowest rates (Jankowska et al. 2009). However, men with the highest E2 levels (> or = 37.40 pg/mL) also had a greater incidence of atherosclerosis (heart disease), diabetes, obesity, stroke, enlarged prostate, breast tissue growth, breast cancer, and other problems.

The Activational Influence of Sex Hormones on Disease Risk: Testosterone (T)

The activational effects of testosterone have also been investigated with respect to modulating disease risk; however, the results are conflicting and may be due to the timing for which the effects are investigated. Lower T levels in middle-aged and older men are associated with insulin resistance, the metabolic syndrome, and diabetes (Navarro et al. 2015). Furthermore, lower T in older men predicts cardiovascular events, including stroke and transient ischemic attack, and is associated with higher CVD and overall mortality (Schwarcz and Frishman 2010). One interventional study using T therapy in men with CVD found beneficial effects on exercise-induced myocardial ischemia (Bhasin et al. 2006). However, in another trial of older men who were randomized to receive a substantial dose of T, the authors reported cardiovascular *adverse* effects (Snyder et al. 2016). Importantly, these effects were not observed in a comparable trial where men received a more conservative dose of T, suggesting that optimal dosing of T in older men with existing CVD is critical. In the Cardiovascular Risk in Young Finns Study, higher levels of T in younger men (24–45 years old) were associated with reduced cardiovascular risk characterized by lower levels of triglycerides, insulin, and systolic blood pressure and higher levels of high-density lipoprotein cholesterol (HDL-c) (Firtser et al. 2012). For women, elevations in T production, as seen with polycystic ovarian syndrome (PCOS), are associated with insulin resistance and CVD risk (Dokras 2013).

The Activational Influence of Sex Hormones Influenced by the Testosterone to Estradiol (T/E) Ratio

As discussed above, T and E reportedly have beneficial, neutral, or harmful effects on disease risks, implying that the timing as well as the relative ratio of T/E might be critical variables. Gong et al. concluded that the T/E ratio is key in the relationship between sex hormones and the risk of cerebrovascular disease (Gong et al. 2013) and that it was the ratio more than the individual sex hormones per se that had a greater impact on predicting outcomes. An additional study focusing on the T/E ratio in postmenopausal women with coronary heart disease (CHD) reported that the T/E ratio was negatively associated with total cholesterol, low-density lipoprotein cholesterol (LDL-c), and the atherogenic index of plasma but positively associated with HDL-c and HDL-c/LDL-c (for all, $p < 0.0001$). Furthermore, the authors concluded that an imbalanced T/E ratio was strongly associated with cardiovascular risk factors in postmenopausal women with CHD (Dai et al. 2012). Another study compared the effects of low T and/or low E in 3000 aging men who were in the age range of 69–80 for 4.5 years (Tivesten et al. 2009). Those men with low T had 65% greater all-cause mortality, but those men with low E2 also had 55% more deaths. Men who had both low E2 and low T had almost twice the risk of dying compared to men with higher E2 and T. While these data suggest that low levels of E and/or T per se increase disease risk, they further strongly suggest that focusing on either hormone in isolation may not adequately provide information as to their impact.

The Metabolic Impact of Gender

In the previous discussion, we focused on the timing and duration of exposure of the cell/organism to sex hormones and how this impacts physiological processes such as metabolism. Here we will discuss the contribution of gender to metabolic processes. As previously mentioned, gender is a psychosocial context and is a continuum, and it is therefore difficult to define because it is not binary. Cultural norms pertaining to gender roles and sex-related behaviors fluctuate and change over time making it even more difficult to factor gender as a variable in metabolic research. Specifically, there are cultures where gender is defined differently than how it is defined in Westernized societies, cultures such as Berdache (a French term for younger partners in male homosexual relationships used by Native Americans), the fa'afafine (Samoan for "the way of a woman") in the Pacific, and the kathoey in Thailand all of which are examples of cultures that define "males" and "females" differently than in Westernized cultures.

Rarely has gender been factored into studies with respect to its influence on biological processes such as metabolism. There are a few studies using Westernized definitions of gender where "men" are often thought to be at an increased risk for cardiovascular disease, in part due to their *gender*-based propensity to engage in

risk-taking behaviors such as smoking or alcohol consumption. Importantly, women who have taken on societal roles associated with the male gender have been demonstrated to have an increased disease prevalence linked with the pressures associated with these gender-defined roles (Izadnegahdar et al. 2014; Sozzi et al. 2007; Kawase et al. 2013; Hausmann et al. 2012; Bekhouche et al. 2015). One of the complexities and barriers associated with studying gender as a biological variable is that the term "gender" cannot be used when referring to in vitro assays or basic science animal research as this psychosocial context can only be studied in humans.

The Metabolic Influence of Gender Identity

Gender is a spectrum influenced by alignment of the perception of self with the sex assigned at birth. Masculine and feminine perception and behaviors may be present within the same individual and may change during life and could be age dependent. Some individuals have incongruence between their gender identity and their natal sex, and this is referred to as gender incongruence (GI). This is in contrast to gender dysphoria, which is a subjective manifestation of gender-sex incongruence. When a person with a normal somatic sexual differentiation is convinced that he or she is actually a member of the opposite sex, this may be associated with an irresistible urge to be hormonally, surgically, and psychosocially adapted to the desired sex as manifested in transgender individuals.

It is important to note that population-based surveys rarely ask questions to identify transgender people and, therefore, cannot be used to provide estimates of the size and characteristics of the transgender population. There are federal government-sponsored national population-based surveys that track the demographics, health, and well-being of US residents; unfortunately, these surveys do not currently measure gender identity. More recently, in an attempt to identify and generate data on the transgender population, several state-level population-based surveys have been conducted and identified transgender respondents. Based on these surveys, the number of individuals for whom GI applies is estimated to exceed 0.6% of the population (~1.4 million people) (Gates 2011; Flores 2016). This new estimate is nearly double the estimate from data generated roughly a decade. There also appear to be differences based on state, with state-level estimates of adults who identify as transgender ranging from 0.3% in North Dakota to 0.8% in Hawaii (Gates 2011). There are several reasons which may account for this robust increase in reported numbers of individuals with GI, and this may be due to the perceived increase in visibility and social acceptance of transgender people which may increase the number of individuals willing to identify as transgender on a government-administered survey. Within the Veterans Health Administration, the incidence of transgender-related diagnoses increased by 76% from 2009 to 2013 (Kauth et al. 2014). Despite this increased awareness and acceptance, the transgender community represents one of the most underserved and marginalized populations in healthcare. The chromosomal configurations, 46 XY in males transitioning to females (henceforth referred

to as transwomen) and 46 XX females transitioning to males (referred to as transmen), obviously remain unchanged (Gooren et al. 2015), yet their endogenous sex hormones are often suppressed, while they are exposed to exogenous sex hormones to enhance their desired secondary sex characteristics. Trans- and gender-diverse individuals undergoing cross-sex hormone therapy are a unique model to study the physiological effects of sex steroids. Unknown are the metabolic implications associated with cross hormone administration within this population. Specifically, the health effects of hormone manipulation on the bone, muscle, cardiovascular risk, cognition, and quality of life are not known.

Environmental/Psychosocial Influence of Gender Identity

In order to understand the genesis of sexual dimorphisms and gender identity, it is important to appreciate that sex-specific development of humans comprises irreversible sexual differentiation of the external genitalia during embryogenesis and sexual maturation of secondary sex characteristics occurs during puberty (e.g., sex-specific body proportions, bone size, pubertal voice change), and this is followed by sex-specific development of extragenital tissues and organs, including the brain. During the developmental window of 2–3 years of age, the perception of gender "identity" ensues. Some children insist from the moment they can speak that they are not the gender indicated by their biological sex. Yet the genesis of GI is not fully understood, and initially prevailing theories on the origins of GI focused on the lack of congruence between natal sex and experienced gender as being psychosocial in nature, suggesting early traumas including dysfunctional family dynamics or childhood sexual abuse as being contributory. This concept has subsequently been refuted. Fully characterizing the biological basis of GI is therefore required to not only advance our knowledge of human sexual biology but also to improve the quality and efficacy of transgender healthcare.

There are data suggesting genetics may be involved in the development of gender identity and GI. Evidence demonstrates monozygotic (MZ) twin pairs show a higher concordance of GI than dizygotic (DZ) twin pairs (Heylens et al. 2012; Sadeghi and Fakhrai 2000). Although the concordance rates of homosexuality in monozygotic twins vary depending on ascertainment methods (Coolidge et al. 2002), twin studies have also suggested a genetic component influencing sexual orientation (Andreazza et al. 2014; Korasz and Simon 2008), and these data would suggest there may be environmental/gene/programming interactions which influence GI, such as epigenetics.

Epigenetics is the language used to translate both intrinsic and extrinsic/environmental cues into molecular signals modifying gene expression patterns. This interaction between genetic, epigenetic, and environmental conditions is what manifests a phenotype (Martin-Subero 2011). MicroRNAs (miRNAs) are a class of noncoding RNAs that regulate posttranscriptional gene expression and influence epigenetics. miRNAs control cell proliferation, metabolism, apoptosis, and

differentiation. miRNAs are stable in the blood where they circulate either in a free/uncomplexed form or in microvesicles, exosomes, or apoptotic bodies and/or are bound to proteins. Because miRNAs are regulatory molecules in all organisms and influence almost every aspect of physiology, including embryogenesis, metabolism, and growth and development, it is important to note that the regulation of miRNAs is affected by sex steroid hormones as well as X sex chromosome-linked genes (Sharma and Eghbali 2014). As an example, estrogens regulate a number of miRNAs, and the X chromosome is enriched in miRNAs (Sosa et al. 2015). Although miRNAs have been widely studied as epigenetic regulators, only a few studies have focused on their role in mediating sexual dimorphisms (Morgan and Bale 2012) and/or their ability to influence sexual identity (Fagegaltier et al. 2014).

Recently, emphasis has been placed on elucidating potential relationships between sex differences and miRNA expression by using a comprehensive analysis of small RNA-sequencing datasets based on different human diseases and tissues. Results suggest some miRNAs exhibit inconsistent and even opposite expression patterns between males and females (Royo et al. 2015; Dai and Ahmed 2014), and these data further support the importance of sex chromosome and sex hormone interactions and their impact on biological systems such as metabolism which can be regulated by brain structure and function.

Brain Function and Size Are Influenced by Gender

Nearly every region in the brain is sexually dimorphic. The total brain volume is larger in men than in women, even when corrected for body size (Smith et al. 2015). Brain regions high in androgen receptors, such as the amygdala, are larger in men, while regions with a higher density of estrogen receptors, such as the hippocampus, are higher in women (Smith et al. 2015). Importantly, sexual differentiation of the brain is traditionally thought to result from organizational effects of fetal testosterone; however, recent findings suggest that sex differences may be present *before* the onset of testosterone production (Lentini et al. 2013). These differences are thought to be due to genes located on the X and Y chromosomes. The timing and origins of brain development may shed light on windows of susceptibility where natal sex and experienced gender may differ, thereby influencing gender identity. There are data to suggest GI results from atypical sexual differentiation where the body and genitals develop in the direction of one sex and the brain and gender in the direction of the opposite sex. This divergence is theoretically possible because windows of sensitivity for sex hormone imprinting during prenatal development are different for the brain and testes/gonads and allow for potential influence due to miRNAs.

Several studies in transgender individuals have demonstrated their brains differ from cis-individuals (individuals who have alignment between natal sex and gender identity) and are more consistent with people of their self-experienced gender (Simon et al. 2013; for a review see Steensma et al. 2013). Specifically, Guillamon et al. used MRI to examine the brains of female-to-male and male-to-female

transgender individuals – both before and after treatment with cross-sex hormones (Zubiaurre-Elorza et al. 2014). The investigators found, even *before* cross hormone treatment, the brain structures of the transgender individuals were more similar to the brains of their experienced gender than those of their natal sex. For example, female-to-male transgender individuals had relatively thin subcortical areas (these areas tend to be thinner in cis-men than in cis-women), whereas male-to-female transgender individuals tended to have thinner cortical regions in the right hemisphere, which is characteristic of the cis-female brain. Importantly, the researchers went on to demonstrate that these differences became even more pronounced after cross hormone therapy (Guillamon et al. 2016).

Additional studies published in 2014, by psychologist Burke and biologist Bakker using functional MRI, examined the response of 39 prepubertal and 41 adolescent boys and girls with gender incongruence to androstadienone, an odorous steroid with pheromone-like properties that causes a sexually dimorphic response in the hypothalamus of cis-men versus cis-women. The researchers found that the adolescent boys and girls with GI responded much like peers of their experienced gender (Burke et al. 2014a). Dr. Baudewijntje Kreukels, an expert on GI, commented on these findings indicating they were critical in establishing a biological basis for GI "because sex differences in responding to odors cannot be influenced by training or environment." In another study, Burke et al. measured the responses of boys and girls with GI to echolike sounds produced by the inner ear in response to a clicking noise. Boys with GI responded more like cis-females, who have a stronger response to these sounds (Burke et al. 2014b).

Although the number of studies examining brains of people with GI is still low, they have taught us that brain phenotypes for transgender individuals seem to exist even prior to hormonal transitioning at puberty and provide evidence for the role of prenatal organization of the brain in the development of GI. Researchers argue that sex differences seen in the brain are due to functional and structural organization of the human brain which is a continuous and dynamic process that persists throughout one's life (experience-dependent plasticity). As an example, the activational influence of fluctuating hormones during puberty, menstrual cycle, menopause, or hormone replacement therapy all influence brain function. Specifically, in the brains of transgender male and females, cortical thickness increases under treatment with testosterone, while estrogen and antiandrogen treatment in male-to-female transgender individuals is associated with a decrease in cortical thickness (Zubiaurre-Elorza et al. 2014). These results indicate that brain structures in adulthood are also subjected to the impact of sex hormones.

The Metabolic Impact of Cross-Sex Hormone Therapy

When the diagnostic criteria are met for GI, individuals opt for interventions and procedures aimed at alleviating the incongruence between their gender identity and their biological sex (American Psychiatric Association 2013). Transgender practice

guidelines were designated by the Endocrine Society in 2009 (Hembree et al. 2009). Two procedures were designed to alter naturally produced sex hormones: cross-sex hormone therapy (CSHT) and sex reassignment surgery. The objectives of CSHT are (1) to induce the appearance of sexual characteristics consistent with gender identity and (2) to suppress endogenous hormone levels and secondary sexual characteristics associated with biological sex. For example, within the first 6 months of CSHT, changes in transwomen include breast growth, decreased testicular volume, and decreased spontaneous erections. Transwomen experience changes in body fat redistribution, muscle mass, and hair growth. Desired effects from CSHT can require between 3 years and 5 years; however, effects that occur during puberty, such as voice deepening and skeletal structure changes and perhaps influences on metabolism, cannot be reversed with CSHT. Sex reassignment surgery is a final step in the transition due to its irreversibility, and not all subjects elect for this procedure.

There is limited knowledge of desired and undesired effects of CSHT, despite the fact that these recommended procedures were established using best practice guidelines. There is a profound lack of well-designed prospective studies of the effects of cross-sex hormone therapy. Importantly, cross-sex hormone therapy provides an opportunity to study sex steroid biology, and therefore quality research is needed to guide evidence-based guidelines to improve healthcare for trans- and gender-diverse individuals. This is important, as individuals often need cross-sex hormone therapy lifelong, and evidence-based treatment guidelines are needed to meet the exponential increase in demand for transgender healthcare. Data from a large gender identity study indicate that hormone therapy taken by transgender individuals is associated with a higher cardiovascular mortality rate among transwomen (male to female) but not among transmen (female to male). However, in both transgender populations, there is a higher incidence of type 2 diabetes than exists in the cis-population (Wierckx et al. 2013). The authors of the study explained that although hormone therapy is part of the established treatment of gender identity disorder, outcome data regarding morbidity and mortality "are scant."

To begin to further understand why cross hormone therapy impacts metabolic disease risk and if the influence of sex hormones is guided by the suppression of endogenous or supplementation of exogenous hormones, we investigated the role of sex hormones and their influence on insulin sensitivity and hepatic steatosis in a population of transwomen with and without testes (Nelson et al. 2016). Despite receiving similar estrogen therapy, transwomen who elected for bilateral orchiectomy had improved metabolic health when compared to those transwomen who retained their testes. More specifically, when the transwomen were stratified according to circulating testosterone levels, those with the highest testosterone also had the highest incidence of hepatic steatosis and insulin resistance (Nelson et al. 2016). These findings are important because they are the first in which the effects of endogenous and exogenous hormones have directly been compared in the transgender population. Furthermore, our data suggest that suppression of endogenous testosterone in transwomen appears to improve insulin sensitivity and reduce hepatic steatosis. More research is needed to illustrate the optimal hormonal milieu for transgender

individuals; importantly, this research will be informative with regard to the optimal sex hormonal and sex chromosomal combination to protect against disease risk in the cis-population as well.

Conclusion

In this chapter, we have discussed many factors and challenges associated with defining the role of sex and gender in disease risk and prevalence. Designing and executing experiments to specifically target the role of sex and gender must take into account the sex chromosomes, the organizational and activational influence of sex hormones, and the ratios of endogenous and exogenous sex hormones. Researchers are encouraged to critically think of the impact that their experimental design has on the sex hormonal profile and to accurately analyze the data focusing on the impact of sex, not only of the individual being studied but also of the cell in a dish. It is no longer acceptable to be blind to the influence of sex and gender, nor is it acceptable to focus research only on one sex or gender to the exclusion of the other. It is also important that when data are interpreted, age, type, route of administration, and timing of the hormonal manipulation must be considered. Statements such as "there are no sex differences" will need to be strongly defended following rigorous characterization of the impact of sex chromosomes and the organizational and activational impact of the sex hormones. Lastly, we would be remiss if we did not also emphasize the need to take into account other fundamental developmental, evolutionary, and life history traits in medical research. There is a bias and tendency to treat all adults as equivalent, drawing conclusions devoid of sex, gender, age, race, and environment. Understanding how these physiological states impact biology is critical for the development of personalized medicine.

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