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Acute and Repeated Ozone Exposures Differentially Affect Circadian Clock Gene Expression in Mice

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Circadian rhythms have an established role in regulating physiological processes, such as inflammation, immunity, and metabolism. Ozone, a common environmental pollutant with strong oxidative potential, is implicated in lung inflammation/injury in asthmatics. However, whether O_3 exposure affects the expression of circadian clock genes in the lungs is not known. In this study, changes in the expression of core clock genes are analyzed in the lungs of adult female and male mice exposed to filtered air (FA) or O_3 using qRT-PCR. The findings are confirmed using an existing RNA-sequencing dataset from repeated FA- and O_3-exposed mouse lungs and validated by qRT-PCR. Acute O_3 exposure significantly alters the expression of clock genes in the lungs of females (*Per1, Cry1*, and *Rora*) and males (Per1). RNA-seq data revealing sex-based differences in clock gene expression in the airway of males (decreased *Nr1d1/Rev-erbα*) and females (increased *Skp1*), parenchyma of females and males (decreased *Nr1d1* and *Fbxl3* and increased *Bhlhe40* and *Skp1*), and alveolar macrophages of males (decreased *Arntl/Bmal1, Per1, Per2, Prkab1*, and *Prkab2*) and females (increased *Cry2, Per1, Per2, Csnk1d, Csnk1e*, *Prkab2*, and *Fbxl3*). These findings suggest that lung inflammation caused by O_3 exposure affects clock genes which may regulate key signaling pathways.

1. Introduction

Ground-level ozone (O_3) is a reactive oxidant gas and a common urban air pollutant generated by the photochemical reaction of several pollutants, particularly volatile organic compounds and nitrogen oxides. O_3 exposure has been largely associated with severe cardiopulmonary disease-related morbidity and mortality globally.[1,2] Exposure to O_3 occurs through inhalation and due to its low water-solubility, inhaled O_3 can penetrate deeply into the airways of the lung, resulting in increased lung inflammation, altered lung function and changes in epithelial barrier function.[3–8] Thus, O_3 exposure often leads to exacerbations in young and old individuals suffering from chronic inflammatory lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD).[9–11] Additionally, chronic exposure to O_3 can potentially lead to the onset of chronic respiratory conditions, such as asthma and COPD in susceptible individuals.[10,12,13] The constantly evolving regulatory policies and preventive measures worldwide have significantly improved the air quality standards and their related adverse health outcomes.[14,15] Nevertheless, exposure to an unhealthy level of ambient O_3 still occurs on a daily basis due to climate change and increasing warm temperature conditions.[16,17]

Accumulating evidence from current literature indicates that O_3 exposure induces oxidative stress, exaggerated airway inflammation, and enhances the innate immune response in the lungs.[18–20] Additionally, epidemiological and clinical studies have shown that sex (biological) and gender (social) factors can affect the susceptibility to negative effects of O_3 exposure.[21–25] The airway epithelial cells and resident alveolar macrophages play important roles during the initiation and resolution of O_3-induced lung injury.[26,27] In recent years, transcriptomic and metabolomic approaches have been widely used to understand the gene-environment interaction that occurs following O_3 exposure in male and female lungs.[7,28–30] Evidence from prior reports show that O_3 exposure transcriptionally affects lung tissues and inflammatory cells in the lungs, altering key canonical signaling pathways and molecules, such as heat-shock proteins, extracellular matrix remodeling proteins, and proin-

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flammatory signaling pathways.[7,18,28,31,32] The lungs are a very complex tissue that consists of a multitude of different cell types, and hence bulk transcriptomics data cannot direct us toward cell-type-specific changes in gene expression contributing to the observed phenotypes.[33] However, several reports from animal models of O_3 exposure have indicated compartment-specific transcriptomic changes in O_3-exposed mice compared to controls exposed to filtered air (FA).[28] These studies have revealed sex differences in the expression of inflammatory genes and microRNAs that could contribute to lung disease development and exacerbation in males and females.[3,34–37]

Circadian rhythms are intrinsic biological oscillations governed by an autoregulatory feedback loop which in turn is controlled by the core clock genes that are virtually expressed in all cells. The central clock in the suprachiasmatic nucleus of the brain entrains the peripheral tissues (lung, heart, liver, spleen, kidney, gut, etc.) via neuronal and hormonal signaling. CLOCK:BMAL1 forms the core clock complex that heterodimerizes to mediate the transcription of core clock genes, such as Period (*Per 1-3*), Cryptochrome (*Cry 1-2*), and *Rev-erbα/β*. Subsequently, PER and CRY proteins negatively regulate CLOCK:BMAL1 heterodimer-mediated transactivation function.[38] The circadian clock plays an important role in mediating several physiological processes, such as innate and adaptive immune response, inflammation, and metabolism.[39–42] It has been proposed that the circadian clock may play a role in immune function during respiratory disease and systemic inflammation.[43] We have previously reported that circadian clock disruption in the lungs occurs following acute and chronic exposure to environmental tobacco smoke (ETS)/cigarette smoke (CS) and in ETS-exposed mice infected with influenza A virus (IAV).[44–46] *Rev-erbα* (*Nr1d1*: Nuclear receptor subfamily 1 group D member 1), a key core circadian clock regulatory gene has been shown to play an important role in CS, allergen, lipopolysaccharide (LPS), and bleomycin-induced lung inflammation and injury.[46–51] Prior studies support that *Nr1d1*-based targeted therapy (e.g., Rev-erbα agonists: GSK4112, SR9009, and SR9011) may be beneficial in chronic lung diseases, such as COPD, asthma, sepsis, and pulmonary fibrosis, highlighting the importance of circadian clock regulation in pulmonary diseases.[46–53] Currently, there are no studies available that demonstrate circadian clock alteration/dysregulation in the lung following O_3 exposure.

In this study, we first aimed to determine whether or not O_3 exposure differentially affects the expression of core clock genes in the lungs of female and male mice. We chose females along with males due to the increased susceptibility to O_3-induced inflammation, based on our prior studies.[35] We next validated our findings using one previously available gene expression omnibus (GEO) dataset, an RNA-sequencing (RNA-seq) dataset from FA and O_3-exposed mice. We focused on 25 selected circadian rhythms associated genes to compare the results of qRT-PCR analysis with the RNA-seq dataset and reinforce the findings from our study (acute O_3 exposure model). This study is the first to provide evidence demonstrating that acute and repeated O_3 exposure alters the lung transcript levels of genes involved in circadian rhythms in a sex- and time-dependent manner.

2. Materials and Methods

2.1. Experimental Animals

Adult female (10 weeks of age, $n = 4–7$) and male (9 weeks of age $n = 3–4$) C57BL/6J mice for acute and repeated ozone exposure (female and male ≈7 weeks of age followed by 3 weeks acclimatization) were purchased from The Jackson Laboratories (Bar Harbor, ME) and maintained in a 12:12 h light-dark cycle, with ad libitum access to food and water. These mice were chosen based on their strain-dependent susceptibility to O_3-induced lung injury as described previously.[5,6] The Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee (for acute O_3 exposure) and Louisiana State University College of Medicine Institutional Animal Care and Use Committee (for repeated O_3 exposure) approved all animal procedures. All animal experiments were conducted as per the ARRIVE guidelines.

2.2. Acute Ozone Exposure

Adult mice were exposed to 2 ppm of ozone (O_3) or filtered air (FA: control); (between 11:00 am and 2:00 pm [Zeitgeber time ZT5-8] in females and between 12:00 pm and 3:00 pm [ZT6-9] in males) for 3 h in chambers as described previously.[35] All mice were euthanized at 21 h post-exposure (11:00 am and 2:00 pm [ZT5-8] in females and 12:00 and 3:00 pm [ZT6-9] in males) on the following day (see acute O_3 exposure model schematic **Figure 1**A). Lung tissue was harvested for gene expression analysis of selected 10 circadian clock genes, including *Clock, Arntl/Bmal1, Nr1d1/Rev-erbα, Nr1d2/Rev-erbβ, Nfil3, Per1, Per2, Cry1, Cry2,* and *Rora* by quantitative real-time PCR (qRT-PCR).

2.3. Repeated Ozone Exposure

Repeated O_3 (0.8 ppm; 4 h/day for 14 days) or FA control exposure was conducted between 6:00 and 10:00 pm [ZT12-16] in female and male C57BL/6J mice (≈7 weeks of age + 3 weeks acclimatization [≈10 weeks of age]; 5 days/week with no exposure during the weekend for the first 2 weeks followed by four consecutive days of O_3 or FA exposure) and euthanized within 12—16 h post-exposure [between ZT4-8] (see repeated O_3 exposure model schematic Figure 1B).[28] Compartment-specific isolation of airways, parenchyma, and alveolar macrophages for qRT-PCR validation of RNA-sequencing data was performed as described.[28] In brief, the extra-pulmonary airway sample includes the trachea (devoid of the first three cartilaginous rings) along with the first and second-generation extralobular airways were dissected using the dissection microscope. For parenchyma samples, 1 mm margins of the left lung lobes were trimmed. Finally, for alveolar macrophages, CD11b- microbeads were used to isolate alveolar macrophages from bronchoalveolar lavage (BAL) fluid of FA versus O_3-exposed female and male mice.

2.4. Total RNA Isolation and qRT-PCR Analysis

Lung tissue was homogenized in TRIzol reagent (LifeTechnologies, Carlsbad CA) and total RNA was extracted using the

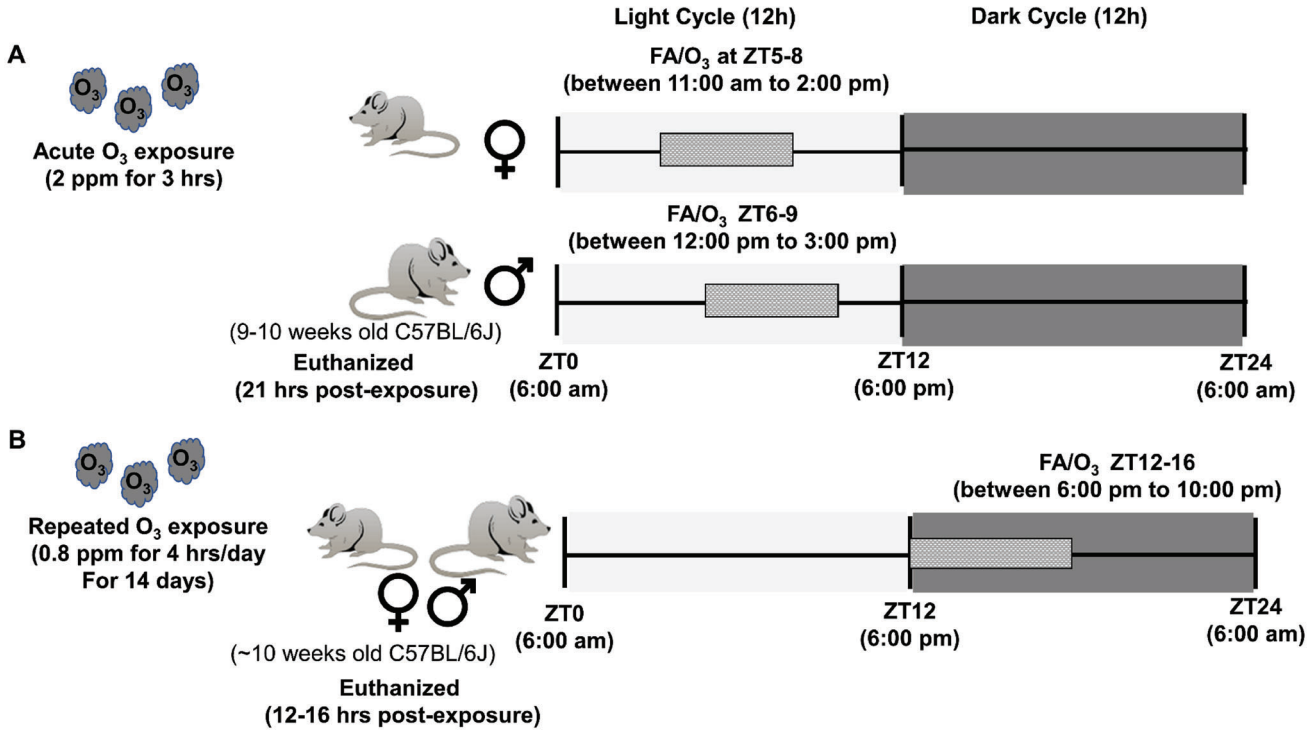

Figure 1. Schematic representation of acute and repeated O_3 exposure models used in this study. WT (C57BL/6J) female and male mice were exposed to FA or O_3 (2 ppm; 3 h during the light cycle between Zeitgeber time [ZT5-8 in females and ZT6-9 in males] for acute O_3 exposure and euthanized 21 h post-exposure) and (0.8 ppm; 4 h day^{-1} during the dark cycle between ZT12-16 [for a total of 14 days] for repeated O_3 exposure and euthanized 12–16 h post-exposure). Lung tissues were harvested to determine gene expression of circadian clock genes from acute and repeated O_3 exposures.

Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. RNA concentration and purity were determined by a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). Mouse circadian gene-specific primers (*Clock, Arntl, Nr1d1, Nr1d2, Nfil3, Per1, Per2, Cry1, Cry2, Rora, Npas2, Bhlhe40, Fbxl3*, and *Skp1*) were synthesized by IDT (www.idtdna.com) (**Table 1**). One microgram of total RNA was used for cDNA synthesis by the RT^2 first strand kit (Qiagen, Valencia, CA) and qRT-PCR runs were analyzed using a CFX Opus 96 Real-Time PCR System (Bio-Rad). Relative expression of target genes was determined by the $2^{-\Delta\Delta Ct}$ method with 18s rRNA (*Rn18s*) as housekeeping gene as described previously.[54,55]

2.5. Repeated Ozone Exposure Dataset Analyses

A dataset available from the Gene Expression Omnibus (GEO) was utilized, from previously published report, to determine the differential expression of core clock genes: Compartment-specific transcriptomics of O_3-exposed murine lungs reveal sex- and cell type-associated perturbations relevant to mucoinflammatory lung diseases GSE156799.[28] In this study, C57BL/6J female and male mice (7 weeks of age) were exposed to filtered air (FA) or repeated O_3 exposure (0.8 ppm; 4 h/night, 5 nights/week, for 3 weeks; exposures were performed between 6:00 and 10:00 pm) and mice were euthanized within 12–16 h post-exposure.[28] To determine differential expression of selected circadian clock genes, the RNA-seq fragment per kilobase per million mapped reads (FPKM) values from the respective GEO dataset and compared values between FA versus O_3-exposed mice. For detailed materials and methods, please refer to the original article.[28]

2.6. Gene Expression Analyses

The dataset with an accession number GSE156799 was downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). This dataset GSE156799 contains compartment-specific transcriptomic analyses of O_3-exposed murine lungs that showed sex- and cell type-specific changes to mucoinflammatory lung diseases. Adult mice (females and males) were repeatedly exposed to 0.8 ppm O_3 or FA between 6:00 and 10:00 pm, and RNA-seq was performed from airway epithelium enriched airways, parenchyma, and isolated alveolar macrophages on a platform of the Illumina NovaSeq 6000 as described previously.[28]

2.7. Online Tools for Hierarchical Cluster Analysis

Hierarchical clustering was performed using the FPKM values for 25 circadian clock genes obtained from GSE156799 dataset with the help of the Morpheus tool (https://software.broadinstitute.org/morpheus/) by selecting the option metric: one minus Pearson correlation, linkage method: average; cluster: rows and columns.

Table 1. qRT-PCR primer pairs for circadian clock and housekeeping genes used in this study.

Genes	Primer sequence
Clock	(F) 5′-GGAGTCTCCAACACCCACAG-3′
	(R) 5′-GGCACGTGAAAGAAAAGCAC-3′
Arntl/Bmal1	(F) 5′-AAGGGCCACTGTAGTTGCTG-3′
	(R) 5′-CTGCAGTGAATGCTTTTGGA-3′
Nr1d1/Rev-erbα	(F) 5′-GAGTCAGGGACTGGAAGCTG-3′
	(R) 5′-AAGACATGACGACCCTGGAC-3′
Nr1d2/Rev-erbβ	(F) 5′-TGGAGGCAGAGCTAGAGGAA-3′
	(R) 5′-ACCCGGTGCTCATGATGT- 3′
Nfil3	(F) 5′-GAACTCTGCCTTAGCTGAGGT-3′
	(R) 5′-ATTCCCGTTTTCTCCGACACG-3′
Per1	(F) 5′-AACGCTTTGCTTTAGATCGG-3′
	(R) 5′-TCCTCAACCGCTTCAGAGAT-3′;
Per2	(F) 5′-CTTGGGGAGAAGTCCACGTA-3′
	(R) 5′-TACTGGGACTAGCGGCTCC-3′
Cry1	(F) 5′-GTCCCCGAATCACAAACAGA-3′
	(R) 5′-TGCGTCTATATCCTCGACCC-3′
Cry2	(F) 5′-TCCCCGGACTACAAACAGAC-3′
	(R) 5′-GTCTACATCCTCGACCCGTG-3
Rora	(F) 5′-TTGCAGCCTTCACACGTAAT-3′
	(R) 5′-AGGCAGAGCTATGCGAGC-3′
Bhlhe40	(F) 5′-ACGGAGACCTGTCAGGGATG-3′
	(R) 5′-GGCAGTTTGTAAGTTTCCTTGC-3′
Fbxl3	(F) 5′-CCTGACTTGTGGCGATGTTTT-3′
	(R) 5′-ACTGTAGGTGGTTTGAGTGCC-3′
Skp1	(F) 5′-ATGCCTACGATAAAGTTGCAGAG-3′
	(R) 5′-TCCATTCCCAAATCTTCCAGC-3′
Rn18s	(F) 5′-GTAACCCGTTGAACCCCATT-3′
	(R) 5′-CCATCCAATCGGTAGTAGCG-3′

2.8. Statistical Analysis

Statistical significance was calculated between the two groups FA versus O_3 using unpaired Student's *t*-test. The probability of significance compared with FA control was based on a two-tail *t*-test. Statistical differences in more than two groups (FA vs. O_3 in Females and Males) were analyzed by two-way ANOVA using Tukey's multiple-comparison test with the GraphPad Prism 9 (La Jolla, CA). For analysis of the dataset from the GEO database, differences among the experimental groups were analyzed using the appropriate statistical test described above based on the FPKM values (RNA-seq) obtained from the GEO dataset. The results were shown as means ± SEM with a $P < 0.05$ considered as statistically significant.

3. Results

3.1. Acute Ozone Exposure Differentially Affects Gene Expression of Circadian Clock Genes in the Mouse Lung

Acute 2 ppm O_3 exposure in females (between 11:00 am and 2:00 pm) showed altered gene expression of core clock genes in mouse lungs compared to FA-exposed control as confirmed by qRT-PCR. qRT-PCR analysis of 10 different clock genes (*Clock, Arntl, Nr1d1, Nr1d2, Nfil3, Per1, Per2, Cry1, Cry2*, and *Rora*) revealed that acute O_3-exposed mice displayed a significant increase in the transcript levels of *Per1, Cry1*, and *Rora* in the lungs compared to FA-exposed controls. However, we observed an increasing trend in the gene expression of remaining circadian genes, such as *Clock, Arntl, Nr1d2, Per2*, and *Cry2* in O_3-exposed female mice compared to FA control (**Figure 2**A). Acute 2 ppm O_3 exposure in males (between 1:00–3:00 and 4:00–6:00 pm) showed altered gene expression of clock genes in the lungs compared to FA-exposed control as confirmed by qRT-PCR. Selected clock genes *Bmal1/Arntl* (Aryl hydrocarbon receptor nuclear translocator like) and *Nfil3* (Nuclear factor, interleukin 3 regulated) showed a trend toward reduced expression and *Per1* was significantly reduced compared to FA-exposed control (Figure 2B). We did not compare the expression of core clock genes between sexes because the FA and O_3 (2 ppm) exposure and tissue harvest time points were different in females and males.

3.2. Repeated Ozone Exposure Differentially Affects Circadian Clock Genes in the Airways, Parenchyma and Resident Alveolar Macrophages Confirmed by RNA-seq Analysis from Existing Dataset

We utilized the existing gene expression RNA-sequencing dataset GSE156799 to analyze sex-dependent difference in the expression of core clock genes in three different compartments of the lung (airways, parenchyma, and alveolar macrophages) in female and male C57BL/6J mice exposed to either FA or O_3. Hierarchical cluster analysis of 25 selected circadian clock target genes in three different compartments of the lung: airways, parenchyma, and alveolar macrophages revealed sex-based differences in clock gene expression in different compartments of the lungs in response to O_3 versus FA exposure (**Figures 3**A–C, **4**A–C, and **5**A–C). The males exposed to O_3 showed significantly reduced expression of *Nr1d1* (*Rev-erbα*) transcript levels in the airways compared to respective FA control ($P < 0.01$). In contrast, O_3-exposed females did not show any significant change in *Nr1d1* expression in the airways compared to respective FA control (Figure 3C). Additionally, *Skp1* (S-phase kinase associated protein 1) gene expression was significantly increased in the airways of O_3-exposed females compared to respective FA control ($P < 0.01$). The males did not show any difference in the expression of *Skp1* in the airways between FA- and O_3-exposed groups (Figure 3C). The basal transcript levels of *Skp1* were significantly higher in the airway of male versus female FA groups ($P < 0.01$) (Figure 3C).

Next, we analyzed the FPKM values from lung parenchyma of FA versus O_3-exposed female and male mice. Hierarchical cluster analysis revealed a difference in the pattern of core clock gene expression among FA versus O_3-exposed female and male mice (Figure 4A,B; Figure S1, Supporting Information). Four of the clock genes *Nr1d1, Bhlhe40* (Basic helix-loop-helix family member E40), *Fbxl3* (F-box and leucine rich repeat protein 3) and *Skp1* showed altered transcript levels among the FA versus O_3-exposed female and male mice (Figure 4A–C). Similar to the findings in the lung airway, males exposed to O_3 showed significantly reduced expression of *Nr1d1*, and females exposed to O_3 showed significantly increased expression of *Skp1* in the parenchyma compared to FA control ($P < 0.01$ and $P < 0.05$ in males and females, respectively) (Figure 4C). We observed a trend

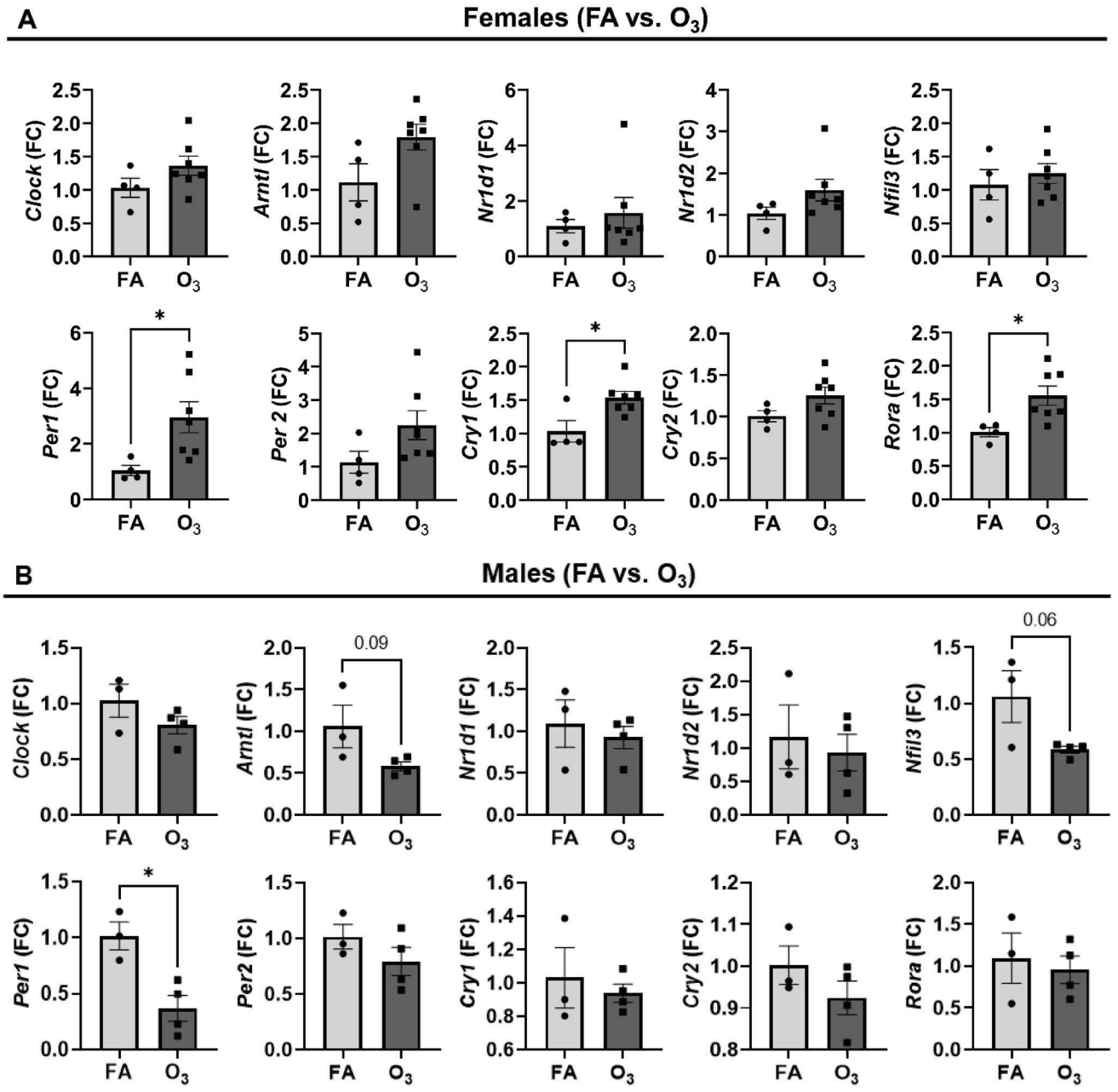

Figure 2. Acute ozone exposure differentially affects the gene expression of circadian clock genes in the lungs analyzed by qRT-PCR. Gene expression of circadian clock genes (*Clock, Arntl, Nr1d1, Nr1d2, Nfil3, Per1, Per2, Cry1, Cry2*, and *Rora*) in A) females and B) males exposed to FA versus O_3 (2 ppm) for 3 h. Data are mean ± SEM (n = 3–4 in FA group and n = 4–7 in O_3 group) and significance determined using Student's t-test. *$P < 0.05$, FA versus O_3.

in reduced expression of *Nr1d1* in females and increased *Skp1* in males exposed to O_3 compared to FA control although these results were not statistically significant. Additionally, female, and male mice exposed to O_3 showed increased transcript levels of *Bhlhe40* in the parenchyma compared to FA control ($P < 0.001$ in females and $P < 0.05$ in males) (Figure 4C). Both female and male O_3-exposed mice show reduced expression of *Fbxl3* in the parenchyma compared to FA control ($P < 0.05$ and $P < 0.01$ in females and males, respectively) (Figure 4C). The transcript levels of other clock genes were highly variable among O_3 versus FA-exposed females and males which did not reach statistical significance.

Additionally, we analyzed the FPKM values from alveolar macrophages (AMs) of FA versus O_3-exposed female and male

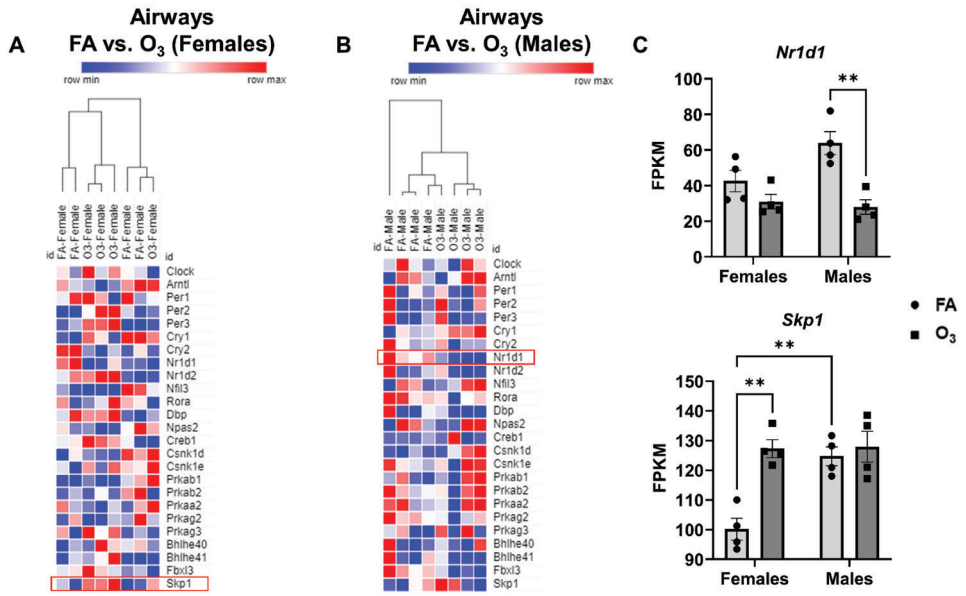

Figure 3. Repeated ozone exposure differentially affects the expression of circadian clock genes in the airways. Hierarchical cluster analysis and Heatmap showing gene expression of circadian clock genes in the airways from FA versus O_3-exposed A) female and B) male mice. C) RNA-seq analysis from GSE156799 showed differential expression of *Nr1d1* and *Skp1* genes based on their FPKM values compared among FA versus O_3-exposed female and male mice. Data are mean ± SEM ($n = 4$/group) and significance determined using 2-way ANOVA. $^{**}P < 0.01$, FA versus O_3 or FA females versus FA males.

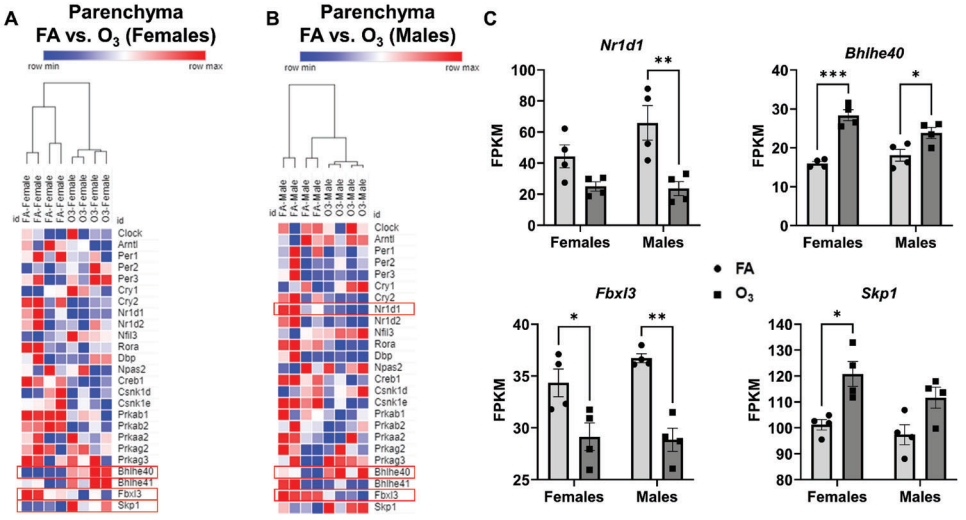

Figure 4. Repeated ozone exposure differentially affects the expression of circadian clock genes in the parenchyma. Hierarchical cluster analysis and Heatmap showing gene expression of circadian clock genes in the parenchyma from FA versus O_3-exposed A) female and B) male mice. C) RNA-seq analysis from GSE156799 showed differential expression of *Nr1d1, Bhlhe40, Fbxl3*, and *Skp1* circadian target genes based on their FPKM values compared among FA versus O_3-exposed female and male mice. Data are mean ± SEM ($n = 4$/group) and significance determined using 2-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, FA versus O_3-exposed females or males.

mice. Hierarchical cluster analysis revealed a distinct difference in the transcript levels of core clock genes between FA versus O_3-exposed female and male mice (Figure 5A,B; Figure S1, Supporting Information). Among the 25 clock genes evaluated, *Bmal1/Arntl, Cry2, Per1*, and *Per2* showed altered expression in the AMs of both females and males exposed to O_3 compared to FA control (Figure 5C). Male mice exposed to O_3 showed reduced expression of *Arntl* in the AMs ($P < 0.01$). The basal transcript levels of *Arntl* in the FA control group were significantly higher in males compared to females in the AMs ($P < 0.01$). Additionally, AMs from females exposed to O_3 showed a significant increase in the expression of *Cry2, Per1*, and *Per2* compared to those from FA control (Figure 5C), whereas AMs from males exposed to O_3 showed significantly reduced expression of *Per1* and *Per2* than

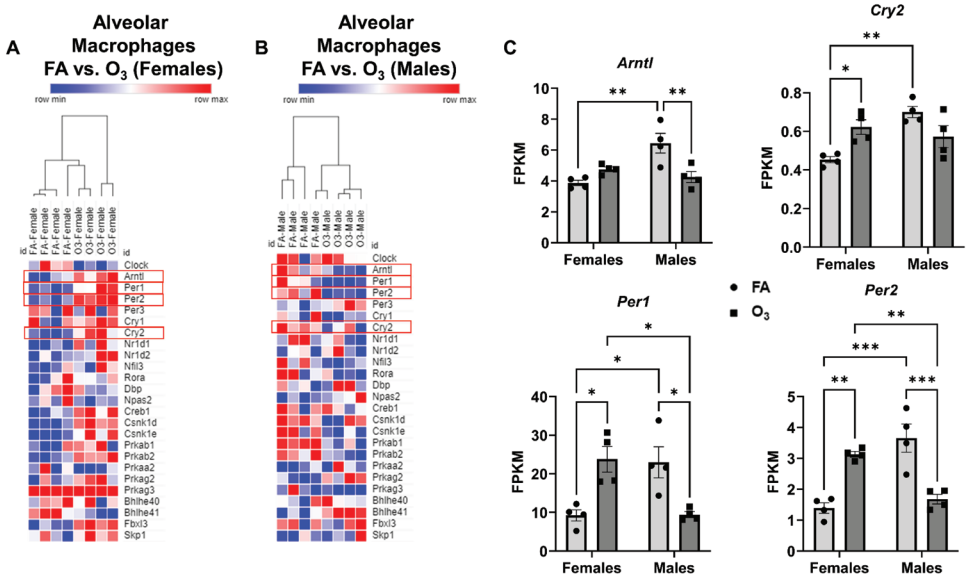

Figure 5. Repeated ozone exposure differentially affects the expression of circadian clock genes in the alveolar macrophages. Hierarchical cluster analysis and Heatmap showing gene expression of circadian clock genes in the alveolar macrophages from FA versus O_3-exposed A) female and B) male mice. C) RNA-seq analysis from GSE156799 showed differential expression of *Arntl* (*Bmal1*), *Cry2, Per1*, and *Per2* circadian target genes based on their FPKM values compared among FA versus O_3-exposed female and male mice. Data are mean ± SEM ($n = 4$/group) and significance determined using 2-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, FA versus O_3-exposed females or males; FA females versus FA males; O_3 females versus O_3 males.

those from FA control mice ($P < 0.05$ and $P < 0.001$ for *Per1* and *Per2*, respectively). The transcript levels of circadian clock genes *Arntl, Cry2, Per1*, and *Per2* were significantly higher in the AMs from FA-exposed male versus FA-exposed female mice (Figure 5C). Furthermore, transcript levels of a few other circadian genes were altered among females and males exposed to O_3 compared to FA control. These include protein kinases (*Csnk1d, Csnk1e, Prkab1*, and *Prkab2*), ligases (*Fbxl3*) and other interacting targets (*Bhlhe41*) (Figure S2, Supporting Information). Among these, the females exposed to O_3 showed a significant increase in the expression of protein kinase genes *Csnk1d, Csnk1e*, and *Prkab2* in the AMs compared to FA control (Figure S2, Supporting Information), while the males exposed to O_3 show reduced expression of *Prkab1* and *Prkab2* in the AMs compared to FA control ($P < 0.01$) (Figure S2, Supporting Information). Interestingly, O_3-exposed females show reduced, while males show increased transcript level of *Bhlhe41* in AMs compared to FA control. Female mice exposed to O_3 also showed increased *Fbxl3* expression in the AMs compared to FA control (Figure S2, Supporting Information). Finally, transcript levels of protein kinase genes *Csnk1d, Csnk1e*, and *Prkab2* and ligase gene *Fbxl3* were significantly reduced in O_3-exposed males versus females demonstrating a sex-based difference in AMs among the O_3-exposed female versus male mice (Figure S2, Supporting Information).

3.3. Repeated Ozone Exposure Differentially Affects Circadian Clock Genes in the Airways, Parenchyma, and Resident Alveolar Macrophages Validated by qRT-PCR Analysis

Total RNA from the airways, parenchyma, and alveolar macrophages of FA and repeated O_3-exposed females and males were used for qRT-PCR validation. Results revealed a sex-based difference in increased expression of *Nfil3, Per1, Per2, Cry1, Cry2*, and *Fbxl3* in the airway of O_3-exposed females compared to respective FA control (**Figure 6**). Additionally, we found that O_3-exposed females showed increased expression of these clock genes compared to O_3-exposed males. However, only two clock genes *Nfil3* and *Bhlhe40* were significantly altered in the lung parenchyma of O_3-exposed males and O_3-exposed females compared to respective FA controls (**Figure 7**). Finally, the expression of clock genes in alveolar macrophages from O_3-exposed females revealed increased expression of *Arntl* and *Nfil3* compared to FA control. Additionally, O_3-exposed males showed reduced expression of *Arntl, Nfil3, Cry1, Per1*, and *Fbxl3* compared to O_3-exposed females (Figure S3, Supporting Information). Together, these findings support the hypothesis that acute, as well as repeated O_3 exposures affect the expression levels of circadian clock genes in the lung. Altered expression of these genes in response to air pollution exposure may play an essential role in altered immune responses, as well as cytokine/chemokine signaling both in the structural and immune cell compartments of the lungs that may contribute to inflammation and injury.

4. Discussion

Ozone is among the major air pollutants that contribute to chronic inflammatory lung diseases, such as asthma and COPD.[1,2,9–15] This study focused on determining mRNA expression of core circadian clock genes and provide experimental evidence to show that sex-specific differences exist in three different compartments of female and male mouse lungs when exposed to O_3. Prior studies have observed sex-based differences in animal models of O_3-induced lung inflammation and injury.[4,28,34–36,56,57] A recent study showed increased total cells and neutrophils in

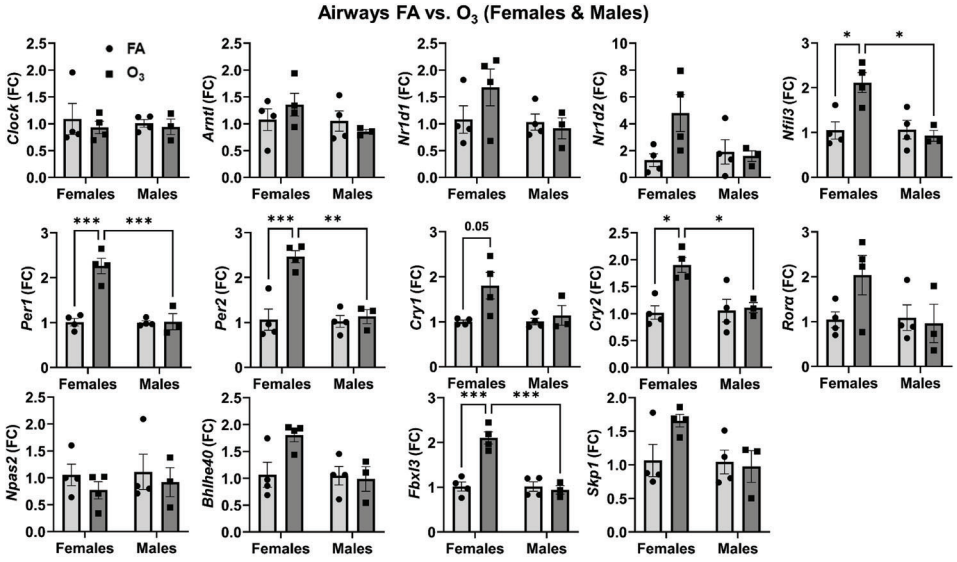

Figure 6. Repeated ozone exposure differentially affects the expression of circadian clock genes in the airways validated by qRT-PCR. Gene expression of circadian clock genes (*Clock, Arntl, Nr1d1, Nr1d2, Nfil3, Per1, Per2, Cry1, Cry2, Rora, Npas2, Bhlhe40, Fbxl3*, and *Skp1*) in females and males exposed to FA versus O_3 (0.8 ppm) for 4 h/night (5 days/week for 3 weeks). Data are mean ± SEM ($n = 4$ in FA group and $n = 3$–4 in O_3 group) and significance determined using 2-way ANOVA. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, FA versus O_3-exposed females; O_3 females versus O_3 males.

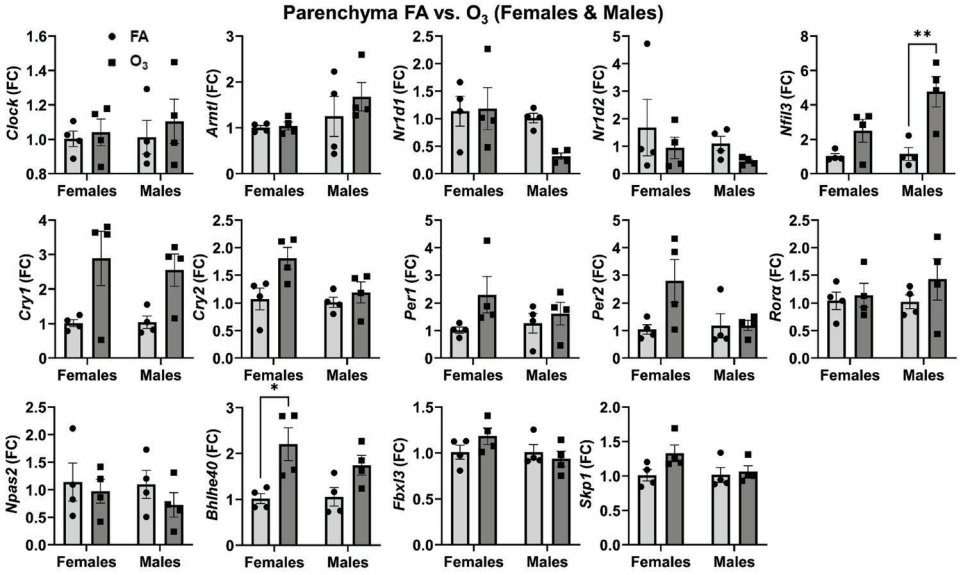

Figure 7. Repeated ozone exposure differentially affects the expression of circadian clock genes in the parenchyma validated by qRT-PCR. Gene expression of core circadian clock genes (*Clock, Arntl, Nr1d1, Nr1d2, Nfil3, Per1, Per2, Cry1, Cry2, Rora, Npas2, Bhlhe40, Fbxl3*, and *Skp1*) in females and males exposed to FA versus O_3 (0.8 ppm) for 4 h/night (5 days/week for 3 weeks). Data are mean ± SEM ($n = 4$ in FA group and $n = 4$ in O_3 group) and significance determined using 2-way ANOVA. $^*P < 0.05$, $^{**}P < 0.01$, FA versus O_3-exposed females and males.

BAL fluid of female mice associated with an increase in acute phase cytokines/chemokines, but different miRNA expression compared to male mice.[34] A more recent report validated some of these findings and identified increased expression of pro-inflammatory eicosanoids and resolvins in female mice.[56]

In this study, we found female and male mice showing a difference in the transcript levels of clock genes in the airways versus parenchyma versus alveolar macrophages may have influenced the mucoinflammation and mucous cell metaplasia-associated pathways affected differentially in repeated O_3-exposed female and male mice. Compartment-specific transcriptomic analysis of O_3-exposed mouse lungs revealed sex differences (O_3-exposed females showed more changes compared to males) in gene expression and mucoinflammatory phenotypes.[28] Pathway analysis of differentially expressed genes (DEGs) revealed cell division and DNA repair genes are among the most enriched in

O_3-exposed airways that was further supported by immunohistochemical analyses. Similarly, in O_3-exposed parenchymal tissues, cell division and DNA repair pathways along with inflammatory pathways were enriched that support the direct role of epithelial and immune cells. Furthermore, in O_3-exposed alveolar macrophages immune response and cytokine–cytokine receptor interactions were enriched.[28] Previously, adult C57BL/6J female mice when exposed to FA, 1 or 2 ppm O_3 showed DEGs analyzed by RNA-seq specifically in the conducting airway (CA) and airway macrophages (AM).[7] These studied revealed DEGs in O_3-exposed CA that were associated with epithelial barrier function (*Gjb3-5, Cldn2/4, Adam12*, and *Tgm1*), detoxification processes (*Mt1, Ugt1a6a/b*, and *Gstm1/2/6*) and cellular proliferation (Pcna and Cdh3), whereas O_3-exposed AM showed genes involved in innate immune signaling, cytokine production (*Ccl17, Slpi*, and *Ccl22*) and extracellular matrix remodeling (*Krt7, Krt8*, and *Krt18*).[7] Our analysis of GEO data from repeated O_3 exposure showed sex-dependent differences in the expression of clock genes in different compartments of FA versus O_3-exposed mouse lungs. Interestingly, *Nr1d1* (*Rev-erbα*), a nuclear receptor and transcription factor that plays an essential role in the circadian timing system, was significantly affected in the O_3-exposed airway of males and parenchyma of both males and females. Recently, we and others have shown *Nr1d1* gene regulates pulmonary inflammation and epithelial-mesenchymal transition during cigarette smoke exposure,[46] and allergen- and LPS-induced lung injury models.[47,52] Hence, the *Nr1d1* gene may have a profound cell type-specific role in O_3-induced lung injury models which need to be further explored.

Additionally, two important genes *Skp1* and *Fbxl3* that are involved in the E3 ubiquitin ligase protein complex activity and subsequent degradation of cryptochromes (CRYs) were differentially affected in the airways of female and parenchyma of females and males. This Skp1-Cul1-Fbxl3 (SCF[Fbxl3] protein complex is well characterized for its ubiquitin ligase E3 activity. Fxbl3 is an F-box protein that exclusively mediates ubiquitination followed by degradation of CRYs and hence regulates circadian oscillation in target tissues and cells.[58] In our analysis, *Skp1* transcript levels were increased in O_3-exposed females in the airways and the parenchyma of females and males compared to FA control. Surprisingly, the expression of *Fbxl3* were only affected in the O_3-exposed parenchymal compartment of the lungs in females and males, but not in the airways. Altered expression of these genes that contribute to the E3 ubiquitin ligase complex formation and regulation might contribute to the altered expression of *Cry1* and *Cry2* observed in O_3-exposed mice compared to FA control. We observed sex differences in the expression of circadian clock genes in alveolar macrophages of females and males exposed to O_3 compared to FA controls. O_3-exposed male mice showed decreased transcript levels of clock genes *Bmal1, Per1*, and *Per2* and protein kinase genes *Prkab1*, and *Prkab2* in alveolar macrophages. However, O_3-exposed female mice showed increased transcript levels of clock genes *Cry2, Per1*, and *Per2* and other protein kinases and ligase genes, such as *Csnk1d, Csnk1e, Prkab2*, and *Fbxl3* in alveolar macrophages. Our data analysis indicate that sex differences could contribute to the altered directionality of clock gene expression in the structural versus immune compartment of the lungs. Future studies determining the status of circadian clock gene expression in the lung myeloid cell types during acute versus chronic O_3 exposure will provide better insights into the molecular mechanisms that lead to lung inflammation and injury.

Our experiments using acute O_3 exposure in female mice showed increased expression of *Per1, Cry1*, and *Rora* genes, but males showed a decreasing trend in *Bmal1* and *Nfil3*, but significantly reduced *Per1* expression in the lungs analyzed by qRT-PCR. qRT-PCR validation using samples from FA and repeated O_3-exposed airways revealed a significant increase in the expression of clock genes in the O_3-exposed females compared to FA control and O_3-exposed males (*Nfil3, Per1, Per2, Cry2*, and *Fbxl3*). Additionally, *Nfil3* was significantly increased in O_3 males versus FA control and *Bhlhe40* was significantly increased in O_3 females versus FA control in the parenchyma. Several clock genes, such as *Nfil3, Cry1, Cry2, Per1*, and *Per2* showed an increasing trend in O_3 females compared to FA control. Similar results (increased expression of *Bmal1* and *Nfil3*) were observed in the alveolar macrophages from O_3 females compared to FA control and O_3 females versus O_3 males (reduced expression of *Bmal1, Nfil3, Cry1, Per1*, and *Fbxl3*) validated by qRT-PCR analysis. Microarray data from wild-type and Tlr4-KO mice exposed to O_3 at the different time points (6, 24, and 48 h) showed Heat-shock protein 70 (HSP70) as an effector molecule downstream of TLR4 that regulates O_3-induced lung inflammation.[31] Prior evidence suggests that acute TLR4-mediated cytokine response following the LPS challenge affects the degradation of repressor protein REV-ERB*α* (NR1D1) in the lung epithelial cells and mouse lungs.[47]

Evidence from the literature indicates that glucocorticoids (GCs: steroid hormones) produced by the adrenal cortex are under the control of circadian rhythm and stress. GCs have been shown to regulate rhythms of innate and adaptive immune responses.[59] Circadian rhythms of tissue inflammation are regulated by GC-mediated inhibition of inflammatory cytokine and chemokine expression at the active phase in mice (dark cycle/night). In mice, T cells accumulate in secondary lymphoid organs (e.g., spleen, lymph nodes, and Peyer's patches) during the active phase. In contrast, T cells in blood accumulate at the resting phase (light cycle/day). The observed diurnal oscillation is lost in T cell-specific glucocorticoid receptor (GR)-deficient and glucocorticoid response element (GRE) mutant mice.[60] CXCR4 expression on T cells show diurnal oscillation similar to IL-7R in mice. This was abolished in T cell-specific CXCR4-deficient mice, suggesting that IL-7R induced by GCs is possibly involved in the diurnal oscillation of the T cell distribution.[60] Earlier reports showed the effect of ozone exposure on lymphoid cells at different times post-exposure.[61,62] They found that lymphocyte number in mediastinal lymph node changes dose-dependently at different times during the 1–28 days period (acute phase: hyperplastic increase in cell mass [days 1–7] and subacute phase: functional changes in lymphocyte reactivity [days 8–28].[61] We speculate that observed lung compartment and sex-specific differences in the gene expression of core clock genes following acute and repeated O_3 exposure conditions may be due to GC-mediated change in innate and adaptive immune response that differentially affects both the structural and immune cells in the lung which has to be systematically explored.

This study has some limitations. First, lung tissues from female and male C57BL/6J mice (9–10 weeks of age) exposed to acute O_3 or FA for 3 h during the light cycle/resting phase and

analyzed 24 h post exposure at different times were used for the qRT-PCR analysis of circadian clock genes from a prior study.[35] Second, we utilized a different repetitive O_3 exposure model (FA or O_3 exposure for 4 h/ night, 5 nights/ week, for a total of 3 weeks during the dark cycle/active phase) from the available GEO dataset to demonstrate the proof of concept that circadian clock genes were altered following repetitive O_3 exposure in mouse lungs.[28] Third, we were unable to perform a comparative analysis of sex-based differences in the expression of clock genes in acute O_3-exposed female and male mice since the experiments were not conducted in parallel (time of exposure and tissue harvest were not the same). In the FA- and repeated O_3-exposed group validated by qRT-PCR, we had limited samples available in alveolar macrophages from females ($n = 2$/group) which limited the statistical analysis of the data presented in Figure S3 (Supporting Information), although the trends in FPKM and qRT-PCR data were similar for specific clock genes (e.g., *Cry2, Per1, Per2*, and *Fbxl3* variably between females and males). Additional data to evaluate whether single versus repeated O_3 exposure develops tolerance in vivo and if there is a correlation in mRNA versus protein levels of altered clock targets in different lung compartments which is beyond the focus of the current study. We strongly suggest based on prior evidence from other lung injury models (e.g., air pollution, cigarette smoke, viral and bacterial infection) that the circadian clock has a profound role in regulating lung inflammation and immune response following O_3 exposure in a cell-type dependent manner. Future studies will investigate on the acute versus chronic O_3 exposure-induced inflammatory response and associated lung pathophysiological changes using transcriptomics and proteomics approaches.

This study for the first time provides evidence that circadian clock genes were altered during O_3-induced lung inflammation and injury in the lungs. Our findings suggest that selected core clock genes, such as *Bmal1, Nr1d 1–2, Per 1–3*, and *Cry 1–2* may have a profound cell type-specific role in modulating inflammation, immune response and DNA repair following acute versus repeated O_3 exposure which need to be further investigated. It is possible that both stress hormones, as well as sex hormones, may have directly or indirectly contribute to the sex difference in circadian clock gene expression observed among female and male mice exposed to O_3 compared to FA control. Additionally, there could be a time-of-day effect in the measured outcomes that may lead to increased expression of certain clock genes whereas reduced expression of other clock genes. Utilizing targeted circadian clock deletion global and lung cell-type specific transgenic mice for acute and chronic studies will provide a better understanding of the novel role of the molecular clock in O_3-induced lung inflammation and injury.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

I.K.S., S.K.D., I.C., Y.S., and P.S.: designed the study and conducted the experiments; I.K.S.: primarily responsible for the experimental design, critical interpretation of the data, preparation of figures, and writing of the entire manuscript. P.S. and Y.S.: provided FA and O_3-exposed mouse lungs from acute and repetitive exposures analyzed in this study. All the authors checked the content and approved the final version of the revised manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

circadian rhythms, lung inflammation, ozone, sex differences, transcriptomics

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