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RESEARCH ARTICLE

17β-Estradiol affects lung function and inflammation following ozone exposure in a sex-specific manner

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Fuentes N, Nicoleau M, Cabello N, Montes D, Zomorodi N, Chroneos ZC, Silveyra P. 17β-Estradiol affects lung function and inflammation following ozone exposure in a sex-specific manner. *Am J Physiol Lung Cell Mol Physiol* 317: L702–L716, 2019. First published September 25, 2019; doi:10.1152/ajplung.00176.2019.—Inflammatory lung diseases affect men and women disproportionately, suggesting that fluctuations of circulating hormone levels mediate inflammatory responses. Studies have shown that ozone exposure contributes to lung injury and impairment of innate immunity with differential effects in men and women. Here, we hypothesized that 17β-estradiol enhances inflammation and airway hyperresponsiveness (AHR), triggered by ozone exposure, in the female lung. We performed gonadectomy and hormone treatment (17β-estradiol, 2 wk) in C57BL/6J female and male mice and exposed animals to 1 ppm of ozone or filtered air for 3 h. Twenty-four hours later, we tested lung function, inflammatory gene expression, and changes in bronchoalveolar lavage fluid (BALF). We found increased AHR and expression of inflammatory genes after ozone exposure. These changes were higher in females and were affected by gonadectomy and 17β-estradiol treatment in a sex-specific manner. Gonadectomized male mice displayed higher AHR and inflammatory gene expression than controls exposed to ozone; 17β-estradiol treatment did not affect this response. In females, ovariectomy reduced ozone-induced AHR, which was restored by 17β-estradiol treatment. Ozone exposure also increased BALF lipocalin-2, which was reduced in both male and female gonadectomized mice. Treatment with 17β-estradiol increased lipocalin-2 levels in females but lowered them in males. Gonadectomy also reduced ozone-induced expression of lung IL-6 and macrophage inflammatory protein-3 in females, which was restored by treatment with 17β-estradiol. Together, these results indicate that 17β-estradiol increases ozone-induced inflammation and AHR in females but not in males. Future studies examining diseases associated with air pollution exposure should consider the patient's sex and hormonal status.

air pollution; estrogen; lung inflammation; sex differences; sex hormones

INTRODUCTION

Air pollution exposure can initiate severe inflammatory responses in susceptible individuals (23, 64). Ground-level ozone is a harmful air pollutant that can trigger health problems, such as throat irritation, airway inflammation, and impaired lung immunity (36). Ozone can also decrease lung function, induce airway hyperresponsiveness (AHR), and worsen asthma symptoms, leading to an increase in hospital admissions, healthcare costs, and morbidity and mortality (2, 37, 40).

It is known that several lung diseases display sexual dimorphism in risk, prevalence, and severity (77). For example, the current incidence of asthma in women (10.4%) is nearly double that of men (6.2%) in the United States (10). Accordingly, the risk for women having an asthma exacerbation requiring hospital admission is also almost double than it is for men (44, 46). These sex differences in exacerbation risk and prevalence, together with the observed crossover in the incidence of asthma versus age, where asthma trends move from higher rates in boys than girls before puberty to higher rates in women than men after puberty, and the new-onset asthma observed in significant numbers in girls during adolescence, suggest that some of these effects may be attributable to hormonal factors (28, 29, 57, 76). To address this phenomenon, clinical studies in women have explored associations of asthma exacerbations and frequency of symptoms with circulating hormone level oscillations occurring throughout the menstrual cycle, pregnancy, oral contraceptive use, and menopause (15, 54, 67, 73). Combined, these studies have shown that sex hormones can play a significant role in differences between male and female inflammatory responses and affect central features of lung disease, such as airway tone, inflammation, and immunity (58, 69, 71). Despite this evidence, only a few studies have addressed the mechanisms by which sex hormones affect the pathogenesis of lung disease.

Sex differences have also been observed in clinical and animal studies where ozone exposure was studied (16, 72). In humans, ozone significantly decreases forced expiratory volume in 1 s in female young adults compared with men (43). In mice, we previously reported that female mice have greater counts of bronchoalveolar lavage fluid (BALF) neutrophils, high expression of acute phase cytokines/chemokines, and different microRNA (miRNA) signatures than male mice (6, 34). Importantly, some of these parameters differ in females exposed to ozone in various stages of the estrous cycle (31, 34). Additional studies have also reported that female hormones, such as estrogens and progestogens, can modify lung immune responses, affect airway tone, and alter gene transcription, whereas androgens such as testosterone may exert an opposite effect (35). Together, the results for these studies suggest that hormonal factors may influence lung inflammatory responses to environmental exposures, such as ground level ozone. How-

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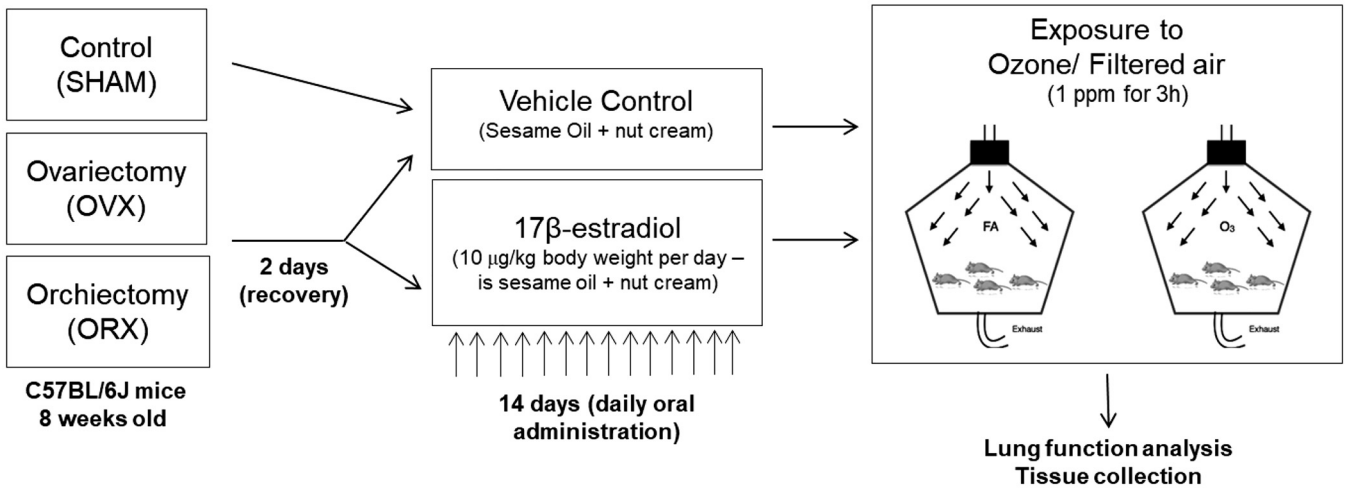

Fig. 1. Experimental design. Sham and gonadectomized [ovariectomized (OVX), orchiectomized (ORX)] female and male C57BL/6J mice received daily 17β-estradiol (10 μg·kg body wt^{-1}·day^{-1}). The hormone or vehicle control was mixed with nut cream and administered orally for 2 wk. Animals were then exposed to ozone (1 ppm; O_3) or filtered air (FA) for 3 h.

ever, there is not a clear understanding about the mechanisms behind these differences and specifically whether sex hormones are involved.

Previous studies in mouse models of allergic asthma have reported protective effects of estrogen against AHR (22, 53, 61) and suggested involvement of the inflammasome and signaling by the estrogen and androgen receptors in this regulation (3, 14, 47). However, the effects of estrogen on airway inflammation and immunity remain highly controversial. While some studies have reported protective effects of estrogen on lung inflammation, the overall effects on lung immunity and beyond airway smooth muscle biology have not been explored in detail. A few studies have reported proinflammatory effects of estrogens in asthma (19, 51), altered phagocytosis of bacteria in mice exposed to ozone (25), and exacerbation of ozone-induced oxidative stress markers in cultured lung epithelial cells (12). Two recent studies have also reported involvement of the microbiome as a mediator of sex-specific inflammatory responses to ozone (16, 17). Due to the complexity of hormone-related physiological mechanisms, experimental models are limited, and the overall effects of gonadal hormones on lung immunity have not been well characterized in the context of ozone exposure.

In this study, we hypothesized that 17β-estradiol circulating levels regulate lung function and inflammation following ozone exposure. To test this hypothesis, we exposed gonadectomized female and male C57BL/6J mice, with or without 17β-estradiol treatment, to ozone or filtered air (FA). We then analyzed the overall inflammatory response among groups by comparing lung cell profiles and inflammatory gene signatures, and we assessed lung mechanics. Our results indicate that pulmonary function and inflammation following ozone exposure can be affected by 17β-estradiol levels in a sex-dependent manner. Together, the results presented here suggest that estrogen can regulate lung mechanics and inflammatory mechanisms initiated in response to air pollution exposure and suggest that future clinical and animal studies should consider hormonal status when evaluating lung function and inflammatory outcomes.

MATERIALS AND METHODS

Animals. For all experiments, we used adult male and female C57BL/6J mice (8 wk old) from JAX Laboratories (Bar Harbor, ME). Animals were housed in a 12:12-h light-dark cycle with food and water *ad libitum* at the Penn State College of Medicine animal facility. The Penn State College of Medicine Institutional Animal Care and Use Committee approved all procedures.

Gonadectomy and hormone treatment. Male and female C57BL/6J mice were gonadectomized or sham operated under a ketamine-xylazine cocktail (90 mg/kg ketamine, 10 mg/kg xylazine, ip) in our research facility. Briefly, a ventral skin incision was made in the lower abdominal area of female mice to provide access to the peritoneal cavity. The ovary and oviduct were exteriorized, and a sterile silk ligature was placed around the oviduct. Each ovary and part of the oviduct were removed with a single cut. The body wall was closed by sterile absorbable vicryl suture. In males, a small incision was made in the scrotum, and testicles were exteriorized. The epididymis, vas deferens, and testicular blood vessels were exposed and clamped while holding the testicular sac with sterile tooth forceps. A sterile silk ligature was placed around the testicular blood vessels to prevent bleeding, and testicles were removed using scissors. The skin was closed with metal clips. Sham animals were subjected to the same procedure, but no gonads were removed. Following surgery, sham and gonadectomized mice were monitored twice daily to ensure optimal recovery, and they received daily 17β-estradiol at a dose of 10 μg·kg body wt^{-1}·day^{-1}. The hormone or vehicle control (sesame oil) were mixed with nut cream (Nutella) at the calculated dose and administered orally following published protocols (66). For this, animals were placed in separate cages and fed the nut-cream mix on small polystyrene weighing boats. After 2 wk, animals were used for exposures. A schematic of the experimental model is provided in Fig. 1. The estrous cycle stage in sham mice was determined by vaginal smear, and

Table 1. *Number of female mice in each estrous cycle stage*

Estrous Cycle Stage (Sham Females)	No. of Animals
Proestrus (P)	4
Estrus (E)	3
Metestrus (D1)	6
Diestrus (D2)	6

animals in all estrous cycle stages were used for ozone exposures to avoid cycle effects (Table 1) (30).

Ozone exposure. We used an ozone exposure chamber, as described in Mishra et al. (56). Briefly, mice were exposed to 1 ppm of ozone or FA (control) for 3 h in adjacent chambers ($n = 4–8$ per group). Both male and female mice were located in glass containers with bedding, food, and water ad libitum and placed in an exposure chamber as described in Cabello et al. (6). Ozone concentration was recorded every 15 min for the duration of the exposure. The system delivers a regulated air flow (>30 air changes/h) at a controlled temperature (25°C) and relative humidity (50%) (70).

RNA preparation. At 24 h following exposure, lungs were collected and snap frozen in liquid nitrogen for downstream purification of nucleic acids. Total RNA was extracted from pulverized lung tissue using the Direct-Zol RNA kit (Zymo Research, Irvine, CA), following the manufacturer's protocol and treated with DNAse I (Zymo Research, Irvine, CA) to remove genomic DNA. RNA concentration was determined by Nanodrop, followed by a Bioanalyzer analysis for RNA quality (RNA integrity number > 7) and purity (6).

Real-time PCR. For gene expression analysis, 2 μg of purified RNA were retro-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Waltham, MA), following the manufacturers' protocol. The expression levels of *Ccl20*/MIP-3 (macrophage inflammatory protein-3) (assay no. Mm01268754), IL-6/interleukin-6 (assay no. Mm00446190), *Cxcl2*/MIP-2 (assay no. Mm00436450), and *Nos2* (assay no. Mm00440502) were then measured by real-time PCR in 25–50 ng of cDNA, using TaqMan assays (Life Technologies, Carlsbad, CA). Results were analyzed as described by us previously and normalized to 18s rRNA expression (assay no. Mm03928990) by the relative quantification method (52).

Lung function. Mice were anesthetized with ketamine, (90 mg/kg), xylazine (10 mg/kg), and vecuronium bromide (1 mg/kg ip) at 24 h postexposure. Animals were connected to a flexiVent system (SCIREQ Inc., Canada) at a respiratory rate of 150 breaths/min and a positive end-expiratory pressure of 3 cmH_2O. Increasing doses of methacholine (MCh; 0–50 mg/mL, Sigma-Aldrich, St. Louis, MO) were administered as described previously (27).

BALF analysis. A solution of 2.5 mL Dulbecco's phosphate-buffered saline and 1 mM EDTA was used to lavage the lungs, following standard protocols (6). Total cell number was assessed using a hematocytometer. A total of 50,000 cells per slide were used for cytospins and stained with a Hema-3 stain kit (Fisher Scientific, Pittsburgh, PA). Slides were independently analyzed under light microscopy for the presence of immune cells. Lipocalin-2/neutrophil gelatinase-associated lipocalin (NGAL) levels were determined by ELISA (R&D Systems, Minneapolis, MN, kit no. MLCN20) in 50 μl of BALF.

Serum hormone determination and protein measurement. To confirm the efficiency of gonadectomies and hormone replacement, serum levels of 17β-estradiol and testosterone were measured by

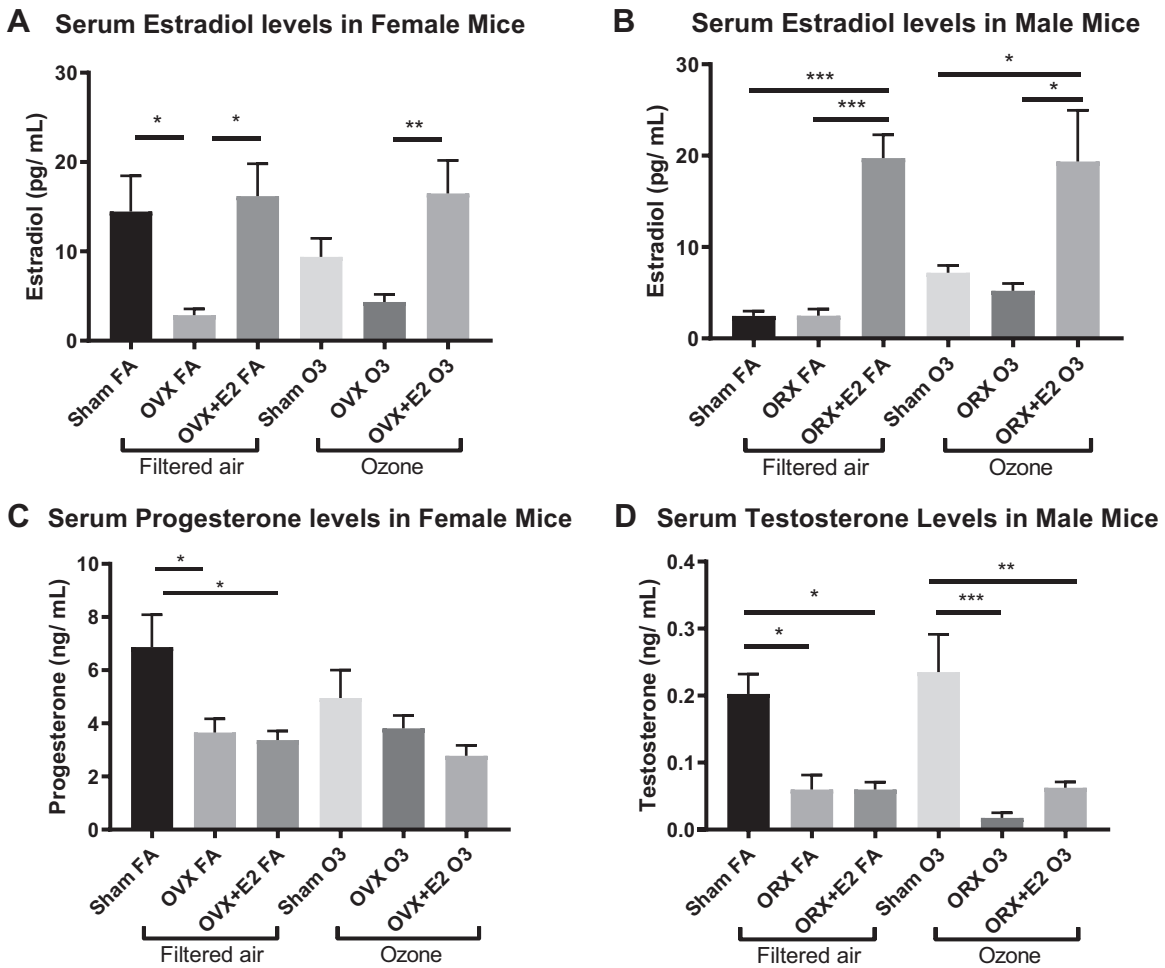

Fig. 2. Serum levels of 17β-estradiol and testosterone measured by ELISA. *A* and *B*: 17β-Estradiol levels in female (*A*) and male (*B*) mice are shown. *C*: testosterone levels in male mice. *D*: progesterone levels in female mice. Animals were exposed for 3 h to 1 ppm ozone (O_3) or filtered air (FA). OVX, ovariectomized mice; ORX, orchiectomized mice; OVX+E2, 17β-estradiol-treated ovariectomized mice; ORX+E2, 17β-estradiol-treated orchiectomized mice. Values are means ± SE of $n = 5–8$ mice per group (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$).

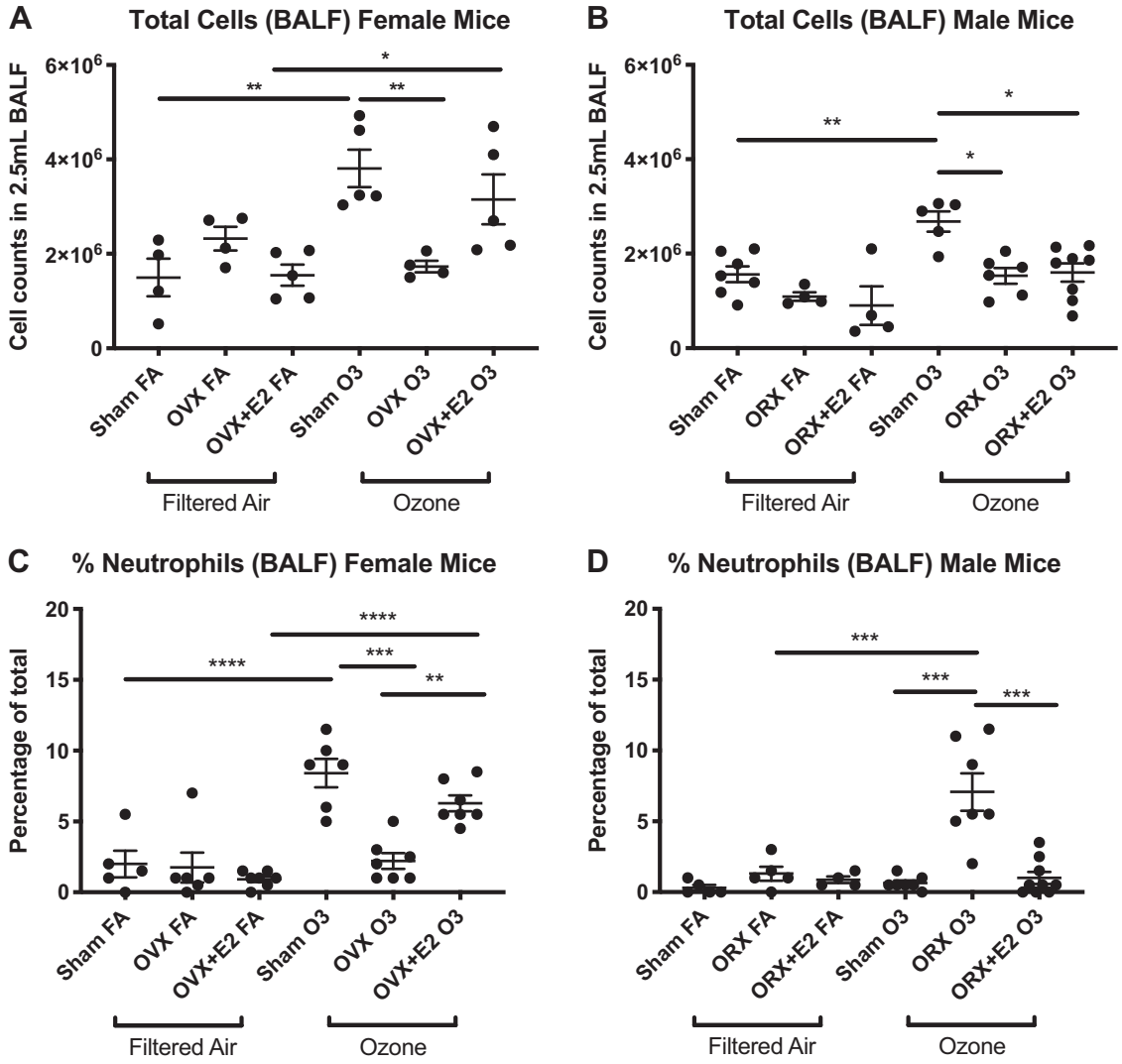

Fig. 3. Bronchoalveolar lavage fluid (BALF) fluid cell counts. Total cells in female (*A*) and male (*B*) mice exposed for 3 h to 1 ppm ozone (O_3) or filtered air (FA). Polymorphonuclear neutrophils (% of total) in females (*C*) and males (*D*) were measured at 24 h postexposure to O_3 or FA. OVX, ovariectomized mice; ORX, orchiectomized mice; OVX+E2, 17β-estradiol-treated ovariectomized mice; ORX+E2, 17β-estradiol-treated orchiectomized mice. Values are means ± SE of $n = 5–8$ mice per group (*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.0001$).

ELISA (catalog nos. ES180S-100 and TE187S-100, Calbiotech, El Cajon, CA).

Data analysis. Interactions of sex, exposure, and gonadectomy were assessed by three-way ANOVA, followed by Tukey's post hoc test ($\alpha = 0.05$) using GraphPad Prism software. Simple main effects of sex, exposure, and hormone status, as well as Pearson correlation calculations, were also analyzed with GraphPad Prism.

RESULTS

Determination of hormone levels. We confirmed the efficiency of ovariectomy (OVX), orchiectomy (ORX) and hormone treatment in both OVX and ORX mice by measuring serum levels of estradiol and testosterone (24 h after exposure

Table 2. *Bronchoalveolar lavage fluid macrophage and lymphocyte cell percentage in experimental groups*

	Female						Male					
	Filtered Air			Ozone			Filtered Air			Ozone		
	Sham	OVX	OVX+E2	Sham	OVX	OVX+E2	Sham	ORX	ORX+E2	Sham	ORX	ORX+E2
Macrophages, %	96.3	97.3	97.6	90.3	96.6	92.7	98.4	97	97.6	98.2	91.9	97.9
Lymphocytes, %	0.48	0.44	0.46	0.55	0.53	0.56	0.40	0.46	0.41	0.49	0.45	0.44

OVX, ovariectomized mice; ORX, orchiectomized mice; OVX+E2, 17β-estradiol-treated ovariectomized mice; ORX+E2, 17β-estradiol-treated orchiectomized mice.

to ozone or FA). As expected, gonadectomies performed in female mice resulted in significantly lower levels of 17β-estradiol and progesterone, and in lower testosterone levels in males compared with control (sham) mice (Fig. 2). Additionally, we found significantly higher circulating estradiol levels in all gonadectomized mice treated with 17β-estradiol (female: OVX+E2, male: ORX+E2), as well as in sham males exposed to ozone (Fig. 2). Circulating 17β-estradiol levels in OVX+E2 mice were similar to those observed in proestrus afternoon (30). Treatment with 17β-estradiol did not affect testosterone or progesterone levels. However, while ozone exposure did not significantly affect circulating estradiol and testosterone levels, it resulted in lower progesterone levels in sham mice (Fig. 2).

17β-Estradiol affects the inflammatory response to ozone. As previously reported (6), exposure to ozone caused an increase in total cell numbers in BALF of both female and male mice, with significantly higher cell numbers and neutrophilia in females than males (Fig. 3). Moreover, a three-way ANOVA revealed a significant interaction of sex, ozone exposure, and gonadectomy status for total cell counts ($P < 0.05$) and neutrophil counts ($P < 0.0001$). Single-effects analysis revealed a decrease in total cell number and neutrophil counts in OVX mice exposed to ozone compared with the ozone-exposed control (sham) group, indicating that ovarian hormones affect this response. Interestingly, when OVX mice were treated with 17β-estradiol (OVX+E2) and exposed to ozone, a significant increase in total cell number and higher neutrophilia was found. In ORX males exposed to ozone, there was a significant reduction of total cells, but a significant increase in neutrophil counts compared with the ozone-exposed sham males. In addition, there were no changes in BALF differential cell counts in ozone-exposed ORX mice treated with 17β-estradiol (ORX+E2) compared with the ORX group. Ozone-exposed ORX+E2 mice, however, had a significant decrease in neutrophils, reaching similar levels to those of the control group. We observed no significant changes in macrophage and lymphocyte cell percentage among experimental groups (Table 2).

17β-Estradiol influences lipocalin-2 expression in ozone-induced lung inflammation. As previously reported by us, the levels of the lung injury marker neutrophil gelatinase-associated lipocalin (lipocalin-2/NGAL) were significantly higher in the BALF of both females and males exposed to ozone (Fig. 4), with higher levels in males than in females. In gonadectomized animals (ORX, OVX), we found a significant reduction of NGAL expression compared with ozone-treated sham mice. Interestingly, this effect was reversed in OVX females treated with 17β-estradiol, but not in ORX males, which experienced a reduction in NGAL levels with hormone treatment. There were no differences in lipocalin levels in BALF of mice exposed to FA. Three-way ANOVA confirmed a significant interaction of sex, ozone exposure, and gonadectomy/estradiol replacement for BALF lipocalin-2/NGAL levels ($P < 0.001$). These results suggest a potential role of 17β-estradiol in sex-specific mechanisms of ozone-induced inflammation and injury. To determine a relationship between neutrophilia and lipocalin-2 expression, we also conducted correlation analyses and found a positive correlation between lipocalin levels and neutrophil counts in females (Pearson $r = 0.9786$, $R^2 = 0.9576$, $P = 0.037$), but not in males (Pearson $r = -0.1085$, $R^2 = 0.01178$, $P = 0.872$). Together, these results indicate that lipocalin-2 expression is influenced by 17β-estradiol in a sex-dependent manner, potentially via neutrophil recruitment to the lung in response to ozone exposure.

Exposure to ozone in gonadectomized mice induces differential inflammatory gene expression signatures in males and females. To test whether 17β-estradiol affected the inflammatory response to ozone, we screened for the expression of inflammatory genes in total lung homogenates from gonadectomized mice exposed to ozone or FA using quantitative PCR. As expected, expression of the *Cxcl2* transcript was elevated in male and female controls exposed to ozone, and this was

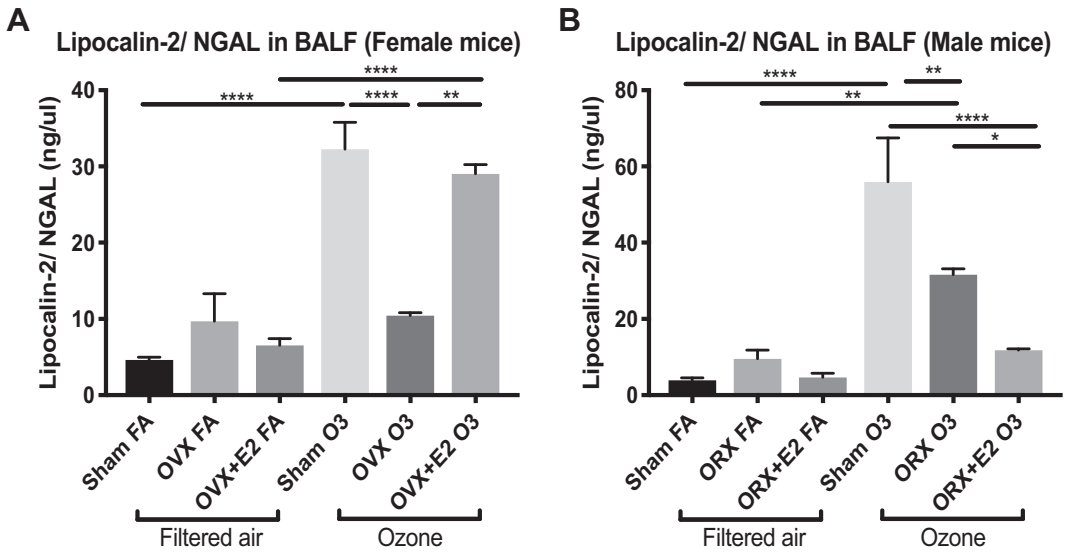

Fig. 4. Lipocalin-2 [neutrophil gelatinase-associated lipocalin (NGAL)] levels in bronchoalveolar lavage fluid (BALF) of treated mice. Lipocalin was measured by ELISA in BALF from female (*A*) and male (*B*) mice exposed to ozone (1 ppm; O_3) or filtered air (FA) for 3 h. OVX, ovariectomized mice; ORX, orchiectomized mice; OVX+E2, 17β-estradiol-treated ovariectomized mice; ORX+E2, 17β-estradiol-treated orchiectomized mice. Values are means ± SE of $n = 5–8$ mice per group (*$P < 0.05$, **$P < 0.005$, ****$P < 0.0001$).

higher in females (Fig. 5). We also observed a decline in *Cxcl2* expression in gonadectomized mice and an increase with 17β-estradiol replacement in females. In addition, we found that *Nos2* expression was significantly higher in ORX mice exposed to ozone compared with ozone-exposed sham controls and FA-exposed ORX mice. However, we found no difference in *Nos2* expression in females (Fig. 5), nor an interaction of sex, exposure, or hormone status by three-way ANOVA for *Nos2* and *Cxcl2* expression. We also confirmed that, in females, exposure to ozone resulted in significantly higher expression of the inflammatory cytokines IL-6 and CCL20 (MIP-3α). Interestingly, expression of both IL-6 and *Ccl20* mouse transcripts was reduced in OVX mice exposed to ozone but not in OVX mice treated with 17β-estradiol (Fig. 6). In males, *Il6* and *Ccl20* levels were significantly higher in ozone-exposed sham versus FA groups. However, ORX males exposed to ozone had increased levels of IL-6, which were not influenced by 17β-estradiol replacement (Fig. 6). Similarly, only sham males exposed to ozone displayed increased levels of *Ccl20* versus FA-exposed males, and gonadectomy abolished this effect. While there was a significant interaction of sex, exposure, and hormone status for both IL-6 and *Ccl20* expression (three-way ANOVA, $P < 0.05$), gonadectomy did not affect expression of inflammatory genes in either male or female groups exposed to FA. Together, these results support the hypothesis of an interaction of gonadal hormones and ozone exposure in the mechanisms leading to the observed sexually dimorphic phenotypes in lung inflammation, with estradiol levels associated with higher inflammation in females, but not males, exposed to ozone.

17β-estradiol treatment alters ozone-induced AHR. To determine whether 17β-estradiol treatment affects ozone-induced AHR differentially in males and females, we compared lung function parameters in OVX- and ORX-treated mice following ozone exposure. As shown above, female (sham) mice exposed to 1 ppm ozone displayed significantly higher total respiratory system resistance (Rrs) at higher doses of methacholine (25–50 mg/mL) than females exposed to FA and ozone-treated males (Fig. 7). On the other hand, OVX female mice exposed to ozone showed a significant decline in Rrs and Newtonian

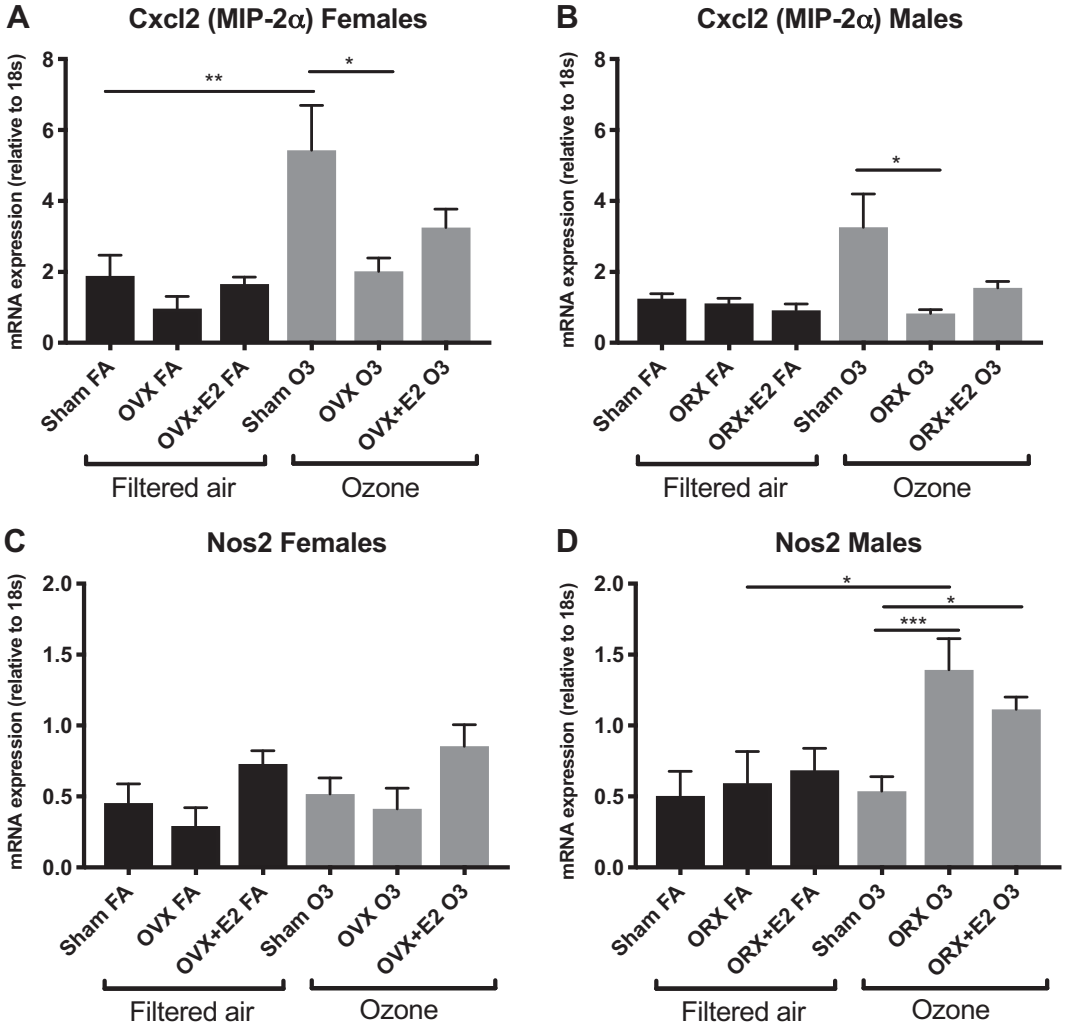

Fig. 5. Relative mRNA expression of *Nos2* and *Cxcl2* (macrophage inflammatory protein-2α, MIP-2α) in lung tissue. Gene expression was analyzed by real-time PCR in whole lung tissue extracts from female (*A* and *C*) and male (*B* and *D*) mice exposed to ozone (O_3) or filtered air (FA). OVX, ovariectomized mice; ORX, orchiectomized mice; OVX+E2, 17β-estradiol-treated ovariectomized mice; ORX+E2, 17β-estradiol-treated orchiectomized mice. Values are means ± SE of $n = 5–8$ mice per group (*$P < 0.03$, **$P < 0.006$, ***$P < 0.001$).

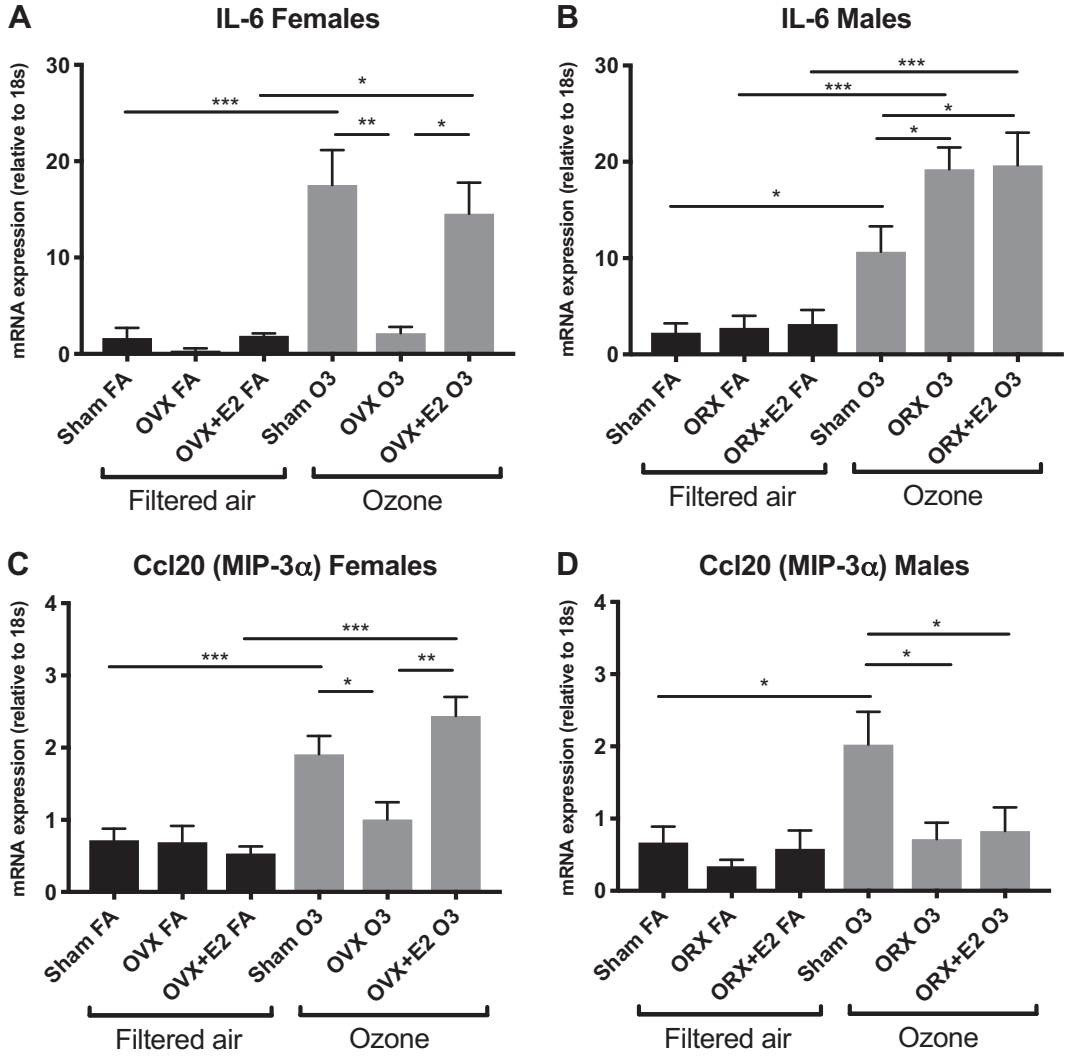

Fig. 6. Relative mRNA expression of IL-6 and *Ccl20* (macrophage inflammatory protein-2α, MIP-2α). Gene expression was analyzed by real-time PCR in whole lung tissue extracts from female (*A* and *C*) and male (*B* and *D*) mice exposed to ozone (O_3) or filtered air (FA). OVX, ovariectomized mice; ORX, orchiectomized mice; OVX+E2, 17β-estradiol-treated ovariectomized mice; ORX+E2, 17β-estradiol-treated orchiectomized mice. Values are means ± SE of $n = 5–8$ mice per group (*$P < 0.05$, **$P < 0.002$, ***$P < 0.001$).

resistance levels compared with the female sham group. This effect was reversed when OVX mice were treated with 17β-estradiol. In males, we did not find differences in control (sham) groups exposed to ozone or FA (Fig. 8). However, ORX males exposed to ozone displayed a significant increase in Rrs compared with sham males, whereas Rrs decreased after 17β-estradiol replacement in ozone-exposed ORX+E2 males. When we compared tissue damping (G), a measurement associated with tissue resistance in small airways (Fig. 9), we found that ORX mice exposed to ozone displayed higher G levels than FA-treated ORX and ozone-exposed ORX+E2. We did not observe any changes in airway resistance in mice exposed to FA. When comparing elastance of the total respiratory system (Ers), we found a significant decrease in sham females exposed to ozone versus FA, and no effect of ovariectomy and hormone replacement (Fig. 10). In contrast, ORX males exposed to ozone showed a significant increase in Ers at higher doses of methacholine than sham males and ORX males exposed to FA, an effect that was ameliorated with 17β-estradiol treatment (Fig. 11), suggesting that estradiol has contextual inflammatory and anti-inflammatory effects. However, these effects were not significant when comparing tissue elastance, a parameter associated with elastance in small airways (Fig. 12).

DISCUSSION

Sex disparities in sensitivity to air pollution exposure and the induction and exacerbation of lung disease by ozone exposure have been identified, but the hormonal basis of these differences has not yet been examined (5, 55, 74). It has also been shown that asthma worsens at differential rates in women versus men, and that this is contingent on their hormonal status, including the menstrual cycle, pregnancy, menopause, and hormone therapy (39, 76). Moreover, postmenopausal women display differing outcomes in lung health when subjected to estradiol replacement therapy (18, 49, 50). Here, we used mouse models of gonadectomy and hormone replacement to

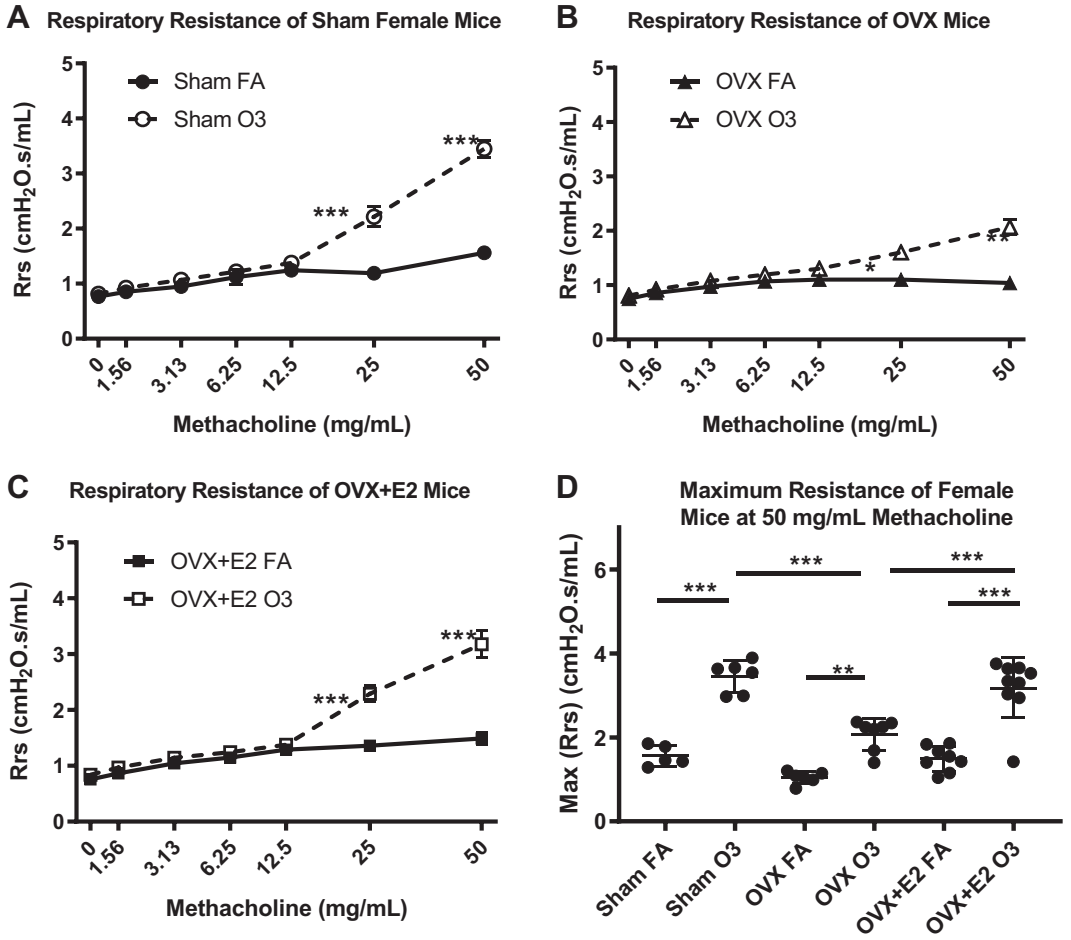

Fig. 7. Effect of ozone exposure and 17β-estradiol treatment on airway hyperresponsiveness in females. Airway resistance was measured by flexiVent in female mice exposed to ozone (O_3; dashed line) or filtered air (FA; solid line). *A–C*: respiratory system (whole lung) resistance (Rrs) in sham (control) (*A*), ovariectomized (OVX) mice (*B*), and 17β-estradiol-treated ovariectomized (OVX+E2) mice (*C*). *D*: comparison of Rrs at 50 mg/mL of methacholine among groups. Values are means ± SE of data from $n = 4–6$ mice per group (*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$).

investigate the mechanisms by which the female gonadal hormone, 17β-estradiol, modulates inflammatory responses to ozone. Specifically, we tested the hypothesis that 17β-estradiol mediates the ozone-induced inflammatory response in the female lung, but not the male lung. To distinguish between 17β-estradiol-mediated effects versus anatomical and/or physiological airway differences in the mouse lung, as well as other sex-specific endocrine effects, we conducted experiments in male and female sham and gonadectomized mice treated only with 17β-estradiol. Our results show that 17β-estradiol affects lung inflammation, AHR, and lung gene expression responses triggered in response to ozone exposure in both male and female mice, suggesting a role of 17β-estradiol in mediating sex-specific mechanisms of ozone toxicity.

Based on our preliminary studies, in which we observed exacerbated inflammation in female mice exposed to ozone in the follicular phase of the estrous cycle (i.e., when estrogen levels are high) versus the luteal phase (when estrogen levels are low), as well as recent studies showing estrogenic regulation of inflammatory responses in the lung (3, 21, 62), our main goal was to determine whether circulating 17β-estradiol levels triggered lung inflammation and AHR on ozone exposure. To test this, we exposed gonadectomized female and male C57BL/6J mice, with or without 17β-estradiol treatment, to ozone or FA. We then analyzed the overall inflammatory response by comparing lung profiles, expression of inflammatory genes, and lung mechanics. Our results show that both pulmonary function and inflammation following ozone exposure are affected by 17β-estradiol levels in a sex-dependent manner. After ozone challenge, OVX developed lower AHR and inflammation than sham females, which was characterized by a decline in airway resistance, total BALF cell numbers, neutrophilia, and lipocalin-2 levels compared with sham-operated mice. Additionally, 17β-estradiol replacement in OVX mice restored the ozone-induced AHR and cell migration to the lungs up to the levels found in sham-operated females. These data strongly suggest that 17β-estradiol exerts an important regulatory role in ozone-induced lung function and inflammation in female mice. On the other hand, we found that ozone-exposed ORX mice displayed a decrease in BALF total cell number and lipocalin-2 levels, and an increase in neutrophils and AHR compared with sham males. While the latter was ameliorated with 17β-estradiol treatment, no other measures were affected by 17β-estradiol, strongly suggesting a sex-specific effect of 17β-estradiol, and a potential involvement of androgens in the response to ozone. While previous studies

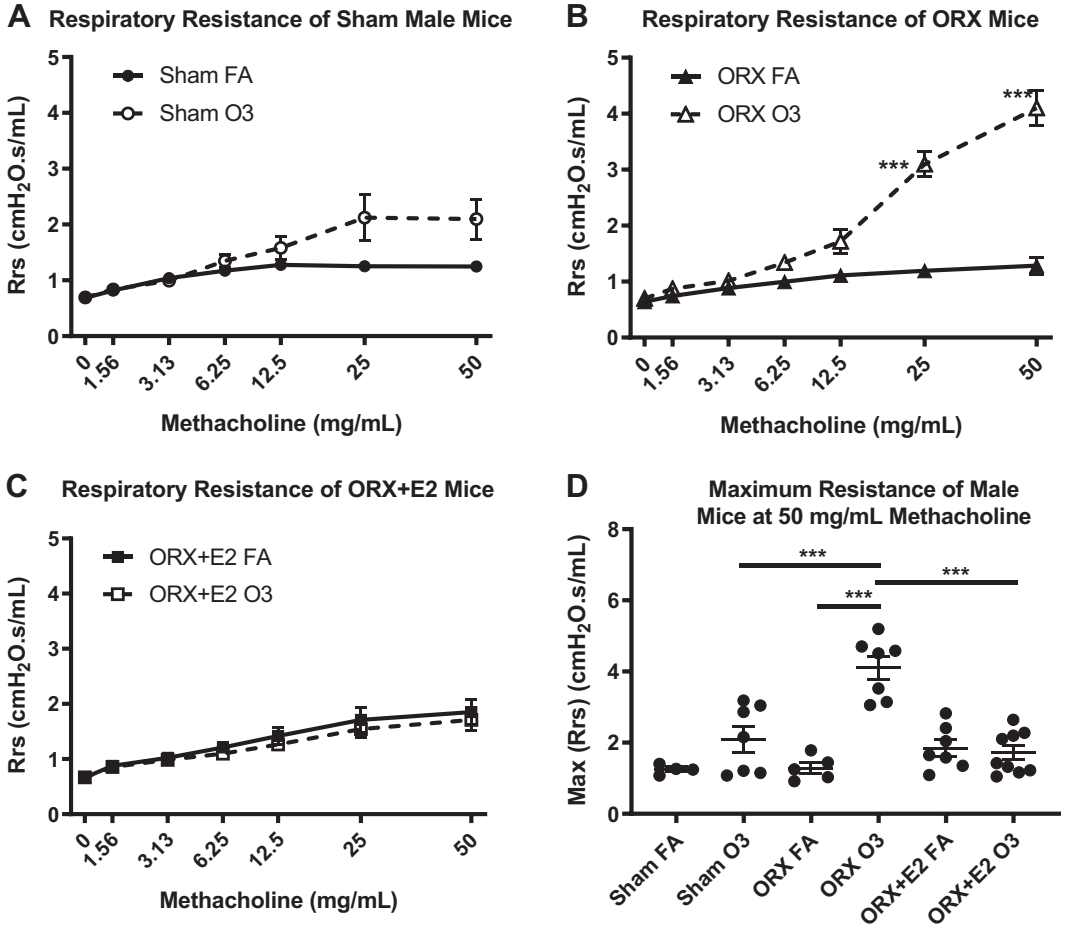

Fig. 8. Effect of ozone (O_3) exposure and 17β-estradiol treatment on airway hyperresponsiveness in males. Airway resistance was measured by flexiVent in male mice exposed to O_3 (dashed line) or filtered air (FA; solid line). *A–C*: respiratory system (whole lung) resistance (Rrs) in sham (control; *A*), orchiectomized (ORX) mice (*B*), and 17β-estradiol- treated orchiectomized (ORX+E2) mice (*C*). *D*: comparison of Rrs at 50 mg/mL of methacholine among groups. Values are means ± SE of data from $n = 4–6$ mice per group (***$P < 0.001$).

have indicated potential roles of androgens in mechanisms of lung inflammation (11, 21, 35), future research in male mice using androgen treatment and appropriate controls may help elucidate these mechanisms.

Studies in animal models of lung inflammation and disease have suggested that estradiol could be associated with disease progression and contribute to AHR development (9). In one study conducted in a rat model, female animals exposed to an allergen had increased airway inflammation compared with males (42). Interestingly, airway inflammation was decreased in allergen-exposed ovariectomized rats, and estrogen replacement reestablished lung inflammation (51). In addition, our group has previously reported sex-specific expression of inflammatory mediators and sex-specific miRNA signatures in response to ozone exposure, as well as a prospective role of circulating hormone levels in the regulation of intracellular signaling pathways (6, 34, 56). Despite this evidence, only a few studies have addressed the mechanisms by which estrogens affect the inflammatory response and lung function in response to ozone. Moreover, it has also been postulated that sex hormones can act as physiological modulators of lung function and immunity in women (1, 8, 24). However, little or nothing is known about the mechanisms by which hormones regulate pollution-induced lung responses in male and female patients.

Analysis of BALF confirmed an increase in total cell number and neutrophils in both female and male control groups, with a pronounced effect in females. OVX mice had a decrease in total cells and neutrophils that was later recovered by 17β-estradiol treatment. Moreover, the same pattern was observed in the expression of *Cxcl2* and *Il6* gene transcripts. The macrophage inflammatory protein, CXCL2, is a chemoattractant for polymorphonuclear neutrophils. Similarly, IL-6 represents a vital checkpoint regulator of neutrophil trafficking during the inflammatory response (26). It has been previously shown in vitro that 17β-estradiol increases the expression of leukocyte adhesion molecules (48, 51). This discovery allowed us to deduce that 17β-estradiol could contribute to cellular trafficking in the airways of ozone-exposed female mice.

We have previously reported that the *Ccl20*, *Il6*, and *Cxcl2* mRNAs were affected by ozone inhalation in both males and females, with higher expression in females than in males (6). Here we further showed that there was a decline in *Il6*, *Ccl20*, and *Cxcl2* transcripts in OVX mice that was partially recovered after 17β-estradiol replacement in ozone groups. This suggests that 17β-estradiol influences the expression of *Il6*, *Ccl20*, and

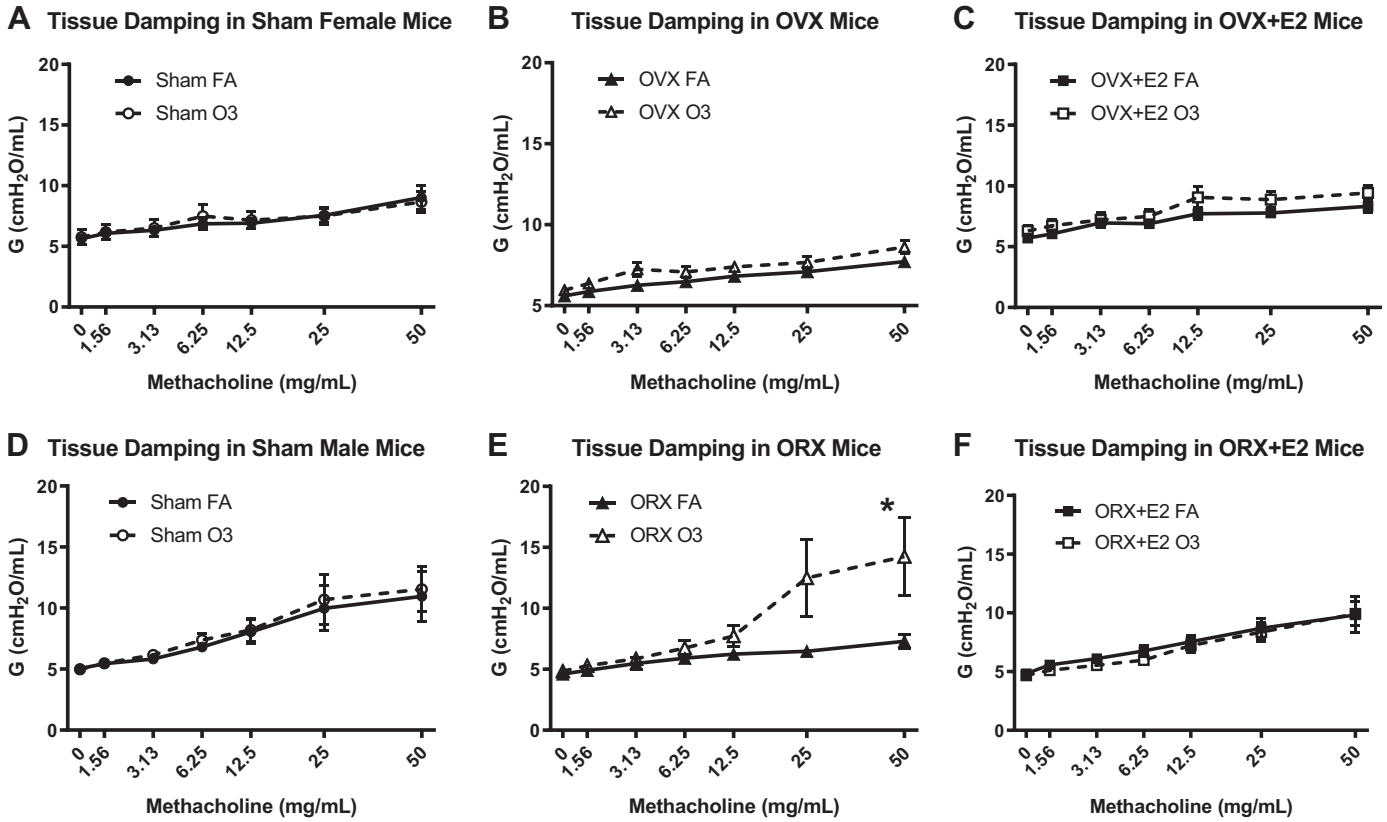

Fig. 9. Tissue damping (G) in male and female mice exposed to ozone (O_3) or filtered air (FA). G (constant phase model) was measured by flexiVent in mice exposed to O_3 (dashed line) or FA (solid line). *A–C*: females: sham (control) (*A*), ovariectomized (OVX) (*B*), and 17β-estradiol-treated ovariectomized (OVX+E2) (*C*) mice. *D–F*: males: sham (control) (*D*), orchiectomized (ORX) (*E*), and 17β-estradiol- treated orchiectomized (ORX+E2) (*F*) mice. Values are means ± SE of data from $n = 4–6$ mice per group (*$P < 0.05$).

Cxcl2 in females. Previous studies in lung cells have described high levels of *Ccl20* on exposure to air pollution and have proposed a role for this inflammatory mediator in the transition from innate to adaptive immunity and in recruitment of dendritic cells (59, 68). The MIP, *Cxcl2*, is secreted by monocytes and macrophages and is chemotactic for polymorphonuclear neutrophils. Interleukin-6 represents a vital checkpoint regulator of neutrophil trafficking during the inflammatory response by orchestrating chemokine production and leukocyte apoptosis (26). It has been previously shown in vitro that 17β-estradiol increases the expression of leukocyte adhesion molecules (48, 51). Thus this discovery allowed us to deduce that 17β-estradiol could contribute to cellular trafficking in the airways of ozone-exposed female mice. One potential mechanism by which 17β-estradiol can affect gene expression is through interaction with nuclear and membrane-bound receptors, a mechanism known to trigger intracellular mechanisms and genomic effects that ultimately affects RNA polymerase activity (33). Our unpublished studies in male and female cultured alveolar macrophages suggest that both nuclear and membrane estrogen receptors contribute to the sex-specific response to ozone and trigger an increase in expression of proinflammatory genes, including cytokines, chemokines, and enzymes involved in macrophage activation, followed by increased neutrophil cell trafficking. While we did not measure additional changes in gene expression, we do expect that multiple gene expression networks are affected by 17β-estradiol treatment due to the pleiotropic effects of estrogens and the presence of estrogen receptor elements in gene promoters (32). Regarding *Nos2* mRNA, we found significantly higher levels in ORX males exposed to ozone versus all other groups, suggesting that this gene may be regulated by androgens. The increase in *Nos2* was independent of 17β-estradiol treatment in ORX males. This is an interesting finding, since prior studies conducted in murine models of ovalbumin sensitization and challenge have reported increased levels of *Nos2* (20, 60), a phenomenon also reported in patients with asthma (41). However, a study conducted in male mice only reported no differences in *Penh* (a marker of AHR) between OVA sensitized WT versus *Nos2* knockout mice (20).

To determine whether ozone-induced lung injury was affected by gonadal hormones, we measured neutrophil gelatinase-associated lipocalin (lipocalin-2) levels in BALF. Lipocalin-2 is produced mainly by neutrophils during lung inflammation/injury (45). We found that lipocalin-2 was increased by ozone exposure in both females and males. However, lipocalin-2 decreased in OVX mice exposed to ozone and was triggered by 17β-estradiol treatment in OVX mice. Therefore, upregulation of lipocalin-2 correlates with the increase in BALF neutrophilia in females, but not males. In addition, the promoter region of the lipocalin-2 gene contains the binding sites of nuclear receptor response elements, including estrogen response elements, suggesting a potential interaction of hormone-receptor complexes with this gene promoter to control

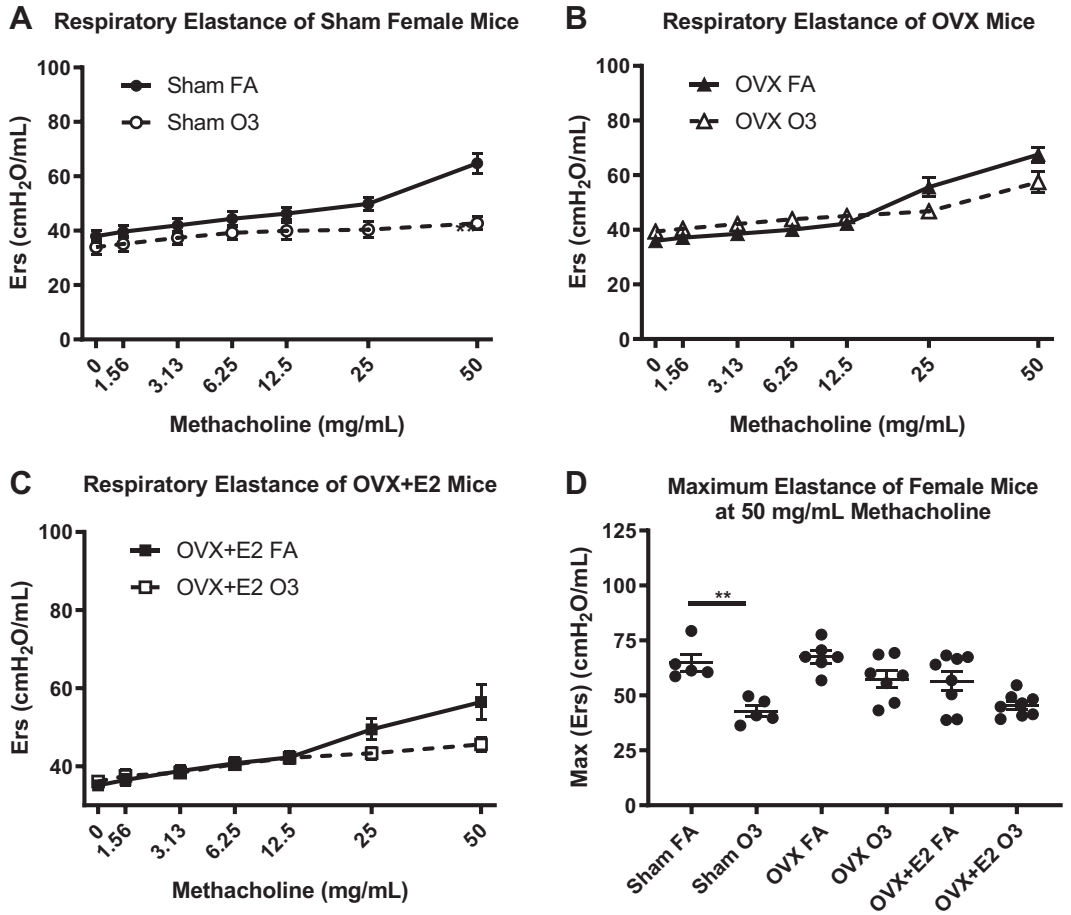

Fig. 10. Respiratory system elastance in female mice. Respiratory system (whole lung) elastance (Ers) was measured by flexiVent in female mice exposed to ozone (O_3; dashed line) or filtered air (FA; solid line). *A–C*: Ers in sham (control) (*A*), ovariectomized (OVX) mice (*B*), and 17β-estradiol-treated ovariectomized (OVX+E2) (*C*) mice. *D*: comparison of maximum Ers at 50 mg/mL of methacholine among groups. Values are means ± SE of data from $n = 4–6$ mice per group (**$P < 0.005$).

lipocalin-2 expression (63). This is dissimilar in males, where ORX displayed an increase in BALF neutrophils, but a reduction in lipocalin-2 levels. Studies have suggested that lipocalin-2 can also be produced by other tissues and cells such as adipocytes, bone marrow, immune cells, liver, spleen, and kidney in mice (78). Overall, lipocalin-2 expression may be influenced by 17β-estradiol in a cell-specific and sex-dependent manner, but more studies are needed to elucidate these mechanisms.

Ozone causes an increase in effective stiffness of the lung because of changes in the conducting airways, with more exacerbations in females (13, 74). These effects have been reported in women exposed to other air pollutants, such as particulate matter and nitrogen dioxide (75). One of the most highly characterized effects of ozone exposure is increased AHR, a main symptom of asthma (38). In this regard, studies conducted in our lab have shown differences in lung mechanics in mice exposed to ozone across the estrous cycle. Female mice exposed to ozone in the follicular phase (i.e., when estrogen levels are high) displayed significantly higher AHR than females exposed to ozone in the luteal phase (i.e., when estrogen levels are low), suggesting a role of sex hormones in altering lung physiology. However, the specific role of 17β-estradiol in controlling AHR remains to be elucidated. Here, we found that ozone was linked to 17β-estradiol changes in respiratory mechanics. Ozone-exposed OVX developed lower AHR than sham females. After 17β-estradiol replacement, OVX mice restored the ozone-induced AHR. In males, ozone-exposed ORX mice exhibited an increase AHR, compared with sham males. Surprisingly, 17β-estradiol treatment in males resulted in reduced AHR. These findings are consistent with prior studies showing that testosterone treatment can relax tracheal smooth muscle and improve lung capacity outcomes (4). Another study reported that the effects of gonadectomy in males could be associated with the role of androgens on vagus nerve-mediated reflex (7). The effect of estradiol treatment on male AHR, however, needs to be further explored. Based on these data, sex differences and sex hormones should be carefully considered when studying air pollution-induced lung function.

In summary, the results presented here suggest that ozone exposure results in sex-specific lung responses, with different thresholds for lung inflammation, lung function, gene expression, and immune cell activation. In addition, several of these actions appear to be mediated, at least in part, by 17β-estradiol, also in a sex-specific manner. Future studies should focus on the mechanisms by which 17β-estradiol, either alone or in combination with other sex hormones, induces inflammation in

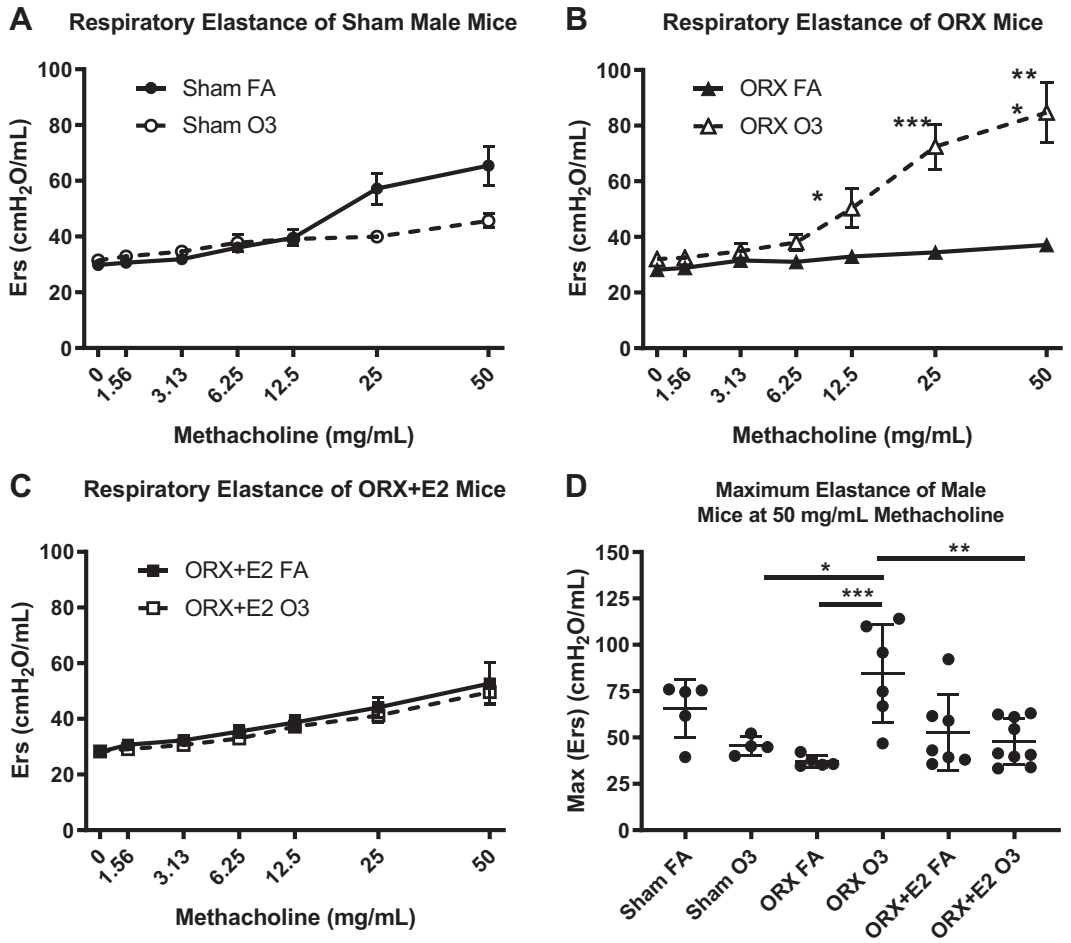

Fig. 11. Respiratory system elastance in male mice. Respiratory system (whole lung) elastance (Ers) was measured by flexiVent in male mice exposed to ozone (O_3; dashed line) or filtered air (FA; solid line). *A–C*: Ers in sham (control) (*A*), ovariectomized (OVX) mice (*B*), and 17β-estradiol-treated ovariectomized (OVX+E2) (*C*) mice. *D*: comparison of maximum Ers at 50 mg/mL of methacholine. Values are means ± SE of data from $n = 4–6$ mice per group ($^*P < 0.05$, $^{**}P < 0.003$, $^{***}P < 0.001$).

the female lung. Potential factors to consider are 17β-estradiol effects in lung epithelial and immune cells, as well as genomic and nongenomic effects of the hormone mediated by the nuclear and membrane estrogen receptors (65). With the upsurge of inflammatory respiratory disorders in women, it is imperative to increase our understanding of sex-specific mechanisms of immune response, as well as to recognize the roles of sex hormones in regulating airway inflammation, innate immunity, and other processes in the lung. Acquiring information on these sex-specific mechanisms will help us elucidate potential therapeutic targets to treat lung inflammation in a more personalized fashion and prevent pollution-induced injury in male and female patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

P.S. conceived and designed research; N.F., M.N., N.C., D.M., N.Z., and P.S. performed experiments; N.F., M.N., N.C., D.M., N.Z., and P.S. analyzed data; N.F., M.N., D.M., Z.C.C., and P.S. interpreted results of experiments; N.F., M.N., D.M., and P.S. prepared figures; N.F. and P.S. drafted manuscript; N.F., M.N., N.C., D.M., N.Z., Z.C.C., and P.S. edited and revised manuscript; N.F., M.N., N.C., D.M., N.Z., Z.C.C., and P.S. approved final version of manuscript.

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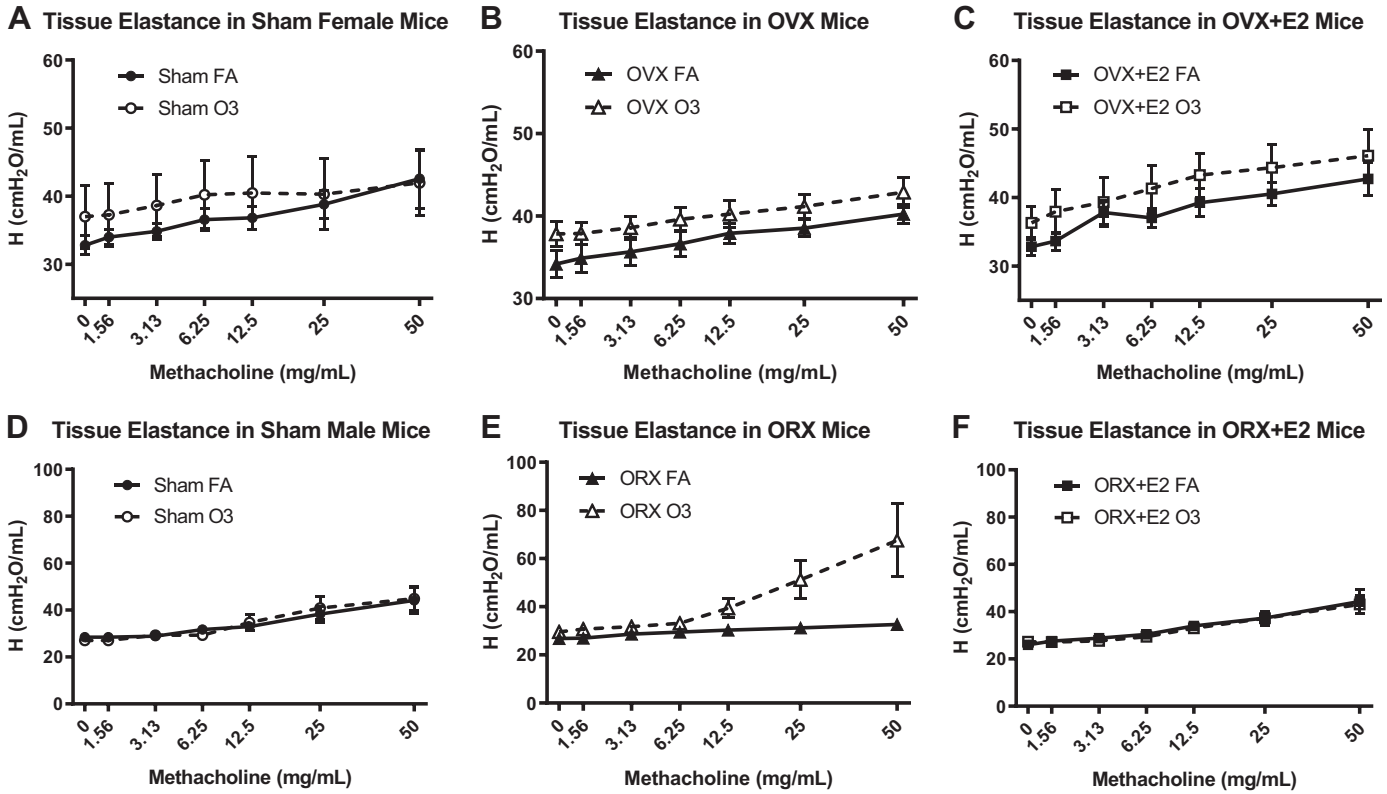

Fig. 12. Tissue elastance (H) in male and female mice exposed to ozone (O_3) or filtered air (FA). H (constant phase model) was measured by flexiVent in mice exposed to O_3 (dashed line) or FA (solid line). *A–C*: females: sham (control) (*A*), ovariectomized (OVX) (*B*), and 17β-estradiol-treated ovariectomized (OVX+E2) (*C*) mice. *D–F*: males: sham (control) (*D*), orchiectomized (ORX) (*E*), and 17β-estradiol- treated orchiectomized (ORX+E2) (*F*) mice. Values are means ± SE of data from 4–6 mice per group.

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