# OZONE-INDUCED LUNG INJURY AND INFLAMMATION ARE MODULATED BY CIRCULATING STRESS HORMONES

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**ABSTRACT**

Andres Ruben Henriquez Coria: Ozone-induced lung injury and inflammation are modulated by circulating stress hormones
(Under the direction of Urmila P. Kodavanti)

Air pollution has been associated with a wide-range of health effects. Pulmonary effects of acute ozone inhalation have been widely characterized in humans and animals. These include decrements in lung function, neutrophilic inflammation, and increased epithelial permeability. Ozone has also been shown to induce cardiovascular, neurological, metabolic and immune effects. It is believed that the “spillover” of bioactive lung-derived molecules to the circulation is responsible for extra-pulmonary effects of ozone. Our lab has recently shown that ozone, in addition to pulmonary effects, induces widespread systemic metabolic and immune effects in rats and humans. Importantly, these systemic changes are associated with increased circulating stress hormones, such as corticosterone and epinephrine, which are known to be involved in the “fight-or-flight” response. In our intervention study, we noted that extra-pulmonary but also pulmonary effects of ozone were dramatically reduced in adrenalectomized rats, indicating that circulating stress hormones contributed to all ozone effects.

Since neuroendocrine stress response targets two essential survival processes, metabolic and immune, in this project we characterized in detail how pulmonary immune response and injury are influenced by the enhanced release of circulating stress hormones after ozone inhalation. In the first study, we demonstrated that the circulating stress hormones act at a transcriptional level to change gene expression after ozone exposure since global pulmonary gene changes induced by ozone exposure were attenuated in adrenalectomized rats. These

ozone-induced expression changes depicted similarity to those predicted by glucocorticoid and adrenergic receptor activation, suggesting the contribution of stress hormones in mediating transcriptional effects. In our second round of studies, we demonstrated that by blocking endogenous stress hormone signaling adrenergic and glucocorticoid receptor antagonists, ozone-induced lung injury and inflammation were reduced in a receptor-specific manner (i.e. β adrenergic blocker inhibited lung neutrophilia while glucocorticoid receptor blocker caused lymphopenia). These results validated the role of stress hormones as bona fide circulating mediators acting directly on the lungs, and provided insights on the role of corticosterone versus epinephrine as individual modulators of ozone effects. In the third study, we combined surgical and pharmacological approaches, and demonstrated that the ozone-injury and inflammation phenotypes can be restored in adrenalectomized rats by providing exogenous glucocorticoid and β-adrenergic receptor agonists. These findings demonstrated unequivocally that stress hormones are the key systemic mediators of ozone-induced lung injury and innate immune effects. Thus, we show that circulating stress hormones, released from adrenal glands in response to ozone exposure, through their effects on β-adrenergic and glucocorticoid receptors, mediate most known pulmonary injury and innate immune effects. Our studies highlight the role of neuroendocrine stress response as a potential key regulator of air pollution health effects. Any perturbations in this neuroendocrine pathway and/or β-adrenergic and glucocorticoid receptor signaling through disease processes or the use of adrenergic or steroidal therapies, are likely to play a central role in susceptibility to air pollution health effects.

Dedicated to Dani, my wife

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## PREFACE

This project was based on the role of neuroendocrine activation in mediating in vivo effects of air pollution, being investigated in the laboratory of Urmila Kodavanti at EPA who served as my mentor. Of the two survival mechanisms induced as a result of neuroendocrine activation, namely metabolic and immune response, this project was designed to identify the role of adrenal-derived stress hormones and their receptors in mediating immune effects of ozone. In the first study, I examined the contribution of adrenal-derived stress hormones in mediating ozone-induced transcriptional response. This collaborative study involved the use of high throughput transcriptomics using Genomics core facility of the US EPA, NHEERL (Anna Fisher, Hongzu Ren and Beena Vallanat) to perform mRNAseq analysis of lung tissues samples derived from prior adrenalectomy study conducted in the laboratory by Dr. Desinia Miller. The bioinformatics of the mRNAseq data was performed by collaboration with Mr. John House and Dr. Fred Wright at the North Carolina State University. Subsequent to this study, I examined the role of stress hormone (adrenaline and glucocorticoid) receptor antagonists and also agonists in mediating ozone-induced lung injury and inflammation through collaboration with NHEERL scientific staff. Mette Schladweiler contributed by performing all general preparations to each study taking the lead in performing necropsies and tissue collection. Mr. Allen Ledbetter contributed by collecting, preparing and performing plethysmography measurements. Drs. Marie McGee/Hargrove and Janice Dye were also involved in whole body plethysmography data collection, analysis and interpretation. Ms. Judy Richards was in charge of the quantification of several biochemical /clinical assays. Ms. Wanda Williams contributed by

performing flow cytometry measurements. Kevin Mauge-Lewis (UNC) contributed by performing PCRs. Dr. Colette Miller was involved in necropsies and the critical revision of the manuscripts. Drs. Urmila Kodavanti and Sam Snow were actively involved in all the steps and tasks of the studies covered in this dissertation, including the advice on study conception and design, acquisition of data, analysis and interpretation of data and critical revision of the manuscripts. Drs. Aimen Farraj, Stephen Gavett, Wayne Cascio, and Ian Gilmour critically reviewed the first two publications included in this dissertation.

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| | |
|---|---|
| AAALAC | Association for Assessment and Accreditation of Laboratory Animal Care |
| Actb | Beta-actin |
| ACTH | Adrenocorticotrophic hormone |
| ADRB2 | $\beta_2$-adrenergic receptor gene |
| ADREX/AD | Adrenalectomy or adrenalectomized |
| ANOVA | Analysis of variance |
| AR | Adrenergic receptor |
| ATS | American Thoracic Society |
| BAL(F) | Bronchoalveolar lavage (fluid) |
| Bhlhe40 | Basic helix-loop-helix family, member e4 |
| bpm | Breaths per minute |
| BSA | Bovine serum albumin |
| CAA | Clean Air Act |
| c-AMP | Cyclic Adenosine monophosphate |
| CCAIR | Coalition for Clean Air |
| CD11b | Integrin alpha M |
| cDNA | Complementary DNA |
| CLEN | Clenbuterol |
| CNS | Central nervous system |
| CO | Carbon monoxide / Corn oil |
| COPD | Chronic obstructive pulmonary diseases |
| COX-2 | Cyclooxygenase-2 |
| CREB-1 | cAMP response element-binding protein |
| CRH | Corticotropin-releasing hormone |

| | |
|---|---|
| CRP | C-reactive protein |
| CT | Cycle threshold |
| CXCL2 | Chemokine (C-X-C motif) ligand 2 |
| CXCR2 | Interleukin 8 receptor, beta |
| DAMPs | Damage asscoiated molecular patterns |
| DEMED | Demedullation or Demedullated |
| DEX | Dexamethasone |
| EDTA | Ethylenediaminetetraacetic acid |
| EGFR | Epidermal growth factor receptor |
| EPA | Environmental Protection Agency |
| ERK5 | Mitogen-activated protein kinase 7 |
| f | Frequency |
| FC | Fold change |
| FEV1 | Forced Expiratory Volume during the first second |
| FMO | Fluorescence minus one |
| Gem | GTP-binding protein overexpressed in skeletal muscle |
| GR | Glucocorticoid receptor |
| H/LMW HA | High/Low Molecular Weight Hyaluronic Acid |
| HBSS | Hank’s Balanced Salt Solution |
| HNE | 4-hydroxynonenal |
| HPA | Hypothalamus-Pituitary-Adrenal |
| Hr/h | Hours |
| i.p. | intraperitoneal |
| IFNγ | Interferon gamma |
| IL-1 | Interleukin 1 |
| IL-10 | Interleukin 10 |

| | |
|---|---|
| IL-13 | Interleukin 13 |
| IL-4 | Interleukin 4 |
| IL-5 | Interleukin 5 |
| IL-6 | Interleukin 6 |
| IL-8 | Interleukin 8 |
| IPA | Ingenuity Pathway Analysis |
| JAK/STAT | Janus kinase/ signal transducer and activator of transcription |
| KC GRO | Chemokine (C-X-C motif) ligand 1 |
| kg | Kilogram |
| LLFL | Lung Lining Fluid Layer |
| LOP | Lipid Ozonation Products |
| MAP | Mitogen-activated protein |
| MIFE | Mifepristone |
| ml | Mililitter |
| mRNA | Messenger RNA |
| MT2A | Metallothionein-2 |
| mTOR | Mechanistic target of rapamycin |
| NAAQS | National ambient air quality standards |
| NAG | N-Acetyl glucosaminidase |
| NF-kB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NHEERL | The National Health and Environmental Effects Research Laboratory |
| $NO_2$ | Nitrogen dioxide |
| Nr3c1 | Nuclear receptor subfamily 3 group C member 1 |
| NRF2 | Nuclear factor (erythroid-derived 2)-like 2 |
| NTS | Nucleus tractus Solitarius |
| $O_3$ | Ozone |

| | |
|---|---|
| PAHs | Polycyclic aromatic hydrocarbons |
| PAI-1 | Plasminogen activator inhibitor-1 |
| Pb | Lead |
| PBS | Phosphate buffered saline |
| PEF | Peak expiratory flow |
| PenH | Enhanced pause |
| PI3K | Phosphatidylinositol-4,5-bisphosphate 3-kinase |
| PIF | Peak inspiratory flow |
| Plk2 | Polo like kinase 2 |
| PM | Particulate matter |
| $PM_{10}$ | PM with an aerodynamic diameter lesser or equal to 10 micrometers |
| $PM_{2.5}$ | PM with an aerodynamic diameter lesser or equal to 2.5 micrometers |
| ppb | Parts per billion |
| ppm | Parts per million |
| PROP | Propranolol |
| PTSD | Post-traumatic stress disorder |
| qPCR | Quantitative polymerase chain reaction |
| RBC | Red blood cells |
| RH | Relative Humidity |
| ROUT | Robust regression and outlier removal |
| RT | Respiration time |
| s.c. | Subcutaneous |
| SAL | Saline |
| SAM | Sympathetic-Adrenal-Medullary |
| Sdpr | Serum deprivation-response protein |
| SEM | Standard error of the mean |

| | |
|---|---|
| SHAM/SH | Sham surgery |
| Slc19a2 | Solute carrier family 19 (thiamine transporter), member 2 |
| $SO_2$ | Sulfur Dioxide |
| Srgn | Serglycin |
| Te | Expiratory time |
| Thbd | Thrombomodulin |
| Ti | Inspiratory time |
| TLR4 | Toll-like receptor 4 |
| TNFR2 | Tumor necrosis factor receptor 2 |
| TNF-α | Tumor necrosis factor alpha |
| TRPA1 | Transient receptor potential cation channel, subfamily A, member 1 |
| Tsc22d3 | Tsc22 domain family protein 3 |
| URTI | Upper Respiratory Tract Infections |
| US /USA | United States of America |
| VOCs | Volatile organic compounds |
| NOx | Nitrogen Oxides |
| WBC | White blood cells |
| WHO | World Health Organization |
| WKY | Wistar Kyoto |
| $\beta_1$AR | Beta 1 adrenergic receptor |
| $\beta_2$AR | Beta 2 adrenergic receptor |
| βAR | Beta adrenergic receptor |
| $\mu g/m^3$ | Micrograms per cubic metter |

# CHAPTER 1: GENERAL INTRODUCTION

## 1.1 Air Pollution and Health

Air pollution has been described as the single largest environmental health risk by the World Health Organization (WHO) ("Burden of disease", 2017). Human morbidity and mortality are increased after acute and chronic exposure to air pollution (Pope and Dockery, 2006; Gotschi et al., 2008; Bell et al., 2013) with outdoor and indoor air pollutants accounting for 7 million premature deaths, or one in eight deaths globally, during 2012 ("7 million premature deaths", 2017). In addition, life expectancy reduction is associated with air pollution levels (Correia et al., 2013), and global disease burden attributable to air pollution has increased during the last 25 years because of recent demographic changes (Cohen et al., 2017).

Historically, air pollution health effects have been reported from antiquity. There is evidence of pneumoconiosis induced by dust exposures in Ancient Egypt (Cockburn et al., 1975) and old texts indicating the dissatisfaction produced by sources of odor or pestilence in Greece and Rome (Sundell, 2004). Transitioning to modernity, the development of technologies, escalation of energy production and consumption exploiting sources such as wood, coal and fossil fuels, the exponential increase in population size, and the growth of urban centers have resulted in the occurrence of extensive episodes of air pollution. "Fogs" described in Meuse Valley, Belgium (1930), Donora, US (1948), Pozo Rico, Mexico (1950), and London, England (1952) are among the most severe episodes reported during the 19$^{th}$ century causing excess human mortality (Holgate et al., [Chapter 2], 1999)

Currently, in the US, a country with relatively low levels of air pollutants ("WHO Global Urban", 2017; Friedrich, 2016), both ambient particulate matter (PM) and ozone pollution are in the top 20 risk factors of death among the population (Murray et al., 2013). In spite of massive

improvements in the air quality and an attempt for compliance with the current national standards (“Air Quality – National Summary”, 2017), air pollutants are still associated with excess mortality (Di et al., 2017) bringing attention on the lack of “safe levels” or a threshold below which air pollution effects are likely to be absent (WHO, 2013).

### 1.1.1 Air Pollutants: Types, Sources and Policies

The term “air pollution” is a wide categorical description to account for the “presence of substances in the air that are harmful to human health or toxic”. Physically, gases and particles can be distinguished in the atmosphere. Particles are microscopic solid or liquid matter forming an aerosol in the air, while compressible toxic gases are mixed in the atmosphere. The interaction and transition between particles and gases is determined by variables such as temperature, pressure, concentration and volume, which altogether determine the occurrence of physical changes and chemical reactions (Seinfeld and Pandis, 2006). Air pollutants are defined as primary when they are directly emitted into the atmosphere or secondary when they are formed in the atmosphere from reactive precursors. The source of the pollutant can be anthropogenic when humans are directly involved in their formation/release or natural such as volcanoes or marine sea salts. Multiple additional classifications have been developed to establish distinctions among pollutants such as point sources or non-point sources, stationary or mobile sources, indoor or outdoor air pollutants, etc. For particles, size becomes a key feature because it determines the penetrability to the respiratory system after inhalation, thus determining the area where these particles are deposited and react. Generally, particles with an aerodynamic diameter larger than 10 μm are retained in the extra-thoracic region such as nasal passages and larynx; particles with an aerodynamic diameter between 2.5 and 10 μm are defined as the “coarse fraction” and are retained in the tracheobronchial region; while particles with an aerodynamic diameter between 2.5 μm to 100 nm (fine fraction) and those less than 100 nm (ultrafine fraction) are retained in the alveolar region (Lippmann, 2010). From the public health perspective, extensive research and regulation has focused on the coarse ($PM_{10}$) and

fine ($PM_{2.5}$) fractions. Gases — unlike particles which are classified purely by a physical property (size) for policy purposes — are diverse, and their availability, reactivity and concentration can determine their chemical nature and toxicological potential.

Government regulations of air pollutants were established initially in developed countries to minimize the noxious effects on the population. The Clean Air Act (CAA) of 1963 was the first piece of major legislation in the US implemented with the intent to monitor and control air pollution levels. After the establishment of the Environmental Protection Agency (EPA) in 1970 and several additional laws, including major revisions to the CAA, the National Ambient Air Quality Standards (NAAQS) were developed to limit the outdoor concentrations of six key ubiquitous pollutants referred to as "criteria pollutants". Sulfur dioxide ($SO_2$), PM, carbon monoxide (CO), nitrogen dioxides ($NO_2$), lead (Pb) and ozone ($O_3$) primary and secondary standards were developed to protect human health and public welfare (Bachmann et al., 2007). NAAQS have been regularly revised based on continued research findings during the past few decades (Owens et al., 2017; Mervis, 2015). Air quality standards have also been established in Europe for benzene, arsenic (As), cadmium (Cd), nickel (Ni) and polycyclic aromatic hydrocarbons (PAHs) (Air Quality Standards, 2017). Air pollution regulations established in the US and Europe are also currently being adopted and implemented in developing countries (Giannadakci et al., 2016). In addition, other institutions (i.e. WHO), scientific associations (i.e. American Thoracic Society (ATS)) and advocacy groups (i.e. Coalition for Clean Air (CCAIR)) have developed air quality guidelines, recommendations, and educational reports on the impacts of air pollution on human health. Recently, the Green Heart Initiative was established by the EPA to educate the general public about the adverse cardiovascular health effects of inhaled pollutants.

1.1.2 Ozone, a criteria pollutant resurfaced

Ozone, at ground level, is a secondary pollutant primarily formed by the reaction of volatile organic compounds (VOCs), nitrogen oxides (NOx) and CO in the presence of sunlight (Haagen-Smit, 1963). These precursors are directly emitted by anthropogenic sources such as automobiles, motor engines, and industrial sources. Therefore, urban settings with hot tropical climates are especially prone to exhibit high ozone concentrations. Cities such as Los Angeles, CA and Houston, TX are known for photochemical smog that is enriched in secondary pollutants such as fine particles and ozone (Haagen-Smit, 1970; Couzo et al., 2013, Berlin et al., 2013). The current NAAQS for ozone is 0.07 ppm, or 140 $\mu g/m^3$, averaged over an 8-hr period ("Table of Historical Ozone National Ambient Air Quality Standards (NAAQS)", 2017). As the EPA ozone 8-hr standard has been reduced several times over the years (1997: from 0.12 to 0.08 ppm; 2008: from 0.08 to 0.075 ppm; and 2015: from 0.075 to 0.07 ppm), many areas within the US remain under nonattainment ("Ozone National Ambient Air Quality Standards", 2017). The standards set by the current European Union are even lower than that of the US (0.06 ppm). As such, ATS has recommended that the US follow similar stringent standards for this gaseous pollutant ("Air Pollution Standards", 2016). The air quality guidelines recommended by the WHO in 2005 calls for even lower standards of ozone to 0.05 ppm (WHO, 2005); however, these levels are difficult to attain in many areas falling within certain zones of the equator.

Based on the 2008 ozone standard, approximately 110 million people live in non-attainment areas in the US ("8-Hour Ozone (2008) Nonattainment Area Summary", 2017) and almost 20 million live in areas classified as extreme (>0.175 ppm) for ozone pollution including Los Angeles- South Coast Air Basin, CA and Son Joaquin Valley, CA ("Ozone Designation and Classification Information", 2017), making ozone the number 1 criteria air pollutant for non-attainment Figure 1.1A. In the international scenario, megacities such as Lagos, Beijing, Paris, Karachi, Delhi and Mumbai exhibit a similar percent of ozone episodes above 0.07 ppm to that of Los Angeles (Kornei, 2017) Figure 1.1B.

Due to their secondary nature, ozone levels exhibit high variability, both temporally and geographically. In urban settings, ozone levels generally peak in the summer and a precise daily cycle shows the occurrence of the peak in the afternoons (Wang et al., 2017; Anderson and Bell, 2010 Figure 1.1C). Although tropospheric ozone levels, and its precursors NOx and VOCs, have been decreasing during over past few years in the US (Simon et al., 2015; “Ozone trends”, 2017), climate change could significantly alter the formation of ground level ozone (Hoag, 2014; Horton et al., 2014) due to increased sunlight to react with anthropogenic and wild-fire precursors (Zhang and Wang, 2016) leading to worsening of ozone episodes (Schnell and Prather, 2016).

**Figure 1.1**

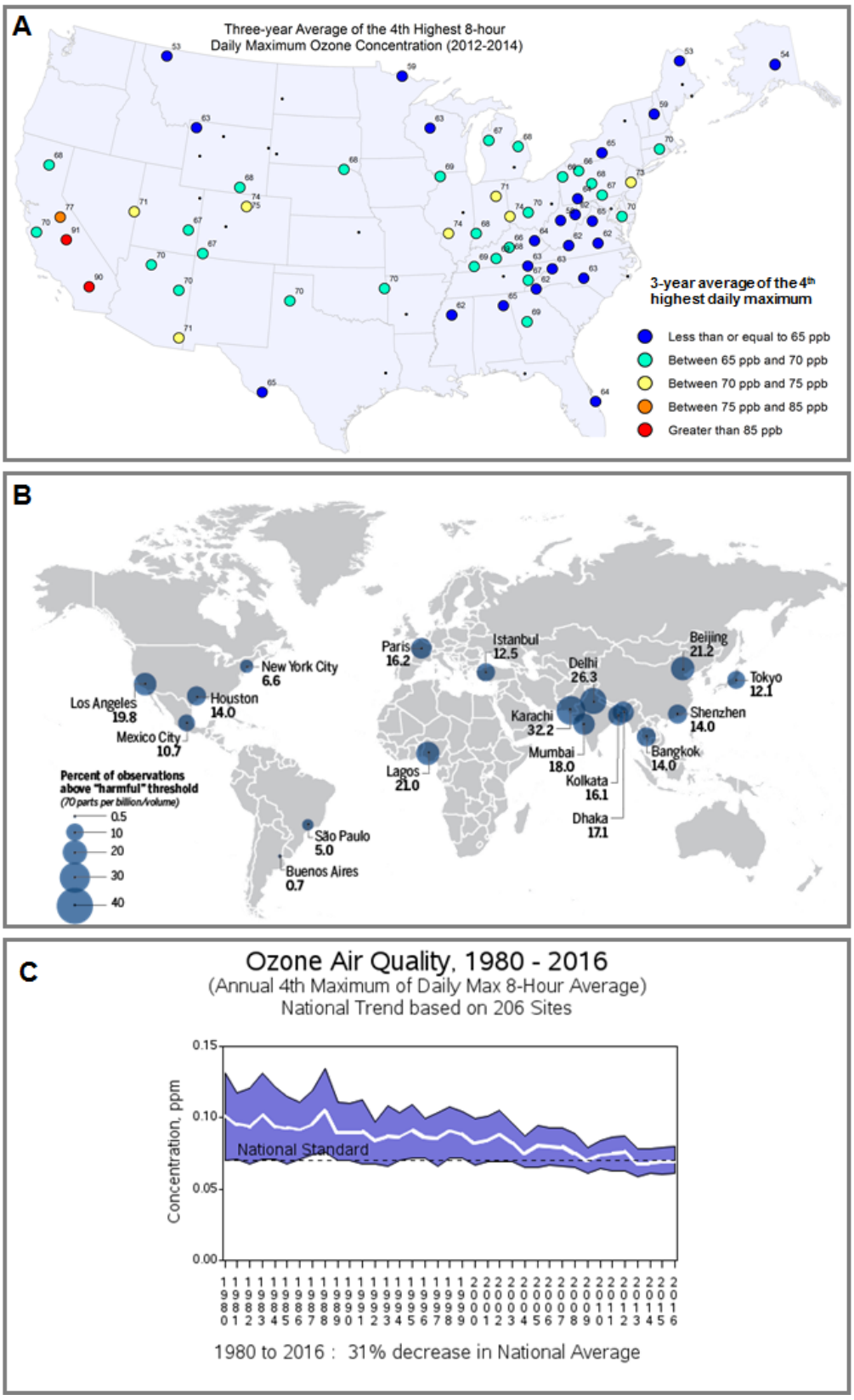

A
Three-year Average of the 4th Highest 8-hour
Daily Maximum Ozone Concentration (2012-2014)
3-year average of the 4th highest daily maximum
Less than or equal to 65 ppb
Between 65 ppb and 70 ppb
Between 70 ppb and 75 ppb
Between 75 ppb and 85 ppb
Greater than 85 ppb
B
New York City 6.6
Los Angeles 19.8
Houston 14.0
Mexico City 10.7
Percent of observations above "harmful" threshold
(70 parts per billion/volume)
0.5
10
20
30
40
São Paulo 5.0
Buenos Aires 0.7
Paris 16.2
Istanbul 12.5
Lagos 21.0
Karachi 32.2
Delhi 26.3
Mumbai 18.0
Kolkata 16.1
Dhaka 17.1
Beijing 21.2
Tokyo 12.1
Shenzhen 14.0
Bangkok 14.0
C
Ozone Air Quality, 1980 - 2016
(Annual 4th Maximum of Daily Max 8-Hour Average)
National Trend based on 206 Sites
Concentration, ppm
0.15
0.10
0.05
0.00
National Standard
1980 to 2016 : 31% decrease in National Average

**Figure 1.1 Levels of ozone in the US and around the world.** A) Categorized three-year average of the 4th highest 8-hour daily maximum ozone concentration in US urban areas (2012-2014), source: https://www3.epa.gov/airquality/greenbook/hnsum.html. B) Worldwide levels of ozone in urban centers as percent of observations above 70 ppb threshold; source: http://www.sciencemag.org/news/2017/03/here-are-some-world-s-worst-cities-air-quality c) Trends of ozone levels in US from 1980 to 2016; source: https://www.epa.gov/air-trends/ozone-trends.

## 1.2 Ozone. Health effects and toxicology

### 1.2.1 Epidemiological and clinical studies involving ozone exposure

Ozone health effects have been extensively investigated at epidemiological and toxicological levels. Epidemiological studies have shown that short- and long-term ozone exposures have been associated with increased cardiopulmonary morbidity and mortality. Short-term effects of ozone exposure have demonstrated that an increase of 10 ppb ozone concentration in the preceding week is associated with a 0.52% increase in daily mortality (Bell et al., 2004). In a database of Medicare recipients (people over 65 years old), Di et al., 2017 found that a 10 ppb increase in ozone was associated with a 1.1% increase in total mortality. Gent et al., 2003 showed that ozone, but not $PM_{2.5}$, concentrations below current NAAQS levels (0.07 ppm) significantly worsened respiratory symptoms in asthmatic children using maintenance medication. Lam et al., 2016 reported that high temperature, humidity and ozone were associated with increased summer-time hospitalizations for asthma while Byers et al., 2016 found associations between ozone exposure and asthma-related emergency department visits in Indianapolis. Similarly, Malig et al., 2016 demonstrated that in California, ozone was associated with increases in emergency department visits for asthma, pneumonia, chronic obstructive pulmonary disease (COPD) and upper respiratory tract infection (URTI). In addition, due to its irritant nature, ozone exposure is associated with increased dry eye disease prevalence in humans (Hwang et al., 2016). Collectively, these studies demonstrate that acute ozone exposure increases the risk of mortality, worsens asthma symptoms, and exerts pulmonary and extra-pulmonary physiological changes.

Controlled human exposures are limited to short-term due to technical and ethical reasons. Humans exposed to ozone at 0.06 ppm, a concentration below the current NAAQS, for 6.6 hr in chambers under controlled conditions demonstrated pulmonary neutrophilic inflammation and significant decrements in forced expiratory volume (Kim et al., 2011). Ozone at 0.08 ppm concentration for 6.6 hr also induced the recruitment of neutrophils, monocytes and

dendritic cells in bronchoalveolar lavage (BAL) fluid 18 hr after exposure (Alexis et al., 2010), while under similar settings, 0.07 ppm levels induced significant decrements in $FEV_1$ in healthy young adults (Schelegle et al., 2009). Ozone exposure at 0.1 and 0.2 ppm for 4 hr significantly changed the expression of oxidative stress response genes in BAL cells from both healthy and asthmatic volunteers (Leroy et al., 2015), and induced systemic inflammation as evidenced by increases in C-reactive protein (CRP) along with cardiac autonomic changes (Arjomandi et al., 2015). Women exposed to 0.4 ppm exhibited increased airway hyperresponsiveness, sputum neutrophils, and plasma IL-6 levels (Bennet et al., 2016). In a review by Bromberg, 2016, acute ozone exposure in humans was found to decrease vital capacity, exacerbate bronchial reactivity, increase airway permeability, induce neutrophilic inflammation, and cause a modest degree of bronchoconstriction, which are reversed completely after hours of exposure without causing chronic edema or lung disease development. These effects of ozone on humans exposed to realistic ambient levels under controlled conditions highlight the importance of further research and support the need for more stringent standards to protect human health. Frampton, 2011 summarized this concept as “this may be the time to reconsider the way we regulate criteria pollutants, and rewrite the Clean Air Act”.

The impact of long-term exposure to ozone on human health has also been studied. Jerret et al., (2009) through the analysis of 18 years follow-up data found that risk of death associated with ozone exposure was significant for respiratory (10 ppb increases the risk of death by 1.040%) but not for cardiovascular causes. A meta-analysis of multiple studies concluded that during the warm season, hazard ratios for cardiovascular and respiratory causes of death increased to 1.01 and 1.03, respectively, with 10 ppb increase in ozone concentration (Atkinson et al., 2016). Conversely, Prueitt et al., (2014) performed a weight of evidence analysis and concluded that “there is no convincing case for a causal relationship between long-term exposure to ambient ozone and adverse effect on the cardiovascular system on humans”. Although epidemiological studies have not been consistent in establishing the role of ozone as a

causal factor of pulmonary and extra-pulmonary effects after long-term exposure, short-term exposures have revealed multiple pulmonary, cardiovascular, cerebrovascular, and systemic effects.

### 1.2.2 Ozone inhalation, distribution, and absorption

Ozone penetrates to the alveoli upon inhalation and therefore, is considered a deep lung irritant. This results in exposure of all encountered sections of the respiratory tract to ozone upon inhalation. Thus, the cellular effects of ozone inhalation are likely influenced by its distribution and deposition in different parts of the respiratory system. Overall, the fate and uptake of ozone after entrance to the respiratory system is dependent on the transport (convection and diffusion), chemical processes (dissolution and reaction) and physical properties (solubility and partition coefficient) (Schlosser et al., 2010). Ozone is relatively insoluble in water and extremely reactive. Several mathematical models have been employed to determine ozone longitudinal distribution in the respiratory tract and realistic simulations have shown that a significant proportion of inhaled ozone reaches the lower respiratory tract (≈60%, Miller, 1995), the conducting lower airways (~65%), and distal areas (≈25%, Hu et al., 1992, 1994). The distribution and absorption of ozone is also changed by anatomical differences in specific subjects and sex (Bush et al., 1996).

Since rodents are obligate nose-breathers and their nasal passages are complex, once inhaled ozone and similar gases are significantly scrubbed off within the nose, their deposition in the lower respiratory tract can be reduced. However, in humans, oral respiration facilitates the direct entrance of ozone to the upper and lower respiratory tract (Kabel et al., 1985). Also, exercise favors the distal distribution of ozone to the respiratory airspaces (Ultman et al., 1994), a critical point to consider since most clinical studies with ozone are conducted during intermittent exercise.

Interspecies comparisons to elucidate the similarities of ozone internal dose among humans and rats demonstrate that resting humans and rats have similar alveolar ozone doses

(Hatch et al., 2013). Also, different publications have asserted that humans are more susceptible to ozone than rats (Gerrity and Wiester, 1987). Hatch et al., 1994 showed that 0.4 ppm of ozone induced higher levels of internal dose and injury endpoints in exercising humans when compared to resting rats. McCant et al., (2017) clarified this often made comparison by demonstrating that intermittent exercise during human exposure results in respiratory rates 4-5 times greater than a resting rat leading to differential activity states between humans and rodents and thus, the tissue dose of ozone. Also, a comparison of ozone-induced pulmonary toxicity between mice, guinea pigs and rats showed that rats were the least responsive of these three species (Dormans et al., 1999). However, often the inter-strain variations within a given laboratory animal species are remarkable, causing a spectrum of effects among all strains within a species (Kodavanti et al., 2015).

Absorption of ozone at the mucosa layer has also been studied. In Sprague-Dawley rats, total ozone uptake was around 40% of total inspired regardless of ozone concentration (Wiester et al., 1987). The penetration of ozone in the lung lining fluid layer (LLFL) is restricted due to its reactivity and limited solubility/diffusivity. Calculations have shown that this “reactive absorption” of ozone might reach the apical side of epithelial cells in the lower airways where patches of relatively thin LLFL are present (Pryor et al., 1992). LLFL presence of phospholipids, cholesterol, and proteins such as mucins will determine the formation of intermediate reactive metabolites, which will mediate the first steps of ozone-induced effects at the pulmonary level (Langford et al., 1995; Pryor et al., 1995).

### 1.2.3 Ozone-induced lung injury and inflammation: mechanisms

Mechanisms of ozone-induced lung injury at the cellular level have been reviewed in detail (Bromberg, 2016; Lippmann, 1993; Mudway and Kelly, 2000; Bhalla, 1999). Ozone exposure generates secondary reactive metabolites or bioactive products by interacting with lung lining components (signal), which are detected or recognized by cells (sensors) in the lung resulting in the changed oxidative status of the cell. This along with stimulated cell signaling

mechanisms involving MAP kinases and NRF2 results in translocation of these factors to the nucleus, DNA binding, and stimulation of pro-inflammatory and phase II metabolism gene transcription (i.e. Inflammatory cytokines and genes involved in antioxidant defense). The inflammatory cytokines and increased expression of cell surface molecules stimulate the rolling of innate immune cells in pulmonary microvasculature and their extravasation into the interstitial and airspaces. Also, it is likely that these reactive byproducts can directly damage tissues by chemical interaction, which might not depend on the recognition of signals by cell surface receptors.

The general ozone-induced lung injury paradigm involves a rapid reaction of ozone with biomolecules in the airway mucosa and alveolar surfactant. LLFL covering the apical side of epithelial cells in the respiratory system is the first substance contacting the inhaled ozone. LLFL contains water, lipids (i.e. phospholipids and cholesterol), surfactant proteins, high molecular weight glycosylated proteins (i.e. mucins), high molecular weight hyaluronic acid (HMW-HA), non-protein antioxidants (i.e. ascorbate and glutathione), iron binding proteins (i.e. transferrin and ferritin), and resident macrophages patrolling the airways and alveoli (George and Hook, 1984; Han and Mallampalli, 2015). The relative proportion and distribution of these components change throughout the respiratory tract. For example, mucus producing cells and surfactant contribute differently to mucosal architecture in the lower respiratory system and alveoli respectively, and the distribution of antioxidant molecules changes through the different stages of the respiratory tract. Also, the presence of hydrophobic surfactant proteins is restricted to alveoli (Parent et al., [Chapter 22-23], 2015)

Ozone interaction with lipids generates ozonation byproducts, which are more soluble/hydrophilic and are proposed to be involved in mediating ozone-induced lung effects. The lipid ozonation products (LOP) could directly contact cell membranes, damage phospholipids, and react with other biomolecules (Pryor et al., 1995). Oxidized lipid molecules can act as bioactive pro-inflammatory molecules. Oxidized phospholipids derived from

arachidonic acid by enzymatic and non-enzymatic reactions have also been proposed as mediators of ozone-induced oxidative stress (Alexis et al., 2000; Schulz et al., 2012). LOP increases the activity of phospholipase C, which catalyzes the conversion of phospholipids to arachidonic acid (Kafoury et al., 1998). In addition, pro-inflammatory lipids such as prostaglandins and leukotrienes are increased after ozone exposure (Hazbun et al., 1993, Alfaro et al., 2007). Leukotriene B4 antagonists were shown to protect against ozone-induced airway hyperresponsiveness (Stevens et al., 1995). Cholesterol oxidation products, known as oxysterols, have also been investigated as a mediator of ozone effects (Speen et al., 2016). Upon exposure, ozone can degrade the surfactant lipids (dipalmitoylphosphatidylcholine [DPPC]) *in vitro* (Qiao et al., 2015). Other ozone-induced lipid peroxidation products are aldehydes, such as 4-hydroxynonenal (HNE), which generates protein adducts (Kirichenko et al., 1996) and can mediate ozone-induced effects (Hamilton et al., 1998). HNE has been used as a surrogate of ozone-exposure in some studies (Hamilton et al., 1996). Ozone oxidation of surfactant proteins may reduce their activity (Hemming et al., 2015; Haque et al., 2009) and oxidized mucin proteins could generate inter- and intramolecular disulfide bonds and the formation of mucin polymers with altered viscoelastic properties (Thai et al., 2008).

Consequently, the biomolecules modified by ozone and the secondary reactive compounds generated from ozone-induced cell injury are detected as damage associated molecular patterns (DAMPs). Reactive compounds and DAMPS can activate membrane receptors such as TLR4 (Connor et al., 2012; Williams et al., 2007), TRPA1 (Taylor-Clark and Undem, 2010), and EGFR (Wu et al., 2014). Subsequent activation of intracellular signaling pathways up-regulate the transcription of pro-inflammatory cytokines with the concomitant release of proteins such as cytokines and recruitment of inflammatory cells such as neutrophils (Alexis et al., 2009; Johnston et al., 2005; Kirsten et al., 2013; Williams et al., 2007). Production of reactive oxygen species (Valavanidis et al., 2013; Wiegman et al., 2014) results in the loss of

epithelial layer integrity with the concomitant vascular leakage (Kadiiska et al., 2011; Kleeberger et al., 2001; Koren et al., 1989). The activation of neutrophils and other leukocytes in the lungs also damages the surrounding tissue inducing a positive feedback loop of pro-inflammatory activation (Auerbach and Hernandez, 2012). The process of inflammatory response once initiated after ozone exposure continues for several hours, which also triggers repair mechanisms and the injury response is fully reversed with reestablishment of normal homeostasis.

Cell-mediated effects of ozone have also been studied at the lung level. For instance, a mast cell stabilizer reduced ozone-induced lung inflammation in mice (Kleeberger et al., 1993, 1999), newly divided eosinophils limited ozone-induced airway hyperreactivity in guinea pigs (Wicher et al., 2017) and macrophage inhibition with gadolinium chloride treatment decreased ozone induced pulmonary injury and inflammation in rats (Pendino et al., 1995). A summary of selected mechanisms involved in ozone-induced injury in the respiratory tract is presented in

**Figure 1.2**

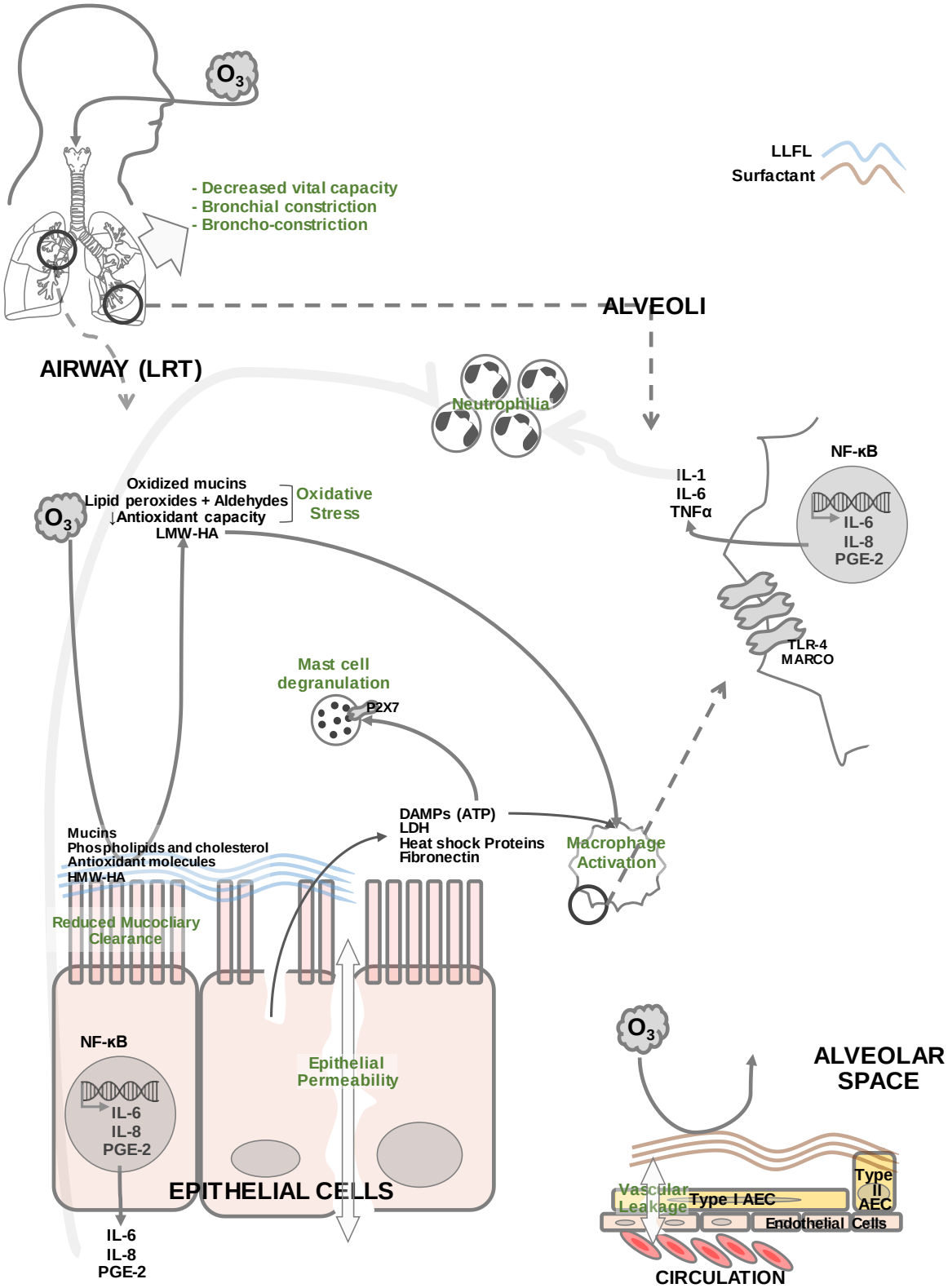

O3
- Decreased vital capacity
- Bronchial constriction
- Broncho-constriction
LLFL
Surfactant
ALVEOLI
AIRWAY (LRT)
Neutrophilia
NF-κB
IL-1
IL-6
TNFα
IL-6
IL-8
PGE-2
Oxidized mucins
Lipid peroxides + Aldehydes
↓Antioxidant capacity
LMW-HA
Oxidative Stress
O3
TLR-4
MARCO
Mast cell degranulation
P2X7
DAMPs (ATP)
LDH
Heat shock Proteins
Fibronectin
Macrophage Activation
Mucins
Phospholipids and cholesterol
Antioxidant molecules
HMW-HA
Reduced Mucociliary Clearance
NF-κB
IL-6
IL-8
PGE-2
Epithelial Permeability
EPITHELIAL CELLS
IL-6
IL-8
PGE-2
O3
ALVEOLAR SPACE
Vascular Leakage
Type I AEC
Type II AEC
Endothelial Cells
CIRCULATION

**Figure 1.2 Known mechanisms of ozone-induced lung injury and inflammation**. Adapted from Auerbach and Hernandez, 2012. AEC: Airway epithelial cell, IL: Interleukin, PGE2: Prostaglandin 2, ATP: Adenosine triphosphate, LDH: Lactate Dehydrogenase, H/LMW HA: High/Low Molecular Weight Hyaluronic Acid, DAMPs: Damage Associated Molecular Patterns, TNF-α: Tumor Necrosis Factor alpha, LRT: Lower Respiratory Tract, LLFL Lung Lining Fluid layer, TLR4: Toll-like receptor 4, P2X7: P2X purinoceptor 7.

### 1.2.4 Ozone-induced pulmonary vascular leakage

Airway permeability is increased after ozone exposure causing protein and albumin to leak from the pulmonary vasculature into the lung and ultimately the BALF, thus allowing researchers to measure the magnitude of epithelial permeability (Reinhart et al., 1998; Kleeberger et al., 2001; Gupta [Chapter 12], 2014). In contrast, specific Clara cell protein CC16 can translocate from lungs to the circulation (Broeckaert et al., 2000; Blomberg et al., 2003), demonstrating that ozone-induced vascular leakage facilitates the bidirectional translocation of biomolecules. A number of mechanisms might be involved in increasing vascular protein leakage to the Lung. Ozone exposure is known to cause epithelial injury as indicated in the earlier discussion. Especially in the alveolar region this injury to type 1 cells can lead to leakage of protein in the alveoli (Banks et al., 1990). Moreover, vascular leakage also can occur through increased activity of sympathetic nerves which may lead baroreflex-induced bradycardia. The increase in the blood flow to the lung can cause vascular congestion, and increase pressure and thus damage to the capillary walls and protein leakage into the alveoli (Šedý et al., 2015). Increased catecholamines have also been postulated to increase pulmonary edema (Krishnamoorthy et al., 2012). Ozone is known to induce bradycardia in rodents (Gordon et al., 2014), decrease blood pressure (Uchiyama et al., 1986) and produce cardiac depression (Wagner et al., 2014). These changes can increase pulmonary vascular pressure and contribute to lung protein leakage.

### 1.2.5 Systemic effects of ozone: inflammation

Extra-pulmonary effects of ozone inhalation have been long recognized (Goldstein, 1978). The range of ozone-induced extra-pulmonary effects reported in laboratory animals englobe virtually all systems. Hepatic dysregulation (Laskin et al., 1994, Theis et al., 2014), metabolomic changes (Miller et al., 2015, Mathews et al., 2017), atherogenic progression (Chuang et al., 2009, Tham et al., 2017), nervous system disruption (Chen et al., 2003,

Chounlamountry et al., 2015) and cardiovascular depression (Wagner et al., 2014, Farraj et al., 2012) represent some of the extra-pulmonary responses occurring after acute ozone-inhalation. Thomson et al., (2013) found that ozone inhalation was sufficient to change the expression of a wide variety of genes in the lung, heart, liver, kidney, spleen, and pituitary tissues. For example, ozone exposure has shown to increase the circulating levels of serum amyloid A in mice (Erickson et al., 2017), while some studies have found increases in circulating IL-8, IL-1β and decreased levels of PAI-1 in healthy volunteers exposed to 0.3 ppm of ozone during 2 hr of intermittent exercise (Devlin et al., 2012). In addition, Bhalla et al., (2002) found that intraperitoneal injection of anti-TNF-α antibody reduced ozone-induced lung injury. Based on ozone and other air pollutant studies it has been believed that the spillover of bioactive compounds from the lungs to the periphery is involved in mediating extra-pulmonary effects.

Systemic Inflammation and vascular impacts have been reported after ozone exposure in humans. Li et al., (2017) found a positive association between ozone exposure and blood levels of tumor necrosis factor receptor 2 (TNFR2) in a Boston area cohort. In a longitudinal study analyzing ozone exposure in controlled scenarios, it was found that short-term exposure was associated with increased blood pressure and platelet activation (Day et al., 2017). Acute ozone exposure has also been associated with increases in relative risk for myocardial infarction (Chiu et al., 2017) and hypertension (Coogan et al., 2017). On the other hand, there are a few negative studies showing no changes in circulating cytokines after ozone exposure in humans and in animals (Erickson et al., 2017; Urch et al., 2010; Bass et al., 2013; Miller et al., 2015).

1.2.6 Ozone effects on the central nervous system (CNS)

Ozone exposure has been recently linked to increased neurological disorders such as dementia, Alzheimer's and Parkinson's disease (Jung et al., 2015; Kirrane et al., 2015; Wu et al., 2015). It has been shown that in rats ozone inhalation promotes hippocampal neurodegeneration and reduced repair (Rivas-Arancibia et al., 2010; Solleiro-Villavicencio and

Rivas-Arancibia, 2010), as well as oxidative stress in the hippocampus mimicking molecular events exhibited in Alzheimer's disease such as amyloid overproduction and mitochondrial abnormalities (Hernandez-Zimbron and Rivas-Arancibia, 2015). Similarly, apoptosis, cell cycle and antioxidant pathways were dysregulated in the hippocampus of rats exposed to ozone (Gomez-Crisostomo et al., 2014). Gonzalez-Guevara et al., (2014) postulated that ozone-induced systemic inflammatory response was accountable for neurological inflammation characterized by high TNF-α and IL-6 levels in the brains of rats. Ozone exposure in rats was also shown to induce cell death in the substantia nigra involved in Parkinson's disease (Rivas-Arancibia et al., 2015). In the same study, the authors found increased levels of oxidized proteins and neuroinflammation associated with increased levels of NF-kB and COX-2, as well as activated astrocytes and microglia. Similarly, Mumaw et al., (2016) demonstrated that serum samples obtained from ozone-exposed rats promoted microglial activation by cytokine-independent factors. These responses were exacerbated in aged glial cells, which highlights the role of the lung-brain axis as an important communication system involved in the development of extra-pulmonary effects of ozone inhalation. Serum amyloid A, a protein capable of crossing the blood-brain-barrier, has been implicated in these ozone effects on the CNS. Martinez-Lazcano et al., (2013) reviewed the basic mechanisms by which ozone exposure may damage the CNS by highlighting the role of neuroinflammation and oxidative stress. Collectively, these studies support the conclusion that ozone exposure is associated with neurological effects although the causal mechanisms still remain speculative.

### 1.2.7 Ozone-induced autonomic effects

Ozone inhalation has been demonstrated to alter autonomic tone. Autonomic control of breathing patterns is mainly regulated by trigeminal nerve afferents, which send signals to the brainstem where breathing centers are located (Sozansky and Houser, 2014). Ozone exposure induces a rapid, shallow breathing (Lee et al., 1979; Alfaro et al., 2007), which results in a reduction in ozone deposition (Alfaro et al., 2004). In agreement with this, trigeminal nerve

irritation has been proposed as a sensory mechanism mediating ozone-induced dysregulation of breathing patterns (Kulle et al., 1975; Shusterman, 2007).

Parasympathetic tone has also been shown to increase after ozone exposure, which is associated with cardiac electrophysiological changes including bradycardia and a decrease in blood pressure in rats (Farraj et al., 2012). Along the same line, several studies have shown decreased heart rate, cardiac depression and bradycardia as a consequence of ozone exposure in animals (Gordon et al., 2014; Arito et al., 1990, 1992; Uchiyama et al., 1986) and humans (Hampel et al., 2012; Jia et al., 2011; Devlin et al., 2012). Epidemiological studies have also found lower blood pressure in ozone exposed populations (Hoffmann et al., 2012), although contradicting effects of ozone increasing blood pressure have also been reported (Coogan et al., 2017, Day et al., 2017). Parasympathetically-mediated pulmonary changes have also been examined after ozone inhalation (Jones et al., 1987). Ozone enhances bronchial reactivity to muscarinic agonists (Roum and Mourlas, 1984), and promotes a loss of M2 muscarinic receptor function (Schultheis et al., 1994; Yost et al., 1999). These studies support the role of autonomic, especially parasympathetic tone, in mediating cardiac electrophysiological changes after ozone exposure.

Ozone-induced stimulation of pulmonary sympathetic tone was postulated to protect from ozone-induced vascular leakage (Delaunois et al., 1997). Graham et al. (2001), using transgenic murine models exhibiting sympathetic hyper- and hypo-innervation, demonstrated that ozone-induced neutrophilia was directly correlated with the magnitude of the innervation. Similarly, the overexpression of $\beta_2$AR in airway epithelial cells decreased ozone-induced vasoconstriction in mice (McGraw et al., 2000). However, another study showed that sympathetic nerve traffic was not changed by acute ozone-induced airway inflammation in humans (Tank et al., 2011), which might be due to the lack of dynamic assessment. Nevertheless, the above studies emphasize the likely involvement of the autonomic system in mediating ozone-induced cardiovascular, and, perhaps also, pulmonary effects.

Another major centrally-mediated mechanism altered during ozone exposure is hypothermia. The hypothalamus plays an important role in thermoregulation (Morrison and Nakamura, 2011). Acute ozone exposure stimulation of hypothermia in rodents (Mautz and Bufalino, 1989) and reptiles (Mautz and Dohm, 2004) is absent in humans (Watkinson and Gordon, 1993). It has been hypothesized that the decrease in core body temperature might serve as an adaptive mechanism to reduce metabolic rate, and thus, the adversity of ozone-induced changes (Gordon et al., 2014).

Finally, behavioral effects of ozone exposure have also been reported (Weiss et al., 1981). Animal experiments have shown dysregulation of sleep patterns (Alfaro-Rodriguez and Gonzalez-Pina, 2005), motor activity (Gordon et al., 2013), decrease exploratory and increased freezing behaviors (Rivas-Arancibia, et al., 2003), as well as overall performance in mice (Sorace et al., 2001) after ozone exposure. Epidemiological studies have shown adverse neurobehavioral effects of ambient air pollutants including ozone (Chen and Schwartz, 2009), and an association between ozone levels and suicides (Biermann et al., 2009). These studies support the involvement of autonomic and other neuronal effects following acute ozone inhalation.

*1.2.7.1 The role of vagal C fibers and peripheral neurons in ozone-induced cardiopulmonary changes*

Pulmonary vagus innervation involving afferent sensory and efferent neurons encompassing both parasympathetic and sympathetic nerves might play an important role in the mediation of ozone-induced responses. Changes in vagal reflex tone are linked to ozone-induced responses in rabbits (Freed et al., 1996). Bilateral vagotomy enhanced peripheral lung reactivity in ozone-exposed rats, suggesting a protective role of vagal reflex stimulation (Ho et al., 1998). In a different strategy, Schelegle et al., (1993) demonstrated that blocking canine vagal C fibers in the lower airways by cooling them abolished ozone-induced effects such as shallow breathing and bronchoconstriction. Autonomic control of blood pressure has also been

studied in relation with ozone exposures. These studies in general support the involvement of autonomic regulation in the cardiophysiological effects of ozone.

Because of its chemical reactivity, ozone is able to activate nerve endings in the airways. Peripheral C fibers located in the respiratory tract are activated by ozone (Jimba et al., 1995; Coleridge et al., 1993; Joad et al., 1996). The depletion of C fibers by capsaicin administration prevented ozone-induced changes in breathing patterns and epithelial injury (Vesely et al., 1999). Ozone-induced chest pain has been correlated with its effect on C fibers causing neuropathic pain (McDonnell et al., 1983) and throat and eye irritation (Tepper and Wood, 1985; Hoppe et al., 1995). C fibers contain ion channels such as TRPA1, which are selectively activated by ozone inhalation (Taylor-Clark and Undem, 2010). TRPA1 knock-out mice were protected against the effects of acrolein, a gas irritant (Kurhanewicz et al., 2017). Neural reflex activation has been proposed as a plausible mode of action determining ozone-induced lung function decrements (Prueitt and Goodman, 2016). Ozone interacts with sensory nerves in bronchial mucosa (Krishna et al., 1997) and triggers the secretion of bioactive neuropeptides such as substance P (Nishiyama et al., 1998; Schierhorn et al., 2002), nerve growth factor (Verhein et al., 2011; Barker et al., 2015), calcitonin gene-related peptide (Oslund et al., 2009; Wu et al., 2007), neurokinin A (Oslund et al., 2008), and tachykinins (Takebayashi et al., 1985). Thus, the experimental evidence shows that ozone is capable of generating sensory signals through the activation of C fibers and the local release of neurokinins and tachykinins, which often have been linked to pulmonary effects of ozone and the generation of reflex cardiopulmonary physiological changes.

*1.2.7.2 Consequences of ozone activation of stress responsive regions in CNS*

The most convincing evidence of an ozone impact on brain regions responsive to stress comes from a number of earlier studies. Neural disturbances associated with catecholaminergic changes in brain areas such as locus coeruleus, striatum and nucleus tractus solitarius (NTS) involved in chemosensory, arousal and motor control were observed after short- and long-term

exposure to ozone (Cottet-Emard et al., 1997; Soulage et al., 2004). Ozone was able to increase catecholamine turnover in sympathetic and central neurons located in the cervical ganglia, NTS and cortex (Soulage et al., 2004). In infant primates, episodic ozone exposure triggered neuroplasticity in the NTS neurons (Chen et al., 2003). Gackière et al., (2011) demonstrated that ozone inhalation caused a time and dose dependent neuronal activation in stress-responsive regions of the brain, specifically the paraventricular hypothalamic nucleus and dorsolateral regions of the NTS overlapping terminal fields of lung vagal afferent nerves, thus providing a possible mechanism by which ozone-induced lung responses are communicated to the brain (i.e. through the vagus nerve). Chounlamountry et al., (2015) described how ozone promotes remodeling of glial coverage and glutamatergic synapses in rat NTS and concluded that "$O_3$-induced pulmonary inflammation results in a specific activation of vagal lung afferents rather than non-specific overall brain alterations mediated by blood-borne agents". These studies support the evidence that neural mechanisms rather than circulating mediators might be involved in ozone-induced cardiovascular and pulmonary changes.

These stress responsive regions stimulated by ozone inhalation can activate the sympathetic-adrenal-medullary (SAM) and hypothalamus-pituitary-adrenal (HPA) axes. HPA and SAM activation culminate with the release of catecholamines and glucocorticoids , respectively, into the bloodstream. Generally, epinephrine and corticosterone synthesized and released from adrenal medulla and cortex, respectively, are considered stress hormones in addition to norepinephrine which is mainly synthesized in postganglionic sympathetic neurons. We and others have recently observed that ozone inhalation activates HPA and SAM axes resulting in increased levels of adrenocorticotropic hormone (ACTH), corticosterone and epinephrine in rodents, and cortisol and corticosterone in humans (Gunnison et al., 1997; Martrette et al., 2011; Thomson et al., 2013; Bass et al., 2013; Miller et al., 2016a, 2016b). In addition, other irritant gases such as acrolein, which primarily induces nasal irritation, has been shown to induce similar responses in rats (Snow et al., 2017). Interestingly, ACTH also has

steroid-independent immunomodulatory properties by binding to melanocortin receptors in leukocytes (Catania et al., 2004). Our studies with ozone and acrolein demonstrate that increases in stress hormones are associated with a variety of metabolic and immune effects in animals (Miller et al., 2015; 2016a, Snow et al., 2017).

*1.2.7.3 Neuroendocrine fight-or-flight stress response is similar to the response induced by ozone exposure*

The physiological changes associated with the fight-or-flight stress response are well characterized. There is a concerted effort of different organs of the body to channel metabolic energy and immune cells to the site of injury or stress such that the stressed portion of the body has resources needed to assure repair and survival, and reestablish homeostasis to a normal level. Stress has been defined as the "nonspecific response of the body to any demand on it" such as injury or fear (Szabo et al., 2017). Taking into account that different fields of biomedicine such as psychology and behavioral sciences also employ "stress" to denote bodily responses, stress has been mechanistically linked to neuroendocrine activation. Eugene Yates defined stress as "any stimulus that will provoke the release of ACTH and adrenal glucocorticoids" and Walter Cannon enriched this definition to include "sympathetic markers of stress" (Fink, 2010 [Chapter 1). Initial observations of stress hallmarks often referred to as "general adaptation system" or a "stress triad" is characterized by hyperemia (enlargement of adrenals), thymus atrophy and hemorrhagic gastric ulcers (Fink, 2010 [Chapter 1). During psychosocial stress, different cues can trigger different responses depending on the ability of the subject to interpret the stress signals coming from the environment. Biogenic or physical stress bypasses the interpretative mechanisms and promotes the neuroendocrine changes (Everly and Lating, 2012).

Stress response, the ultimate survival mechanism, is regulated by the neuroendocrine system of the body. Neuroendocrine responses activated after stressful stimuli are conserved from an evolutionary point of view and are present in all living organisms starting from uni- and

multicellular organisms to humans (Fink, 2010 [Chapter 1]). “Fight or flight” or “acute stress response” has been defined as a rapid reaction to any perceived threat. In this scenario, rapid changes increase the blood concentration of stress hormones such as catecholamines (epinephrine and norepinephrine) and glucocorticoids. These circulating hormones prepare the subject to an eventual danger by mobilizing energy sources from their depots to escape or face the threat, redirecting blood flow to muscles, promoting tunnel vision, increasing blood clotting function, among others. This neural and endocrine activation response initiates changes in virtually all organ systems of the body and based on the type of stress being encountered, these changes vary between organs (Everly and Lating, 2012).

Acute stress is characterized by the activation of both HPA and SAM axes. HPA axis activation starts with the release of corticotropic release hormone (CRH) from the hypothalamus, which activates the pituitary gland to release ACTH which in turn activates melanocortin receptor type 2 located in the adrenal cortex. ACTH promotes the release of glucocorticoids from the adrenal cortex, in humans this is primarily cortisol while in rodents it is corticosterone (Koren et al., 2012). SAM axis activation precedes HPA activation as a rapid response and is controlled by the direct sympathetic innervation of the adrenal medulla which causes the release of epinephrine and norepinephrine into the bloodstream (Ranabir and Reetu, 2011). While epinephrine is primarily made in adrenal medulla, only a small portion of circulating norepinephrine is derived from the adrenal medulla (~10-20%). Sympathetic nerve endings distributed in all organs of the body produce norepinephrine locally, which in a paracrine manner induce rapid local responses upon stimulation of the sympathetic system.

### 1.2.8 Ozone and stress response

#### *1.2.8.1 Ozone exposure induces a neuroendocrine stress response*

Although ozone exposure has been previously linked to stimulation of catecholaminergic neural mechanisms, it is only recently that our lab in a series of studies characterized this response as the same neuroendocrine stress response described above and linked it to a variety of systemic metabolic and immune changes that are often observed after an air pollution exposure. Our lab demonstrated that ozone acts as a stressor and, upon inhalation, consistently activates processes like fight-or-flight response not only in animals but also in humans (Bass et al., 2013; Miller et al., 2015, 2016a; Snow et al., 2017). For example, ozone-induced HPA axis activation characterized by increasing levels of circulating glucocorticoids (Miller et al., 2016a); SAM axis activation characterized by high levels of circulating epinephrine, hyperglycemia, and glucose intolerance (Bass et al., 2013; Miller et al., 2015), degradation of rich energy sources through catabolic reactions such as adipose lipolysis and muscle protein breakdown (Miller et al., 2015), acute phase response (Bass et al., 2015; Miller et al., 2015), and lung innate immune response (Miller et al., 2016b, 2016c).

#### *1.2.8.2 Neuroendocrine stress response and immunomodulation by ozone exposure*

Lymphoid organs are essential for leukocyte maturation. Spleen, thymus and bone marrow have been studied in relation with the regulation of ozone-induced inflammation. Thymus atrophy as a consequence of ozone exposure has been reported (Dziedzic and White, 1986). Li and Richters, (1991) have shown that short-term ozone inhalation reduced thymocytes and spleen T-lymphocytes. Chronic exposure to ozone has also been shown to induce splenomegaly (Hassett et al., 1985) and altered maturation of myelopoietic progenitors in the spleen (Goodman et al., 1989). Holz et al., (2010) hypothesized that a CXCR2 antagonist inhibited ozone-induced neutrophilia by blocking the egress of neutrophils from the bone marrow, while Kenyon et al., (2006) demonstrated that bone marrow-derived neutrophils are recruited into the lungs after ozone inhalation. Francis et al., (2017) showed that the spleen is a

source of lung inflammatory macrophages after ozone exposure and splenectomized rats exhibited decreased levels of ozone-induced pulmonary vascular leakage and cellular infiltration. The mobilization of immune cells has been observed after ozone exposure. Peripheral blood neutrophilia (Bosson et al., 2013), lymphopenia (Miller et al., 2016b), and an increase in CD11b+ circulating leukocytes (Alexis et al., 2004) have been associated with ozone inhalation in vivo. Although ozone-induced innate immune responses are fairly well characterized, no studies have addressed the mechanisms by which these responses are induced and especially the role of neuroendocrine stress axes activation in immune responses induced by air pollution exposure.

Dhabhar et al., (2012) using a rat model of restraint-induced stress characterized dynamic immune responses occurring minutes and hours after the encounter of stressful stimuli. Immediately after stress, the levels of circulating stress hormones were increased as expected. These increases were first associated with the mobilization of lymphocytes, monocytes and neutrophils from “barracks” (marginated pools such as bone marrow, spleen, or lymph nodes) to the circulation. This change was attributed to the rapid increase of epinephrine and norepinephrine peak minutes after the stimulus. After this period, a reduction of circulating leukocytes is observed due to their egress; this time from the circulation to peripheral organs. Dhabhar explained that neutrophils mobilize to the “battlefields” where injury or infection is occurring while lymphocytes and monocytes mobilize to areas of surveillance, such as skin or digestive tract, or return to the “barracks”. This delayed effect is attributed to corticosterone and epinephrine (Figure 1.3). These studies were followed with examining the effects of stress hormones directly by injecting these hormones in rats (Dhabhar et al., 2012; Dhabhar, 2014). Based on this evidence, we postulated that ozone-induced innate immune response might involve dynamic changes in circulating stress hormones. The changes observed after ozone exposure seem to mimic the changes induced by a stress response in these Dhabhar studies.

**Figure 1.3**

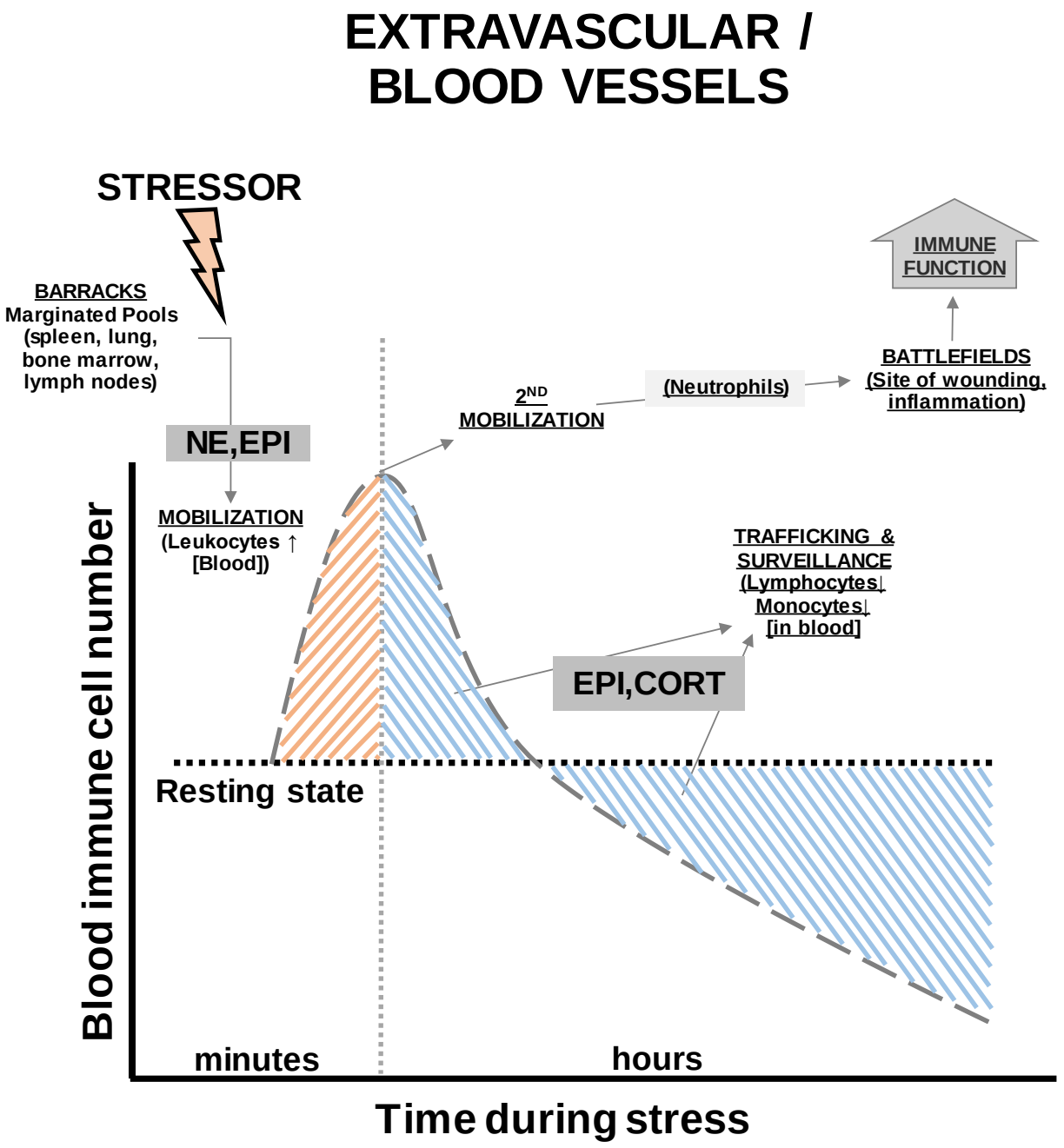



**Figure 1.3 Stress induced leukocyte distribution.** NE: Norepinephrine, EPI: Epinephrine, CORT: Cortisol. Source: Adapted and simplified from Dhabhar et al., 2012

1.2.9 Air pollution, psychosocial stress and therapeutic manipulation of stress hormone receptors

Psychosocial stress potentiates air pollution induced health effects in controlled *in vivo* and epidemiological studies (Cloughery et al., 2010; Fuller et al., 2017), and it is postulated that this interaction involves activation of neuroendocrine system involving the SAM and HPA axes. Therefore, psychosocial and physical stressors could worsen health outcomes. Alternatively, it is also possible that neuroendocrine stress responses triggered by the inhalation of air pollutants can additively or synergistically lower the threshold for psychosocial or physical stressors to induce deleterious health effects. Interestingly, Gent et al., (2003) found that the reported respiratory symptoms in asthmatic children were exacerbated by ozone at levels under NAAQS only in those using maintenance medication. This maintenance therapy is vastly based on the activation of GR to minimize chronic inflammation and bronchodilators such as long acting $\beta_2$AR agonists. Because $\beta_2$AR agonists relax constricted airways, they are widely used for bronchoconstriction occurring in asthma and COPD, often in conjunction with anti-inflammatory glucocorticoids (Cazzola et al., 2012).

Since adrenergic and glucocorticoid agonists mimic the activity of stress hormones and air pollution exposure is associated with increases in stress hormones, it is possible that air pollution effects are exacerbated in those receiving bronchodilators and steroids. Adrenergic receptor (AR) subtypes are selectively located in different tissues. Smooth muscle cells in the airways primarily express $\beta_2$ adrenergic receptors ($\beta_2$AR), whereas vascular smooth muscle cells express alpha receptors, each of which likely having different affinities for epinephrine and norepinephrine. Glucocorticoids activate glucocorticoid receptors (GR) which are located virtually in all cells and are generally used to diminish inflammation in a wide range of pro-inflammatory conditions (Barnes, 1995), and insensitivity to their anti-inflammatory properties has been widely reported (Barnes and Adcock, 2009). Psychosocial stress has shown to induce low grade inflammation (Rohleder, 2014), which is linked to glucocorticoid insensitivity (Li et al.,

2014). In addition, research linking psychosocial stress with exacerbation of asthma episodes, increase in asthma morbidity, deteriorating asthma severity, and development of pediatric asthma in children are being widely explored (Yonas et al., 2012, Booster et al., 2016).

## 1.3 Dissertation goals: Linking ozone-induced lung injury and inflammation to activation of neuroendocrine stress axes (SAM and HPA).

Our laboratory has studied ozone for over a decade in the context of host disease susceptibility where ozone is used as a prototypic oxidant air pollutant with the understanding that any inhaled pollutant that will cause lung injury and inflammation will be comparable to the response induced by ozone (Dye et al., 2015a). While trying to understand how an inhalant encountered by the lung might increase the susceptibility of those having underlying diseases of non-pulmonary origin (i.e. diabetes, cardiovascular, neurobehavioral, reproductive) and influence developmental processes, we observed that ozone exposure induces a myriad of extra-pulmonary effects in healthy animals. More recently, we have identified a putative link involving the activation of neuroendocrine stress axes that allow us to connect air pollutant-induced lung injury and inflammation to a mechanism common to many types of stressors - physical or psychosocial – and link those to multi-organ metabolic and immune effects as discussed above.

Generally, it is believed that extra-pulmonary effects induced by the inhalation of air pollutants involve the spillover of bioactive mediators from the lung to the circulation. Pro-inflammatory molecules such as cytokines, bioactive lipids, or oxidation byproducts originated in the lungs have been proposed to migrate to extra-pulmonary organs and act in the periphery to promote local effects as explained earlier. A large research effort is invested in identifying circulating mediators that induce extra-pulmonary effects after air pollution exposure. Often these unknown circulating mediators have been referred to as “molecular shrapnel”, without having identity of any specific mediator of group of mediators. Our studies, using the prototypic air pollutant ozone, identified this “molecular shrapnel” as hundreds of metabolites, acute phase proteins and more importantly stress hormones, and provided mechanistic understanding that these molecules did not originate from the lung (Kodavanti, 2016). Our studies, thus, offered an alternative mechanistic explanation as to how inhaled pollutants might induce extra-pulmonary

effects by activating neuroendocrine axes in rodents and humans and what circulating factors might be playing a role. Based on the studies from our laboratory, we believe that the activation of neuroendocrine stress axes (SAM and HPA) after air pollution exposure regulates two fundamental survival processes, namely metabolism and immune response, and in doing so, this activation affects virtually all organs in the body. Since air pollution-induced SAM and HPA activation is linked to lung injury and inflammation, the goal of this project was to understand how stress hormones regulate lung injury and inflammation induced by ozone exposure. Based on a series of publications from our lab which supports the involvement of neuroendocrine stress axes (Bass et al., 2013; Miller et al., 2015; 2016a; 2016b; 2016c; Kodavanti, 2016; Snow et al., 2017), we propose a model in which stress hormones are critical circulating players modulating both pulmonary and extra-pulmonary effects observed after ozone inhalation. We sought to provide an alternative mechanistic explanation that circulating mediators released in response to neuroendocrine activation (stress hormones) play a critical role in mediating local pulmonary effects of ozone.

We hypothesized that the CNS-mediated release of stress hormones, epinephrine and corticosterone, through their action on AR and GR, respectively, modulate ozone-induced vascular leakage and inflammation. We addressed this hypothesis by doing a series of experiments that are divided in three specific aims:

1.3.1 Examine if depletion of circulating stress hormones (epinephrine and corticosterone) inhibit ozone-induced lung injury and inflammation through their effects on lung transcriptome and cytokine expression profile

In a previous publication, we demonstrated that adrenal demedullation (the source of epinephrine) and total bilateral adrenalectomy (the source of both epinephrine and corticosterone) diminished ozone-induced lung injury and neutrophil inflammation in rats (Miller et al., 2016b). Based on this observation, we hypothesized that the lack of epinephrine and corticosterone will result in reduction of pulmonary transcriptome changes induced by ozone,

and thus, chemotactic factors involved in neutrophilic inflammation, while changing the balance of specific cytokine pools involved in the innate immune response. In the second chapter, we examined the lung transcriptome and the role of cytokines and chemotactic factors in ozone-induced lung inflammation in control, demedullated and adrenalectomized rats. By assessment of global mRNA sequencing and specific cytokine characterization of archived lung and lavage fluid samples, we examined functional processes differentially impacted by ozone in rats that have depleted circulating epinephrine or epinephrine plus corticosterone.

1.3.2 Assess the role of βAR and GR in mediating ozone-induced lung injury and inflammation using a pharmacological approach

Epinephrine and corticosterone induce their effects on different organs by binding to their respective receptors. A number of AR subtypes exist and display tissue-specific receptor distribution while the affinity of each receptor for epinephrine varies. This variability is essential for mediating tissue-specific effects during stress to assure homeostasis. Airway smooth muscles express $\beta_2$AR while αAR are distributed in the vasculature. In the third chapter, we focused on understanding the role of βAR and GR blockers in mediating ozone-induced lung injury and inflammation *in vivo*. We hypothesized that blocking these receptors will inhibit ozone-induced lung injury and inflammation.

1.3.3 Examine if agonists of epinephrine and corticosterone will rescue ozone-induced lung injury and inflammation in AD rats, while exacerbating effects in control rats

In the fourth chapter, we examined if βAR and GR agonists can rescue ozone-induced injury and inflammation in adrenalectomized (AD) rats, and exacerbate effects in normal rats that have undergone sham surgery (SH). Although βAR and GR agonists are widely used in chronic inflammatory conditions, their contribution to the effects produced by endogenously stress hormones during acute stress has not been delineated. It is not known if ozone-induced lung injury/inflammation and mobilization/extravasation of inflammatory cells from the bone marrow to the lung involve circulating stress hormones. No prior studies have used the

combination of surgical interventions involving adrenalectomy and pharmacological agonists to understand how innate immune response is generated after air pollution exposure. We hypothesized that treatment of AD rats with βAR and GR agonists will reverse the protective effect of adrenalectomy on ozone-induced lung injury and inflammation. Pharmacological agonists, Clenbuterol and dexamethasone, widely used in research were employed to address this hypothesis in conjunction with surgical intervention of AD and SH in rats.

## CHAPTER 2: ADRENAL-DERIVED STRESS HORMONES MODULATE OZONE-INDUCED LUNG INJURY AND INFLAMMATION

### 2.1 Introduction

Ozone is a ubiquitous gaseous air pollutant and one of the major components of smog in urban areas (Cooper et al., 2014; Haagen-Smit, 1952). Due to its reactive nature, acute ozone inhalation causes oxidation of biomolecules such as proteins (Hemming et al., 2015; Kim et al., 2010) and lipids (Kadiiska et al., 2013; Thompson et al., 2013) in lung lining fluid and epithelial cells. An imbalance in the lung oxidant/antioxidant ratio has been widely reported after ozone exposure (Bromberg, 2016; Wiegman et al., 2014). Subsequent activation of pro-inflammatory signaling cascades involving mitogen-activated protein kinases (MAPK), phosphoinositide 3-kinase- protein kinase B (PI3K-AKT), and nuclear factor erythroid 2-related factor 2 (NRF2) (Yan et al., 2016) have been shown in vitro and in vivo, to increase transcription of cell adhesion molecules and pro-inflammatory cytokines (Bromberg and Koren, 1995; Chen et al., 2007; Montuschi et al., 2002). This activation results in the extravasation of innate immune cells, including neutrophils, to the lungs within hours after ozone exposure (Kim et al., 2011; Kirsten et al., 2013; Cabello et al., 2015; Williams et al., 2007; Auerbach and Hernandez, 2012). Ozone exposure has been shown to alter the balance between Th1 and Th2 phenotypes (Steerenberg et al., 1996). Th1-type cytokines are involved in immunity against intracellular pathogens where the effector cytokine IFNγ plays a pivotal role. Th2-type cytokines are involved in the immunity against extracellular parasites as well as development of type 1 hypersensitivity response seen in allergic asthma and[1] are driven mainly by IL4 and IL13 (Berger, 2000). In mice, an ozone-

[1] This chapter previously appeared as Henriquez, A. et al. October 2017. Adrenal-derived stress hormones modulate ozone-induced lung injury and inflammation. Toxicol. Appl. Pharmacol. 329, 249-258.

induced Th2 phenotype shift was shown to depend on innate lymphoid cells (Kumagai et al., 2016).

Adverse ozone effects are not restricted to the lungs and multiple extra-pulmonary alterations have also been reported (Watkinson et al., 2001; Thomson et al., 2013). Ozone inhalation activates stress-responsive regions of the brain, including the nucleus tractus solitaries (NTS) where terminal fields of the lung vagal afferents overlap (Gackière et al., 2011). Ozone exposure also induces reflexively-mediated cardiovascular alterations, such as bradycardia and hypothermia, and decreases blood pressure (Akcılar et al., 2015; Uchiyama and Yokoyama, 1989; Gordon et al., 2014), indicating activation of the autonomic nervous system (Watkinson et al., 1996). It is postulated that nociceptive bronchial C-fibers stimulated by ozone inhalation transmit sensory information to the CNS and mediate systemic responses. These C-fibers modulate ozone-induced airway hyperresponsiveness but not hypothermia or bradycardia in rats (Jimba et al., 1995; Taylor-Clark and Undem, 2011).

Acute ozone inhalation induces systemic metabolic alterations including hyperglycemia, glucose intolerance, release of free fatty acids, activation of acute phase response, and muscle protein catabolism associated with a rise in circulating epinephrine and corticosterone levels (Bass et al., 2013; Miller et al., 2015, 2016a, 2016b). Further, performing an adrenal demedullation (DEMED) or adrenalectomy (ADREX) in rats, which diminishes the source of circulating catecholamines (synthesized in adrenal medulla upon sympathetic stimulation) and catecholamines plus steroid hormones (later synthesized in the adrenal cortex upon stimulation of HPA-axis), respectively, inhibits ozone-induced systemic metabolic impairment (Miller et al., 2016c). Importantly, pulmonary injury and inflammation induced by ozone exposure in rats that have undergone sham surgery (SHAM) are also diminished by ADREX (Miller et al., 2016c), highlighting a potential role of stress hormones in mediating ozone-induced pulmonary injury and inflammation. Although the mechanisms by which ozone induces local lung injury and inflammation are fairly well characterized, the role of circulating factors, such as stress

hormones, in modulating pulmonary vascular leakage and extravasation of circulating immune cells has not been studied. We hypothesized that analysis of the expressed lung transcriptome in SHAM, DEMED, and ADREX rats exposed to ozone would elucidate potential mechanisms by which these hormones contribute to inflammation and injury in the lung following ozone exposure. Further, that decreased circulating levels of epinephrine and corticosterone in ADREX and DEMED rats would transcriptionally inhibit inflammatory modulators involved in ozone-induced neutrophilic inflammation while altering the balance of specific cytokine pools involved in immune function.

## 2.2 Materials and Methods

### 2.2.1 Animals, surgeries and exposure

Lung tissue samples from healthy, male Wistar Kyoto (WKY) rats aged 12–13 weeks (Charles River Laboratories Inc., Raleigh, NC) were used from Miller et al., 2016c. SHAM, DEMED and ADREX surgeries were performed using aseptic sterile technique. Briefly, ketamine plus xylazine (50 mg plus 4 mg/kg body weight, i.p.) were used for anesthesia. Prior to surgery, rats received buprenorphine (0.02mg/kg, subcutaneous) as an analgesic. Charles River Surgeons performed three types of sterile surgeries: control sham surgeries where all procedures were identical to the ADREX surgery except for the removal of adrenal gland (SHAM), bilateral adrenal demedullation where only the medulla portion of the adrenal glands was removed while the cortex was kept in place (DEMED), or bilateral total adrenalectomy where whole adrenal glands were removed (ADREX). Following 4 days of recovery, rats were exposed to either air or 1 ppm ozone, 4 h/day for 1 day or 2 consecutive days (1-D or 2-D) in whole body chambers under controlled flow, temperature and relative humidity. Ozone was generated by a silent arc discharge generator (OREC, Phoenix, Arizona). The chambers (Rochester style “Hinners”) flow was controlled by mass flow controllers (Coastal Instruments Inc., Burgaw, North Carolina) and the ozone concentrations were recorded using photometric ozone analyzers (API model 400, Teledyne Instruments; San Diego, California). Within 1 h of the final exposure, animals were euthanized via an overdose of pentobarbital (>200 mg/kg, i.p.). Bronchoalveolar lavage (BAL) was performed on the right lungs and left lung tissues were snap frozen in liquid nitrogen for storage at−80 °C for later analysis. Cell free BALF aliquots were stored at −80 °C for further analysis.

### 2.2.2 RNA sequencing

mRNAwas isolated from total RNA (500 ng each) using prepX polyA mRNA Isolation Kit (Wafergen Biosystems, Fremont, CA). Samples were run using manufacturer's protocol on the Apollo324 automated sample processing system for mRNA selection and continued on RNA-

Seq library prep with Wafergen's PrepX mRNA 48 Protocol. The resulting cDNA libraries were PCR amplified for 15 cycleswith indexing primers according to Wafergen's protocol. One microliter was taken from each library for quantitation by Qubit dsDNA HS Assay kit (Molecular Probes, Eugene, Oregon). The quality of libraries was checked by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the molar concentration of each library was estimated by using average molecular sizes fromBioanalyzer data and the concentration fromQubit measurement, and each library was diluted to 4 nM accordingly. The diluted libraries were again checked by Qubit to confirm the working concentrations and pooled to make the sample for sequencing run. The pooled libraries were denatured and diluted according to Illumina NextSeq 500 protocols (Illumina Inc., San Diego, CA). The final concentration for sequencing was 1.8 pM + 5% PhiX from Illumina. The sequencing data were stored in Illumina's BaseSpace-cloud.

2.2.3 Real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

For qPCR, each RNA sample was diluted to 10 ng/μL and kept at −80 °C until the day of the experiment. One-step qPCR (SuperScript III, Invitrogen, Grand Island, NY) was run on an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) using 50 ng of RNA. Primers containing a 6-carboxy-fluorescein (FAM dye) label at the 5′ end were purchased from Applied Biosystems (Foster City, CA) for the following genes: β-actin (Rn00667869_m1), tumor necrosis factor alpha (Tnfα, Rn99999017_m1), interleukin 6 (IL-6, Rn01410330_m1), interleukin 1 beta (IL-1β, Rn00580432_m1), interleukin 4 (IL-4, Rn01456866_m1), interferon gamma (Ifnγ, Rn00594078_m1), interleukin 5 (IL-5, Rn01459975_m1), and interleukin 13 (IL-13, Rn00587615_m1). Data were analyzed using ABI sequence detection software, version 2.2 using β-actin as an endogenous control. Expression of each sample was calculated as relative fold change over air-SHAM group at each time point (1-D or 2-D) using the $2^{-\Delta\Delta CT}$ method.

### 2.2.4 Cytokine protein quantification

BALF levels of cytokine proteins (IL-1β, IL-4, IL-5, IL-6, IL-10, IFN-γ, KC-GRO, TNF-α) were quantified using the V-PLEX proinflammatory panel 2 (rat) kit per manufacturer's protocol (Meso Scale Discovery, Gaithersburg, MD). The resulting electrochemiluminescence signals for each target protein in sample wells were detected using Meso Scale Discovery® electrochemiluminescence (MSD-ECL) platform (Mesoscale Discovery Inc., Rockville, MD). The values below the limit of detection were substituted with the lowest quantified value for each cytokine. Measurement of BALF inflammatory mediator proteins was restricted to 2-D only since ozone-induced inflammation peaks at this time point (Ward et al., 2015).

### 2.2.5 Statistics

#### *2.2.5.1 Analysis of lung mRNA sequencing data*

For mRNA sequencing, sequenced reads were mapped to the rat genome (rn6) using ensemble release 83 in the Partek® Flow suite. R version 3.2.3 (2015-12-10) was used for subsequent analyses of gene expression changes. The count matrix was rounded to the nearest integer and assessed for differential gene expression using DESeq2 (v. 1.10.1) (Love et al., 2014). Gene expression was considered different when fold change (FC) was >1.5 (upregulation) or <0.667 (downregulation) and the adjusted p value was ≤0.05. Pathway analysis was conducted using the log2 (fold change) and adjusted p-values from DESeq2 with Ingenuity Pathways Analysis® (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) using an adjusted p-value cut-off of 0.10 (Krämer et al., 2014). Pathway heat maps of normalized counts from DESeq2 were generated with the heatmap.2 function of the g-plots package (Warnes et al., 2015). For glucocorticoid responsive genes, fold changes in ozone-exposed rats relative to air control are reported for each surgery group. To validate qPCR and RNAseq data, correlations between selected genes analyzed using qPCR are shown in Appendix, Supp Fig. 2.1

*2.2.5.2 Analysis of RT-qPCR and BALF cytokine protein data*

Relative gene expression and BALF cytokine data were analyzed using a two-way analysis of variance (ANOVA) with the 1-D and 2-D exposure groups treated as independent experiments. The two independent variables were exposure (air or 1 ppm ozone) and surgery (SHAM, DEMED or ADREX). The Holm-Sidak's test was used to correct for all multiple comparisons and significant differences were considered when a p-value of ≤0.05 was achieved. All data (n = 4–6 animals/group; 4 for SHAM air and ozone groups and 5–6 for ADREX and DEMED groups) are expressed as mean ± SEM. GraphPad prism 6.07 software was used for statistical analysis.

## 2.3 Results

### 2.3.1 Ozone-induced transcriptome changes in SHAM rats are diminished in DEMED and ADREX rats

To determine the impact of DEMED and ADREX-induced depletion of stress hormones on ozone-induced global transcriptional changes, total RNA from lung tissue was assessed with RNA sequencing (RNAseq). For 1-D time point, air and ozone-exposed SHAM, DEMED and ADREX groups were analyzed, while for 2-D time point only air and ozone-exposed SHAM and ADREX groups were analyzed. DEMED groups for 2-D were not analyzed due to constraints in the number of wells in the plate design. Ozone exposure at 1-D time point induced significant changes ($p \leq 0.05$) in the expression of 2337 genes in SHAM rats (Fig. 2.1A). The number of genes changed by ozone were dropped by over 5-fold in DEMED (461 genes) and ADREX (463 genes) rats exposed for 1 day (1-D). Of 1452 genes uniquely changed by ozone in SHAM rats at 2-D time point, 716 were upregulated and 736 were downregulated. Ozone exposure in ADREX rats at 2-D time point resulted in 74 unique significantly changed genes (36 upregulated and 38 downregulated). The number of genes changed by ozone were dropped by over 15- fold ADREX rats exposed for 2 days (2-D). Only 26 genes were commonly changed by ozone between SHAM and ADREX rats at 2-D time point (Fig. 2.1B). Changes in lung gene expression of SHAM rats after ozone exposure were reflective of changes in inflammatory, oxidative stress, steroid metabolism and cell cycle control processes known to be altered after ozone exposure (Ward and Kodavanti, 2015). Ozone-induced expression changes were greatly diminished in DEMED and ADREX rats (Appendix, Supp Tables 2.1 and 2.2). Genes that were induced after ozone exposure in SHAM rats were either not induced or only moderately induced in DEMED and ADREX rats, whereas those that were inhibited in SHAM rats were not inhibited or moderately inhibited in DEMED and ADREX rats. The relative fold change in expression of forty most induced and forty most inhibited genes in SHAM rats exposed to ozone, and their relative expression changes in DEMED and ADREX rats are shown in Appendix, Supp Tables 2.1A and

2.1B. These genes are reflective of cellular changes related to oxidative stress, acute phase response, inflammatory cell signaling and metabolism processes in SHAM rats.

To assess ozone-induced gene changes not affected by DEMED and ADREX, we examined unique and shared genes (Appendix, Supp Table 2.2 A–D) within the Venn diagram (Fig. 2.1). At 1-D, there were 235 genes uniquely changed by ozone in DEMED rats (145 upregulated and 90 down regulated) and 250 genes in ADREX rats (53 upregulated and 197 down regulated). Genes upregulated in DEMED by ozone included those involved in DNA replication processes while genes downregulated in ADREX by ozone were related to processes involved in acquired immunity (Appendix, Supp Table 2.2). There were 193 genes changed by ozone in common between SHAM and DEMED rats, and 180 genes were shared between SHAM and ADREX rats. Those in common with SHAM and DEMED included genes increased in expression related to IL-1 signaling and those involved in temperature and hypoxia or mechanical stimulation response. Whereas, those in common between SHAM and ADREX included genes with increased expression of those involved in protein metabolic processes and decreased expression of those in immune processes (Appendix, Supp Table 2.2). Only 86 genes changed by ozone were shared between, DEMED and ADREX rats whereas 53 significant ozone -induced gene changes were shared by all; SHAM, DEMED and ADREX rats.

Using IPA software, canonical pathways differentially regulated by ozone in SHAM, DEMED and ADREX rats (Tables 2.1 and 2.2; Appendix, Supp Table 2.3 and 2.4) were identified. The top 20 significantly upregulated (activation score > 0) and down-regulated (activation score < 0) canonical pathways after ozone exposure in SHAM rats and their relative changes in DEMED and ADREX rats are presented in Tables 2.1A and 2.1B. Notable pathways activated by ozone in SHAM rats on 1-D and/or 2-D included ERK5 signaling, acute phase response, cell cycle, ceramide signaling, p38 MAPK signaling and Notch signaling while those inhibited include interferon signaling, mTOR signaling, aryl hydrocarbon receptor signaling, iNOS signaling, JAK/Stat signaling, and IL-2 signaling (Tables 2.1A and 2.1B; Appendix, Supp

Table 2.3 and 2.4). In most cases pathway alterations induced by ozone in SHAM rats were abolished or mitigated by both DEMED and ADREX surgeries at 1-D (Table 2.1) or at 2-D time points (Table 2.1B). Gene specific responses in the selected pathways known to be upregulated by ozone such as acute phase response, NRF-2 mediated oxidative stress and PI3K/AKT pathways are displayed as heat maps in Fig. 2.2A–C to get insights into how adrenergic and steroidal hormones regulate expression of specific genes within a given pathway. The individual changes in SHAM rats for specific genes in these pathways are in agreement with our previously published data on ozone effects determined using Affymetrix platform (Ward et al., 2015). Visual display of expression changes for each animal in genes related to specific functional category indicates that the pattern of genes changed by ozone exposure in SHAM rats are lost in DEMED and ADREX rats (Fig. 2.2A–C).

Using the IPA comparison of gene expression signatures with previously known xenobiotics in its database, xenobiotics known to cause similar expression changes were compared to our experiment. Changes in gene expression determined in 1-D ozone exposure in SHAM rats were similar to those induced, among others, by methylprednisolone, dexamethasone, forskolin, and CREB-1 (Table 2.2), suggesting the significant contribution of glucocorticoids and adrenergic mechanisms in ozone-induced changes in lung tissue gene expression. The predicted activation scores in 1-D ozone vs. air exposed SHAM rats for these compounds were attenuated in animals that underwent either DEMED and ADREX surgeries (Table 2.2).

**Figure 2.1**

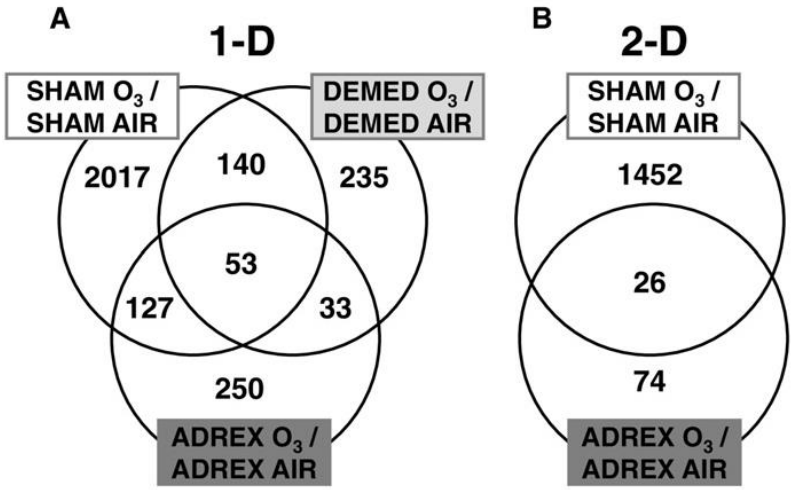



**Figure 2.1 Venn diagrams showing the number of significantly changed genes in lungs of SHAM, DEMED and ADREX rats after ozone exposure.** Gene expression differences were considered significant when the log2 Fold Change (FC) was ≥0.585 or ≤−0.585 [if FC was higher than 1.5 (upregulation) or lesser than 0.667 (downregulation)] and the adjusted p value was ≤0.05. A) SHAM, DEMED and ADREX rats were assessed for 1-D time point while B) only SHAM and ADREX were assessed at 2-D time point.

## Table 2.1

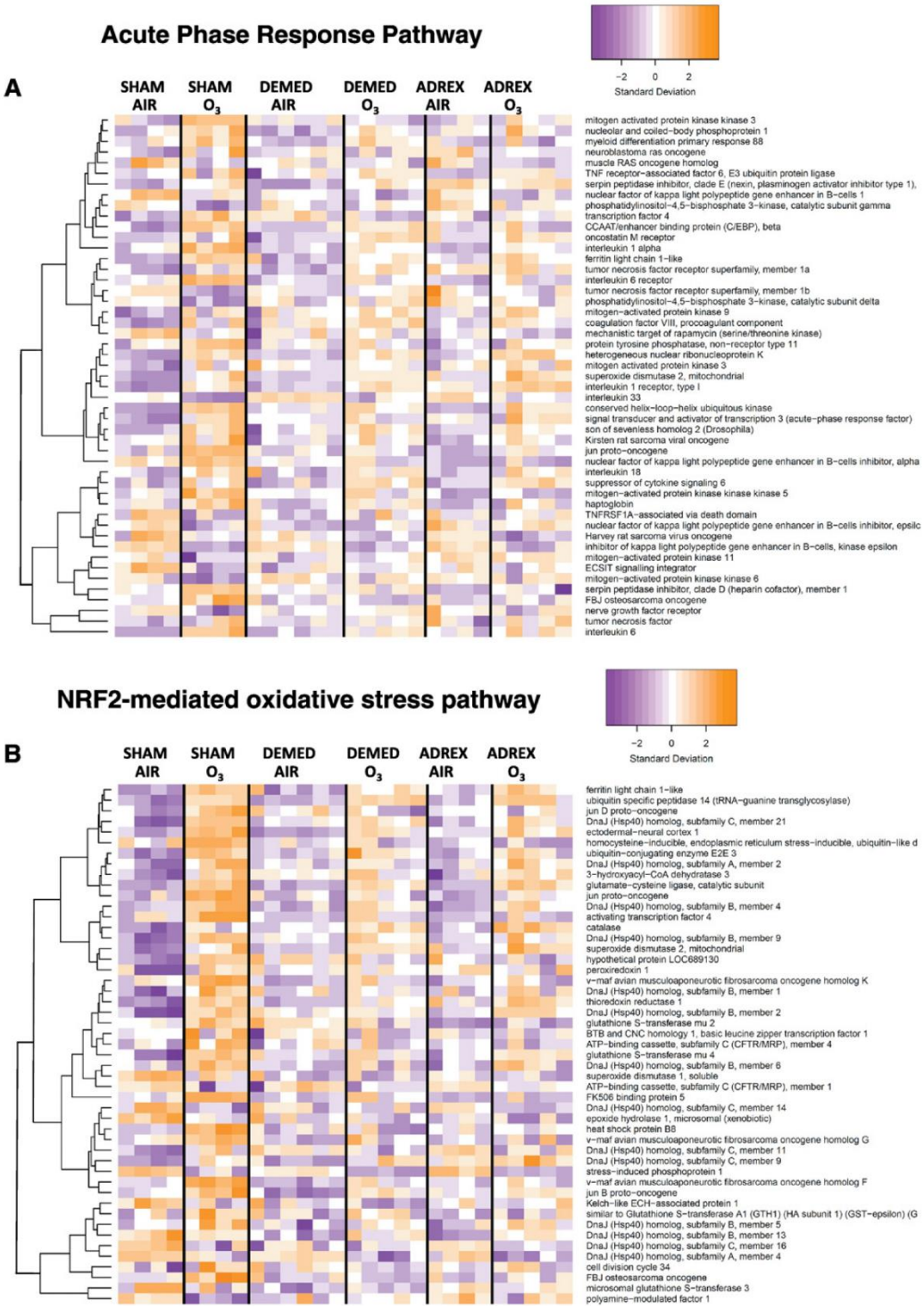
| Canonical pathway | Activation score | | |
|---|---|---|---|
| | SHAM $O_3$/SHAM air | DEMED $O_3$/DEMED air | ADREX $O_3$/ADREX air |
| Ephrin receptor signaling | 2.65 | 0.00 | 0.24 |
| ERK5 signaling | 2.40 | 1.90 | 1.90 |
| Acute phase response signaling | 2.31 | 0.00 | −0.78 |
| VDR/RXR activation | 2.12 | 0.00 | 0.00 |
| 14–3-3-mediated signaling | 2.11 | 0.00 | 0.00 |
| PCP pathway | 1.89 | 0.00 | 0.00 |
| Actin nucleation by ARP-WASP complex | 1.88 | 0.00 | 0.00 |
| Ceramide signaling | 1.76 | 0.00 | −1.60 |
| p38 MAPK signaling | 1.76 | 2.00 | 0.26 |
| CD27 signaling in lymphocytes | 1.70 | 0.00 | −1.13 |
| NRF2-mediated oxidative stress response | 1.68 | 0.26 | 0.00 |
| Agrin interactions at neuromuscular junction | 1.63 | 0.00 | 0.00 |
| nNOS signaling in neurons | 1.63 | 1.00 | 0.00 |
| Gαi signaling | 1.46 | 0.00 | 0.00 |
| Apoptosis signaling | 1.44 | 0.00 | 1.29 |
| Ephrin B signaling | 1.41 | 0.00 | 0.00 |
| Hypoxia signaling in the cardiovascular system | 1.41 | 0.00 | −0.45 |
| PI3K/AKT signaling | 1.41 | −0.24 | −1.40 |
| Cell cycle regulation by BTG family proteins | 1.26 | 2.00 | 0.00 |
| PTEN signaling | 1.26 | 0.00 | 0.24 |
| P2Y purigenic receptor signaling pathway | −1.95 | 0.00 | 0.26 |
| Macropinocytosis signaling | −2.00 | 0.00 | 1.26 |
| PEDF signaling | −2.00 | 0.00 | −0.26 |
| Fc epsilon RI signaling | −2.06 | 0.00 | 0.00 |
| Growth hormone signaling | −2.12 | 0.00 | 0.90 |
| Role of NFAT in regulation of the immune response | −2.12 | 0.00 | −0.73 |
| IL-9 signaling | −2.13 | 0.00 | 0.00 |
| Role of pattern recognition receptors in recognition of bacteria and viruses | −2.14 | 0.00 | −1.21 |
| Role of NFAT in cardiac hypertrophy | −2.17 | 0.00 | 0.00 |
| VEGF family ligand-receptor interactions | −2.20 | 0.00 | 0.28 |
| Type II diabetes mellitus signaling | −2.24 | 0.00 | −0.50 |
| FcγRIIB signaling in B lymphocytes | −2.27 | 0.00 | 0.00 |
| TREM1 signaling | −2.27 | 0.00 | −2.89 |
| Neuropathic pain signaling in dorsal horn neurons | −2.29 | 0.00 | 0.00 |
| Glioma signaling | −2.38 | −0.83 | 0.50 |
| Tec kinase signaling | −2.39 | 0.00 | −0.89 |
| ErbB4 signaling | −2.47 | 0.00 | 0.58 |
| eNOS signaling | −2.48 | 0.00 | 0.00 |
| NF-κB activation by viruses | −2.59 | 0.00 | −1.15 |
| iCOS-iCOSL signaling in T helper cells | −2.92 | 0.00 | −1.21 |

| Canonical pathway | Activation score | |
|---|---|---|
| | SHAM $O_3$/SHAM air | ADREX $O_3$/ADREX air |
| LPS/IL-1 mediated inhibition of RXR function | 2.18 | 0 |
| Role of BRCA1 in DNA damage response | 2.11 | 0 |
| ERK5 signaling | 1.96 | 0 |
| Acute phase response signaling | 1.62 | 0 |
| CD27 signaling in lymphocytes | 1.39 | 0 |
| Cell cycle: G1/S checkpoint regulation | 1.39 | 0 |
| Ceramide signaling | 1.34 | 0 |
| Mitotic roles of polo-like kinase | 1.29 | 0 |
| Ephrin receptor signaling | 0.85 | 0 |
| p38 MAPK signaling | 0.76 | 0 |
| Notch signaling | 0.71 | 0 |
| CDK5 signaling | 0.69 | −0.45 |
| Cdc42 signaling | 0.65 | 0 |
| p53 signaling | 0.65 | 0 |
| GNRH signaling | 0.65 | 0 |
| Rac signaling | 0.60 | 0 |
| B cell receptor signaling | 0.59 | 0 |
| Toll-like receptor signaling | 0.53 | 0 |
| ILK signaling | 0.51 | 0 |
| TGF-β signaling | 0.45 | 0 |
| Regulation of cellular mechanics by calpain protease | −1.73 | 0 |
| Macropinocytosis signaling | −1.79 | 0 |
| PI3K/AKT signaling | −1.80 | 0 |
| Growth hormone signaling | −1.88 | 0 |
| iCOS-iCOSL signaling in T helper cells | −1.88 | 0 |
| IL-2 signaling | −1.89 | 0 |
| JAK/Stat signaling | −1.89 | 0 |
| eNOS signaling | −1.98 | 0 |
| Gαq signaling | −2.00 | 0 |
| iNOS signaling | −2.00 | 0 |
| Fc epsilon RI signaling | −2.12 | 0 |
| UVA-induced MAPK signaling | −2.13 | −1.34 |
| mTOR signaling | −2.19 | 1 |
| Aryl hydrocarbon receptor signaling | −2.27 | 0 |
| IL-9 signaling | −2.31 | 0 |
| Antiproliferative role of somatostatin receptor 2 | −2.32 | 0 |
| Leukocyte extravasation signaling | −2.34 | 0 |
| Tec kinase signaling | −2.40 | 0 |
| NF-κB activation by viruses | −2.45 | 0 |
| Interferon signaling | −3.46 | 0 |

Values indicate the activation score for canonical pathways and were ranked for significant changes determined in SHAM rats. When the *p* values were not significant the activation score indicated is zero.

**Table 2.1 Up and down – regulated canonical pathways** A) Left: Twenty most up-regulated and twenty most down-regulated canonical pathways after ozone exposure in SHAM, DEMED and ADREX rats (1-D time point). B) Right: Twenty most up-regulated and twenty most down-regulated canonical pathways after ozone exposure in SHAM and ADREX rats (2-D time point).

**Figure 2.2**


Acute Phase Response Pathway
A
SHAM AIR
SHAM O3
DEMED AIR
DEMED O3
ADREX AIR
ADREX O3
−2 0 2
Standard Deviation
mitogen activated protein kinase kinase 3
nucleolar and coiled−body phosphoprotein 1
myeloid differentiation primary response 88
neuroblastoma ras oncogene
muscle RAS oncogene homolog
TNF receptor−associated factor 6, E3 ubiquitin protein ligase
serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1),
nuclear factor of kappa light polypeptide gene enhancer in B−cells 1
phosphatidylinositol−4,5−bisphosphate 3−kinase, catalytic subunit gamma
transcription factor 4
CCAAT/enhancer binding protein (C/EBP), beta
oncostatin M receptor
interleukin 1 alpha
ferritin light chain 1−like
tumor necrosis factor receptor superfamily, member 1a
interleukin 6 receptor
tumor necrosis factor receptor superfamily, member 1b
phosphatidylinositol−4,5−bisphosphate 3−kinase, catalytic subunit delta
mitogen−activated protein kinase 9
coagulation factor VIII, procoagulant component
mechanistic target of rapamycin (serine/threonine kinase)
protein tyrosine phosphatase, non−receptor type 11
heterogeneous nuclear ribonucleoprotein K
mitogen activated protein kinase 3
superoxide dismutase 2, mitochondrial
interleukin 1 receptor, type I
interleukin 33
conserved helix−loop−helix ubiquitous kinase
signal transducer and activator of transcription 3 (acute−phase response factor)
son of sevenless homolog 2 (Drosophila)
Kirsten rat sarcoma viral oncogene
jun proto−oncogene
nuclear factor of kappa light polypeptide gene enhancer in B−cells inhibitor, alpha
interleukin 18
suppressor of cytokine signaling 6
mitogen−activated protein kinase kinase kinase 5
haptoglobin
TNFRSF1A−associated via death domain
nuclear factor of kappa light polypeptide gene enhancer in B−cells inhibitor, epsilc
Harvey rat sarcoma virus oncogene
inhibitor of kappa light polypeptide gene enhancer in B−cells, kinase epsilon
mitogen−activated protein kinase 11
ECSIT signaling integrator
mitogen−activated protein kinase kinase 6
serpin peptidase inhibitor, clade D (heparin cofactor), member 1
FBJ osteosarcoma oncogene
nerve growth factor receptor
tumor necrosis factor
interleukin 6
NRF2-mediated oxidative stress pathway
B
SHAM AIR
SHAM O3
DEMED AIR
DEMED O3
ADREX AIR
ADREX O3
−2 0 2
Standard Deviation
ferritin light chain 1−like
ubiquitin specific peptidase 14 (tRNA−guanine transglycosylase)
jun D proto−oncogene
DnaJ (Hsp40) homolog, subfamily C, member 21
ectodermal−neural cortex 1
homocysteine−inducible, endoplasmic reticulum stress−inducible, ubiquitin−like d
ubiquitin−conjugating enzyme E2E 3
DnaJ (Hsp40) homolog, subfamily A, member 2
3−hydroxyacyl−CoA dehydratase 3
glutamate−cysteine ligase, catalytic subunit
jun proto−oncogene
DnaJ (Hsp40) homolog, subfamily B, member 4
activating transcription factor 4
catalase
DnaJ (Hsp40) homolog, subfamily B, member 9
superoxide dismutase 2, mitochondrial
hypothetical protein LOC689130
peroxiredoxin 1
v−maf avian musculoaponeurotic fibrosarcoma oncogene homolog K
DnaJ (Hsp40) homolog, subfamily B, member 1
thioredoxin reductase 1
DnaJ (Hsp40) homolog, subfamily B, member 2
glutathione S−transferase mu 2
BTB and CNC homology 1, basic leucine zipper transcription factor 1
ATP−binding cassette, subfamily C (CFTR/MRP), member 4
glutathione S−transferase mu 4
DnaJ (Hsp40) homolog, subfamily B, member 6
superoxide dismutase 1, soluble
ATP−binding cassette, subfamily C (CFTR/MRP), member 1
FK506 binding protein 5
DnaJ (Hsp40) homolog, subfamily C, member 14
epoxide hydrolase 1, microsomal (xenobiotic)
heat shock protein B8
v−maf avian musculoaponeurotic fibrosarcoma oncogene homolog G
DnaJ (Hsp40) homolog, subfamily C, member 11
DnaJ (Hsp40) homolog, subfamily C, member 9
stress−induced phosphoprotein 1
v−maf avian musculoaponeurotic fibrosarcoma oncogene homolog F
jun B proto−oncogene
Kelch−like ECH−associated protein 1
similar to Glutathione S−transferase A1 (GTH1) (HA subunit 1) (GST−epsilon) (G
DnaJ (Hsp40) homolog, subfamily B, member 5
DnaJ (Hsp40) homolog, subfamily B, member 13
DnaJ (Hsp40) homolog, subfamily C, member 16
DnaJ (Hsp40) homolog, subfamily A, member 4
cell division cycle 34
FBJ osteosarcoma oncogene
microsomal glutathione S−transferase 3
polyamine−modulated factor 1

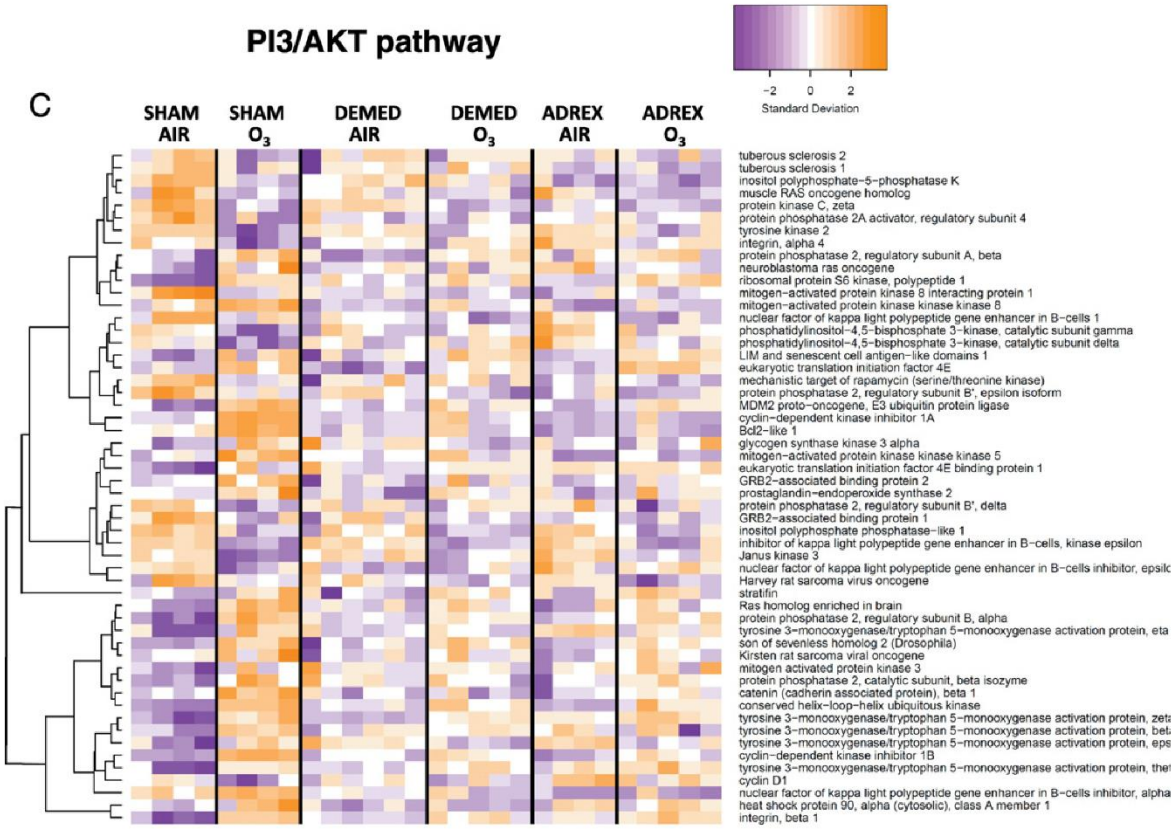



**Figure 2.2 Heatmaps showing hierarchical clustering of selected genes within given signaling pathways after 1-D air or ozone exposure in SHAM, DEMED or ADREX rats.**

Pathway heat maps of row scalled counts from DESeq2 were generated with the heatmap.2 function of the g-plots package. Increased and decreased expression of genes are shown by orange and purple color combination, respectively. Heatmaps were for A) Acute phase response genes, B) NRF2-mediated oxidative stress response genes and C) PI3K-AKT pathways genes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 2.2**

| Predicted Targets | Activation score | | |
|---|---|---|---|
| | SHAM $O_3$/SHAM air | DEMED $O_3$/DEMED air | ADREX $O_3$/ADREX air |
| Forskolin | 5.84 | 3.82 | 1.60 |
| methylprednisolone | 4.97 | 1.81 | −0.74 |
| CREB1 | 4.84 | 2.02 | 1.62 |
| PGR | 4.57 | 1.68 | 1.27 |
| XBP1 | 4.54 | 1.23 | −1.86 |
| PDGF BB | 4.51 | 3.40 | 0.78 |
| dexamethasone | 4.51 | 0.03 | 2.35 |
| gentamicin | 4.49 | 2.97 | −0.64 |
| NUPR1 | 4.45 | −1.12 | 1.80 |
| Salmonella enterica serotype abortus equi lipopolysaccharide | 4.41 | 4.07 | 0.00 |

Values indicate the activation score for predicted upstream targets determined by IPA. This analysis is based on the previous knowledge/published database of how known targets act as a transcription regulator considering the direction of change. Values were ranked for the magnitude of the change in SHAM rats and when the *p* values were not significant or unchanged the activation score indicated is zero.

**Table 2.2 Identification of 10 most predictive targets changed by ozone exposure** (1-D) in SHAM, DEMED and ADREX rats using ingenuity upstream regulator analysis.

### 2.3.2 Ozone exposure increased expression of glucocorticoid responsive genes in SHAM but not ADREX rats

RNA sequencing results were useful in determining if glucocorticoid responsive genes were changed after ozone exposure. A list of 8 genes upregulated by glucocorticoid receptor activation were selected (Bhlhe40, Tsc22d3, Thbd, Sdpr, Slc19a2, Gem, Plk2 and Srgn) (Wang et al., 2004) to determine if the lungs were an active target of the increased glucocorticoid activity driven by ozone-induced increases in circulating corticosterone (Miller et al., 2016a, 2016c). SHAM rats exposed to ozone (1-D) compared with air exposed animals displayed significantly increased lung expression (in all but Thbd (Table 2.3)). These ozone-induced increases were not apparent in ADREX rats demonstrating that ozone activates the expression of glucocorticoid responsive genes in SHAM but not in ADREX rats. As expected, ADREX in air-exposed rats did not change the expression of these genes. On day 2 of exposure, although the induction of glucocorticoid responsive genes in SHAM rats was reduced when compared to day 1 changes, the increases in some of these genes still persisted in ozone-exposed SHAM rats and these effects were diminished in ADREX rats (Table 2.3).

## Table 2.3

| | 1-D | | | 2-D | |
|---|---|---|---|---|---|
| | SHAM ozone/SHAM air | DEMED ozone/DEMED air | ADREX ozone/ADREX air | SHAM ozone/SHAM air | ADREX ozone/ADREX air |
| Tsc22d3 | 2.32* | 1.34 | 1.16 | 1.25 | 0.77 |
| Thbd | 1.23 | 0.92 | 1.16 | 1.04 | 0.98 |
| Sdpr | 1.24* | 1.05 | 1.2* | 0.93 | 0.92 |
| Slc19a2 | 1.38* | 1.21 | 1.25 | 1.76* | 1.11 |
| Gem | 2.73* | 1.80* | 1.10 | 1.88* | 0.82 |
| Plk2 | 2.17* | 1.56* | 1.3* | 1.20 | 0.91 |
| Srgn | 1.49* | 1.11 | 1.03 | 1.10 | 1.08 |
| Bhlhe40 | 2.07* | 1.34 | 1.32 | 1.53* | 0.80 |

Glucocorticoid responsive genes were identified from the master list of ozone-induced differentially expressed genes in the lung. TSC22 domain family protein 3, Tsc22d3 also known as Gilz; thrombomodulin, Thbd; serum deprivation-response protein, Sdpr; thiamine transporter 1, Slc19a2; GTP-binding protein, Gem; serine/threonine-protein kinase PLK2, Plk2; serglycin, Srgn; and class E basic helix-loop-helix protein 40, Bhlhe40. These genes have been shown to be transcriptionally up-regulated after activation of glucocorticoid receptors (Wang et al., 2004). Values indicate mean fold change by ozone when compared to air.

* Indicates significant ozone effect (adjusted $p$ value < 0.05).

**Table 2.3 Ozone-induced fold change in the expression of glucocorticoid responsive genes in SHAM, DEMED and ADREX rats.**

2.3.3 Modulation of lung innate immune response genes (qPCR) and BALF proteins by ozone in SHAM, DEMED and ADREX rats

In order to validate the results obtained using RNAseq, we performed qPCR for innate immune genes known to be induced by ozone exposure. Inflammatory gene expression changes in the lung were determined at both time points, 1-D and 2-D. Scatter plots of relative expression by qPCR and RNAseq in all samples in general, showed significant correlation (Appendix, Supp Fig 2.1), indicating that the expression levels obtained using RNAseq are reflective of the actual changes in the gene expression. In general, the qPCR of key inflammatory genes in ozone-exposed SHAM rats was reflective of changes noted previously in other publications (Ward et al., 2015) (Fig. 2.3). There was a trend for increased expression of Tnfα after ozone exposure in SHAM rats ($p = 0.21$) at 2-D time point (Fig. 2.3A). Il-6 was markedly induced by ozone exposure in SHAM rats at both time points (Fig. 2.3B) and for Il1-β at the 2-D time point (Fig. 2.3C). These ozone-induced changes were maintained for Tnfα (2-D) in DEMED rats, while for Il-6 and Il-1β the changes were not observed in DEMED or ADREX rats.

Expression of Tnfα, Ifnγ and Il-5 (Fig. 2.3A, E, F) as determined using qPCR were increased at 2-D time point, while the expression of Il-13 was increased at both time points in ADREX rats exposed to air (Fig. 2.3G). The expression of Il4, Ifnγ, Il-5 and Il-13 (Fig. 2.3D, E, F, G) was increased in ADREX rats exposed to ozone (2-D only). DEMED did not have significant effect on the expression of any of these genes in air and ozone-exposed rats.

BALF proteins were analyzed only for 2-D time point since ozone induced inflammation peaks at this time. While neither ozone nor ADREX or DEMED changed the levels of IL-1β and IFN-γ (Fig. 2.4C, E), compared to air, ozone exposure resulted in increased levels of IL-6, IL-4, IL-5 and IL-13 proteins in BALF of SHAM rats (Fig. 2.4B, D, F, G). Significant ozone-induced increases of IL-6, IL-4, and IL-13 proteins were not observed in ADREX or DEMED rats (Fig.

2.4B, D, G). Ozone-induced IL-5 increase was not affected by DEMED. In ADREX rats increases in IL-5 were noted in both air and ozone groups (Fig. 2.4F). ADREX also increased BALF protein levels of TNFα (Fig. 2.4A, F).

To explore changes in Th1 and Th2modulation after ozone exposure in SHAM, DEMED and ADREX rats, BALF protein levels of IFNγ and IL4 and their ratio were examined. Increased expression of IFNγ preferentially drives a Th1 response while increased IL4 drives a Th2 response (Serrano et al., 1997). Although IFNγ protein levels were not significantly changed after ozone exposure in SHAM rats, the BALF IL-4/IFNγ ratio was increased (Fig. 2.4H) suggesting that ozone appears to preferentially drive a Th2 response over Th1. This effect was not observed in DEMED and ADREX rats.

**Figure 2.3**

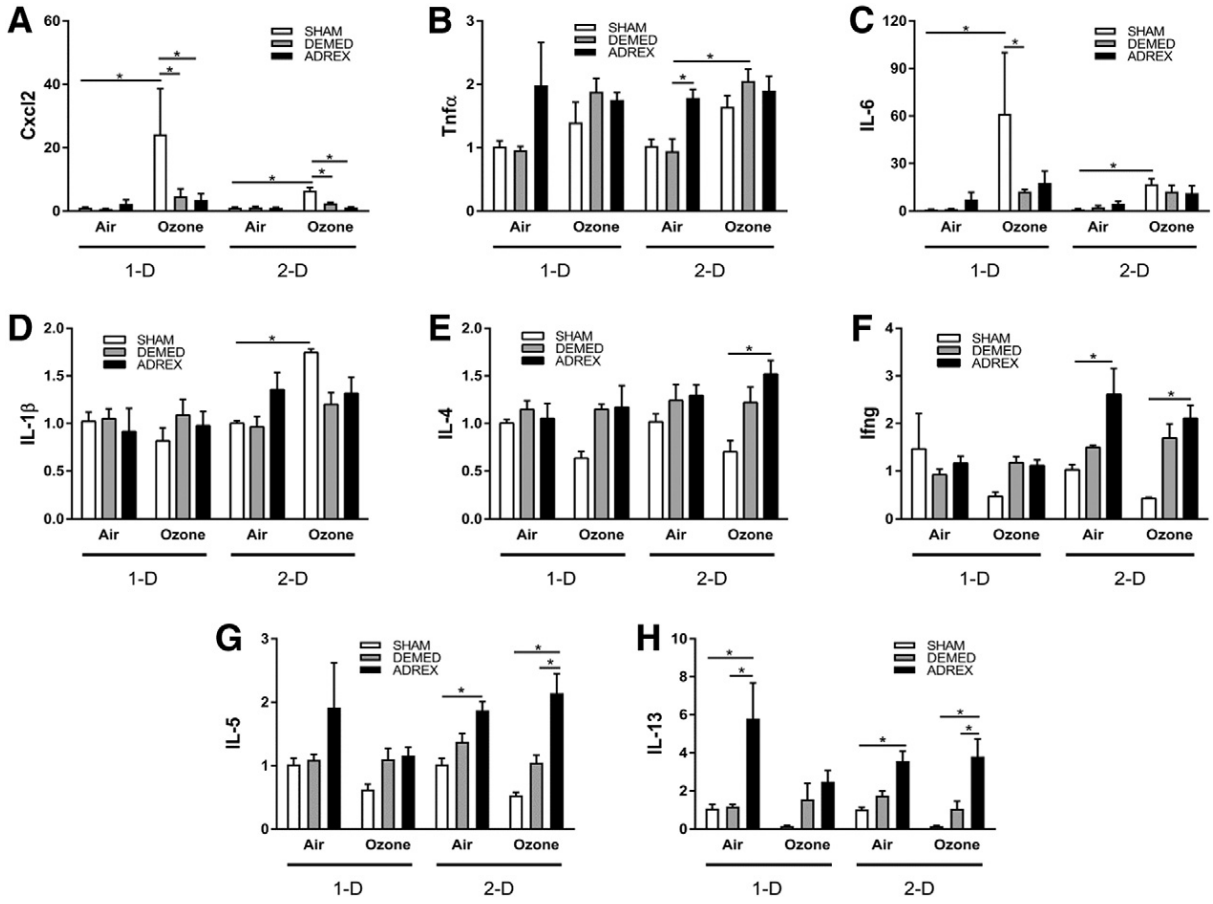



**Figure 2.3 The expression of selected inflammatory cytokine genes in lungs of SHAM, DEMED, and ADREX rats after exposure to air or ozone as determined using qPCR.**

Values represent mean ± SE of relative fold change from each day SHAM-air control (n= 3–4 animals for SHAM air or ozone, and n= 5–6 for other groups). Comparisons between air and ozone under each surgery condition and the comparisons between different surgeries under the same exposure condition are shown in graphs (* indicate p value ≤ 0.05 with respect to surgery or ozone exposure). A) tumor necrosis factor alpha (Tnfα), B) interleukin 6 (Il-6), C) interleukin 1 beta (Il-1β), D) interleukin 4 (Il-4), E) interferon gamma (Ifnγ), F) interleukin 5 (Il-5) and G) interleukin 13 (Il-13).

**Figure 2.4**

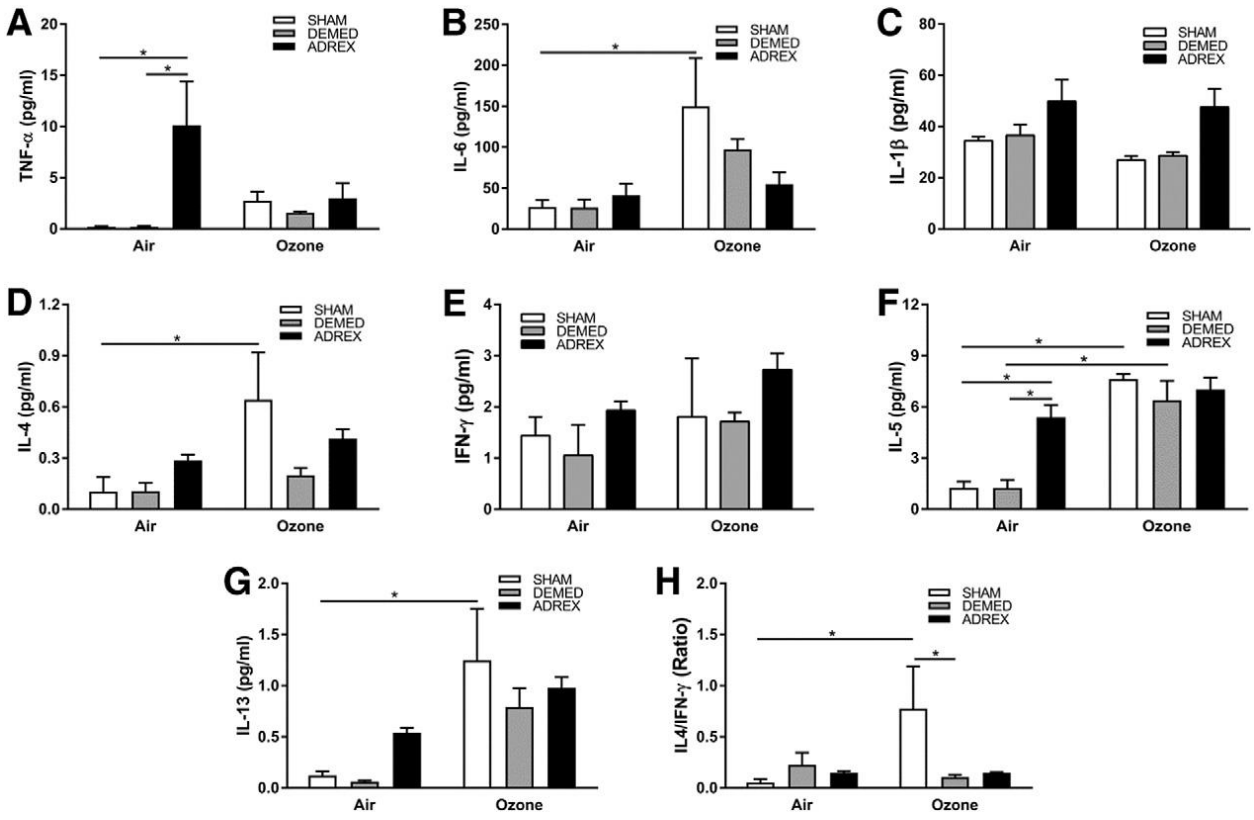



**Figure 2.4 Inflammatory cytokine proteins in bronchoalveolar lavage fluid (BALF) of SHAM, DEMED and ADREX rats after exposure to air or ozone.** Values represent mean±SE (n=4 animals for SHAMair or ozone, and n=5–6 for other groups). Comparisons between air and ozone under each surgery condition and the comparisons between different surgeries under the same exposure condition are shown in graphs (* indicates p value ≤ 0.05with respect to surgery or ozone exposure). A) Tumor necrosis factor alpha (TNFα), B) interleukin 6 (IL-6), C) interleukin 1 beta (IL-1β), D) interleukin 4 (IL-4), E) interferon gamma (IFNγ), F) interleukin 5 (IL-5), G) interleukin 13 (IL-13) and H) IL4/IFNγ ratio.

## 2.4 Discussion

We have recently shown that circulating stress hormones are increased after ozone exposure in rats and humans, and are linked to both systemic and pulmonary effects of ozone exposure (Bass et al., 2013; Miller et al., 2015, 2016a, 2016b, 2016c). In this study, a transcriptional Approach was used to understand the molecular underpinnings of ozone-induced acute lung injury and inflammation in SHAM, DEMED and ADREX rats. The goal was to elucidate potential mechanisms and roles of adrenergic and steroidal hormones in ozone-induced lung injury and inflammation. Ozone-induced changes in expression of number of genes in the lungs of SHAM rats were markedly diminished in DEMED and ADREX rats (5-fold decrease from over 2300 genes). Moreover, pathways involved in ozone-induced inflammatory and oxidative stress responses, such as NRF2, acute phase response, PI3K/AKT and p38 MAP-kinase were up-regulated after ozone exposure in SHAM rats but were not changed in DEMED or ADREX rats. Genes regulating the neutrophilic innate immune response and associated proteins favoring Th2 phenotype induced after ozone exposure in SHAM rats were diminished in DEMED and ADREX rats. When predictive pathways were examined, it was evident that ozone-induced transcriptional changes were similar to those induced by glucocorticoid-like compounds such as dexamethasone and prednisolone and to those induced by forskolin. Ozone exposure was associated with increases in the expression of glucocorticoid-responsive genes in the lungs of SHAM rats but not DEMED or ADREX rats. Together, these findings show that adrenergic and steroidal hormones modulate ozone-induced global gene expression changes in the lung. Hormonal regulation of air pollution-induced injury and inflammation has not been well studied even though steroidal and adrenergic interventions are widely used in treatment of lung diseases (Barnes, 2013; Fuso et al., 2013).

Gene expression outputs obtained by RNAseq were adequate in quantifying the magnitude of changes induced by ozone and were generally consistent with those reported in previous publications (Gohil et al., 2003; Ward et al., 2015). Eighty percent of the top ozone-

induced changed transcripts in the lung as measured by an Affymetrix rat panel (Ward et al., 2015) were altered in the same direction in SHAM rats. The diminution of ozone-induced lung transcriptome changes in the absence of circulating stress hormones in DEMED and ADREX rats could be characterized using RNAseq. Since we were not able to remove the cortex while keeping the medulla intact in these animals, it was not possible to determine if corticosterone plus mineralocorticoids and epinephrine played independent roles in ozone-induced changes. Future work with pharmacological interventions is planned to address the role of individual stress hormones.

Oxidative stress responsive pathways have been shown to be upregulated in the lungs after ozone exposure including NRF2 (Kim et al., 2004) and acute phrase response (Bass et al., 2013; Laskin et al., 1994) which are presumed to counter ozone-induced lung injury and inflammation through induction of genes involved in these pathways (Cho et al., 2013). Moreover, it has been shown that steroid-induced NRF2 activation is a key event induced by oxidant injury and enhances airway epithelial barrier integrity (Shintani et al., 2015). Adrenal-derived stress hormones have been shown to independently increase plasma levels of acute phase proteins (Eastman et al., 1996; Merchant et al., 2010; Schade et al., 1987). The upregulation of NRF2 and acute phase pathways in ozone-exposed SHAM rats but their inhibition in DEMED and ADREX rats suggests that NRF2 nuclear translocation and activation of acute phase response in the lung cells may require the presence of circulating epinephrine and/or corticosterone.

We have shown that ozone exposure induces systemic metabolic changes (Miller et al., 2015). PI3K/AKT is an intracellular signaling pathway involved in metabolism, cell cycle control, and proliferation (Hassan et al., 2013). The activation of selected genes in PI3K/AKT pathway by ozone in SHAM rats at 1-D suggests that the transcriptional changes observed after ozone exposure are not only restricted to inflammatory mechanisms, but also involve other signaling processes including metabolic (Miller et al., 2015; Ward and Kodavanti, 2015). The activation of

adrenergic receptors has been shown to induce PI3K/AKT pathway (Nakaoka et al., 2015) while blockade of these receptors by propranolol has shown downregulation (Pan et al., 2015). Thus, the diminution of the pathway activation score in DEMED and ADREX rats emphasizes the contribution of stress hormones in interactively modulating multiple biological processes.

Based on known glucocorticoid responsive genes (Wang et al., 2004), we identified gene signatures in ozone-exposed SHAM, DEMED and ADREX rats. Although ozone did not change the expression of the glucocorticoid receptor Nr3c1 in SHAM rats (data not shown), the expression of glucocorticoid target genes - Tcs22d3 (also known as Gilz), Bhlhe40, Srgn, Plk2 and Gem - were significantly increased by ozone. These ozone-induced changes in glucocorticoid responsive genes have been shown previously (Thomson et al., 2016) and suggest that the lung is an active target for corticosterone action. The magnitude of this effect was greater on day 1 than on day 2, perhaps suggesting a degree of adaptation to the ozone exposure as has been described for lung injury and inflammation (Kirschvink et al., 2002; Iwasaki et al., 1998).

Although the cellular signaling induced by epinephrine is mediated primarily by posttranslational events, some signature changes known to be mediated by adrenergic receptor activation were found. The predicted gene signature included the activation of Forskolin pathway in ozone-exposed SHAM but not in DEMED or ADREX rats. Forskolin raises the levels of cAMP which is also downstream of β adrenergic receptor activation (Wallukat, 2002). The diminution of ozone effects in DEMED rats supports the role of epinephrine as a modulator of ozone induced lung injury/inflammation.

Ozone-induced innate immune response has been shown to be associated with increases in the neutrophil chemo-attractant IL6 (Gabehart et al., 2015; Johnston et al., 2003; Krishna et al., 1998). We noted that Il6 mRNA and proteins were up-regulated in SHAM but not in ADREX and DEMED rats, suggesting that adrenergic and steroidal hormones transcriptionally regulate innate immune response normally activated by ozone. Increased levels

of epinephrine, and cortisol favors the development of the Th2 response (Spellberg and Edwards, 2001). Ozone exposure has also been shown to promote Th2 phenotype shift in nasal and airway tissues (Kumagai et al., 2016; Wu et al., 2014). Similarly, we observed that there was an increase in BALF IL4/IFN-γ ratio in SHAM but not in DEMED and ADREX rats exposed to ozone suggesting that stress hormones may be involved in preferentially mediating the Th2 shift.

Since stress hormones play a fundamental role in homeostatic balance, ADREX and DEMED surgeries likely change some of the basic physiological processes in tissues where stress hormone receptors are expressed. It is also likely that the lack of circulating epinephrine, mineralocorticoids and glucocorticoids in ADREX rats may impact lung epithelial integrity. ADREX increased the expression of several lung cytokines, including Il-5, Il-13, and Tnfα, as determined using qPCR and immunoassays, in air and ozone exposed animals. ADREX was associated with inhibition of many genes regulating acquired immunity even in air-exposed animals suggesting a key role of adrenal-derived hormones in regulation of immune response upon encountering injury. Surprisingly, DEMED alone, associated with depletion of only epinephrine (Miller et al., 2016b), did not alter the expression of these genes, suggesting that the lack of glucocorticoids but not epinephrine, likely contributes to immune homeostasis. Increased expression of beta-2 adrenergic receptors in alveolar type II cells has been shown to increase alveolar fluid clearance (McGraw et al., 2001), while glucocorticoids have been reported to improve epithelial barrier function (Kielgast et al., 2016).

Ozone remains a potential health hazard as its levels could exceed >0.2 ppm in areas with hot climate and heavy industry (The Royal Society, 2008). Human clinical studies often use 0.2–0.4 ppm concentration with intermittent exercise which is comparable to 1.0 ppm exposure in the rats during rest (Hatch et al., 1994, 2013). The level of ozone used in our study (1.0 ppm) is much higher than what is expected in the polluted air. However, our goal was to achieve

detectable lung injury and inflammation in SHAM rats, such that any potential protective effects of DEMED and ADREX could be reliably detected.

This study did not allow assessment of contribution of individual hormones in ozone-induced lung injury and inflammation since it is not possible to surgically remove only the cortex. Although, the role of stress hormones in the development and magnitude of immunological responses has been extensively studied (Dhabhar et al., 2012), it has not been examined in the context of inhaled pollutants. The temporality of sequential events after the activation of a stress response was not taken into account and the role of ozone-induced hormonal changes in this context will need to be further examined.

In summary, ozone-induced transcriptional changes in the lung are greatly mitigated by both DEMED and ADREX surgeries demonstrating the key roles played by circulating adrenergic as well as steroidal hormones in the ozone-induced injury and inflammation. Ozone-induced increases in innate immune genes and proteins were markedly attenuated, including an increase in the ratio of IL-4/IFN-γ, in DEMED and ADREX rats, suggesting immune modulation by stress hormones. Immune, metabolic and oxidative stress pathways were induced in the lungs by ozone in SHAM rats while ADREX and DEMED conferred protection against these changes. In addition, upstream analysis by IPA showed that global gene changes induced by ozone in the lungs of SHAM rats were similar to steroidal chemicals and adrenergic influence.

From the public health perspective, glucocorticoid and adrenergic receptors are widely targeted to combat pulmonary chronic disease conditions such as asthma and COPD. The dynamic role of environmental stressors and the use of therapeutic approaches targeting steroidal and adrenergic mechanisms should be investigated in the treatment of lung inflammatory conditions.

# CHAPTER 3: ADRENERGIC AND GLUCOCORTICOID RECEPTOR ANTAGONISTS REDUCE OZONE-INDUCED LUNG INJURY AND INFLAMMATION

## 3.1 Introduction

Ozone is a reactive secondary pollutant which oxidizes biomolecules in the respiratory tract upon inhalation (Bromberg, 2016). The accepted paradigm of ozone-induced lung injury and inflammation involves its direct interaction with lung lining components and generation of oxidized lipid and protein byproducts (Auerbach and Hernandez, 2012), which are responsible for activation of the inflammatory signaling cascade and mediating downstream effects such as lung function decrement, increased vascular permeability, and neutrophilic inflammation. These ozone-induced effects are reversible even if daily exposure continues over several days suggesting tolerance or adaptation (Miller et al., 2016c). The oxidatively-modified reactive byproducts act as signaling factors locally within the lung, stimulating the release of cytokines and chemokines to promote recruitment of neutrophils and activation of NRF2 and NFκB pathways in the lung (Hollingsworth et al., 2007). However, the contribution of circulating biomolecules in ozone-induced lung injury and inflammation has only been recently examined (Kodavanti, 2016).

Acute ozone exposure has also been shown to induce pulmonary sensory irritation and C-fiber activation. Upstream events that are involved in this response include neuron firing of the nucleus tractus solitarius (NTS) (Gackière et al., 2011), increases in circulating adrenocorticotropic hormone (ACTH) (Thomson et al., 2013), and cardiac changes through autonomic reflex mechanisms (Arjomandi et al., 2015; Gordon et al., 2014). These events suggest that ozone inhalation is capable of triggering a centrally-mediated neuroendocrine

stress response which results in increased release of stress hormones into the systemic circulation (Kodavanti, 2016; Snow et al., 2017). Indeed, we have recently shown that circulating stress hormones such as epinephrine and cortisol/corticosterone rise rapidly after acute ozone exposure in both rodents (Bass et al., 2013; Miller et al., 2015, 2016b) and humans (Miller et al., 2016a). Subsequent ozone-induced lung global gene expression changes mimic those induced by downstream events promoted by $\beta_2$ adrenergic and glucocorticoid receptor activation, further suggesting that circulating stress hormones play a key role as mediators of pulmonary responses (Henriquez et al., 2017a). The role of circulating adrenal gland-derived stress hormones in ozone-induced lung injury and inflammation in rats was further confirmed by the evidence that bilateral adrenalectomy diminished these ozone effects (Miller et al., 2016b) and associated global lung transcriptional changes (Henriquez et al., 2017a).

Adrenergic receptors (AR) are widely distributed throughout the body and although epinephrine is a prototypical agonist for all types of AR, the selective activation of specific AR subtypes determines the physiological organ-specific response. $\beta_1$AR are mainly expressed in the cardiac tissue and are central in maintaining cardiac output and contractility of cardiac muscle (Kurz et al., 1991), hence βAR blockers are widely used to reduce blood pressure. On the other hand, $\beta_2$AR are primarily distributed in the smooth muscles of bronchi and blood vessels (Morgan and Laufgraben, 2013). Airway smooth muscle relaxation by $\beta_2$AR agonists is a common pharmacological intervention used for bronchodilation in asthma and chronic obstructive pulmonary disease (Cazzola et al., 2013). Propranolol (PROP) is a non-selective βAR antagonist capable of blocking both $\beta_1$AR and $\beta_2$AR and, unlike epinephrine, it readily crosses the blood brain barrier (Olesen et al., 1978).

Circulating cortisol/corticosterone binds to glucocorticoid receptors (GR) that are present in virtually all cells in the body. Nuclear translocation of these receptors up-regulates a variety of genes involved in homeostatic response(s) (Oakley and Cidlowski, 2013). Non-genomic actions of glucocorticoids on GR have also been identified (Duque and Munhoz, 2016). Although the

anti-inflammatory and immunosuppressive actions of glucocorticoids are not completely understood, potent GR agonists are commonly used to treat inflammatory conditions (Petta et al., 2016). By contrast, other studies have shown pro-inflammatory actions of GR activation (Cruz-Copete and Cidlowski, 2015). Mifepristone (MIFE) is a GR antagonist used to examine cellular effects of GR (Kakade and Kulkarni, 2014).

The goal of this study was to use a targeted pharmacological approach to examine the role of βAR and GR in mediating ozone-induced lung injury and inflammation. PROP, a non-selective βAR antagonist, was used to antagonize the activity of epinephrine while MIFE, a GR antagonist, was used to antagonize the activity of corticosterone. We hypothesized that the blocking of βAR and/or GR would produce selective inhibition of lung injury and/or inflammation and associated signaling events caused by exposure to ozone in rats.

## 3.2 Materials and Methods

### 3.2.1 Animals

Male Wistar Kyoto (WKY) rats (10 weeks of age), purchased from Charles River Laboratory (Raleigh, NC) were housed in pairs in polycarbonate cages containing beta chip bedding under controlled conditions (21°C, 50-65% relative humidity and 12h light/dark cycle). Rats were provided with standard Purina (5001) rat chow (Brentwood, MO) and water *ad libitum,* and housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved animal facility. Animal procedures were approved by the US Environmental Protection Agency, National Health and Environmental Effects Research Laboratory Institutional Animal Care and Use Committee. Because we previously determined that 5-10% of male WKY rats develop spontaneous cardiac hypertrophy (Shannahan et al., 2010), all animals were evaluated for evidence of cardiac hypertrophy relative to body weights at necropsy. Those with 20% or greater increases in the heart to body weight ratio were removed from further data analysis.

### 3.2.2 Drug pretreatments and ozone exposures

Three studies were conducted for each protocol involving PROP, MIFE, and PROP followed by MIFE (PROP+MIFE) pretreatments (Fig. 3.1). For each study, rats were randomized by body weight into four groups (vehicle/air, drug/air, vehicle/ozone, drug/ozone) and time point (one day, D+1; or two days, D+2) (n=8/group). In the first study, rats were injected i.p. with sterile saline (1 mL/kg) or propranolol hydrochloride (PROP, Sigma-Aldrich, St Louis, MO; 10 mg/kg in saline). In the second study, rats were injected s.c. with pharmaceutical grade corn oil (1 mL/kg) or mifepristone (MIFE, Cayman Chemical Co., Ann Arbor, MI; 30 mg/kg in corn oil). In the third study, control rats were injected with saline (1 mL/kg, i.p.) followed by corn oil (1 mL/kg, s.c.) while the drug-treated group was injected with PROP (10 mg/kg, i.p.) followed by MIFE (30 mg/kg, s.c.) (PROP+MIFE). For all three studies, the vehicle/drug pretreatments began seven days prior to the start of air or ozone exposure (from Day-7 to Day-1 in the morning) and

continued each day of air or ozone exposure (D+1 and D+2) (Fig. 3.1). Rats were exposed to either filtered air or ozone (0.8 ppm) for 4h during D+1 and/or D+2 in individual wire-mesh cages placed in exposure chambers.

Ozone was generated using a silent arc discharge generator (OREC, Phoenix, AZ) and delivered to 1000 L Hinners-type chambers using mass flow controllers. $O_3$ concentration was recorded continuously by photometric analyzers (API Model 400, Teledyne, San Diego, CA). Chamber temperature (average $^{o}$F) and relative humidity (RH: average %) were measured continuously and recorded hourly. The mean ozone chamber concentration was 0.803±0.002 ppm (Mean ± standard deviation). The RH for control chamber was 49±4 and ozone chamber was 46±3. The temperature of control chamber was 72.8±0.3 $^{o}$F and ozone chamber was 74.2±0.4 $^{o}$F. The control chamber flow was maintained at 256±24 while ozone chamber flow at 261±3 liters per minute).

**Figure 3.1**

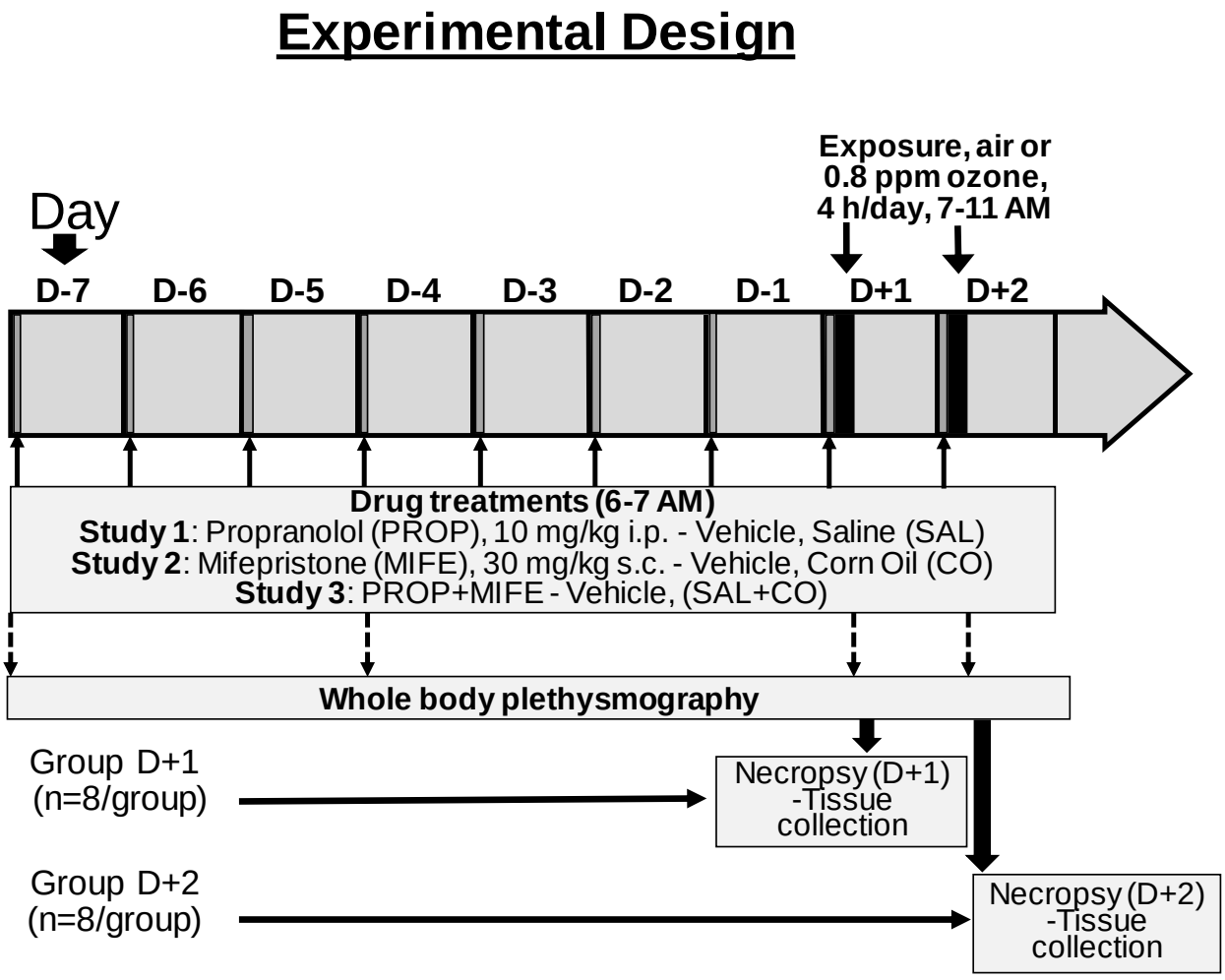



**Figure 3.1 Schema of the experimental design.** For all three studies, the timing for drug pretreatments and the information on air or ozone exposure, plethysmography and necropsies are indicated by corresponding arrows. Animals assigned to 1 day (4 hr) air or ozone exposure are referred as group D+1 and those assigned to 2 consecutive days of exposure are referred as group D+2. Animals assigned to group D+2 were subjected to plethysmography prior to the start of drug pretreatment, after 3 days of drug pretreatment (D-4) and immediately after each day of air/ozone exposure. Necropsy and tissue collection were performed immediately after exposure (D+1) or after exposure and plethysmography (D+2) (within 1-2 hours of exposure). Vehicles: SAL, saline; CO, corn oil; drugs: PROP, propranolol; MIFE, mifepristone.

3.2.3 Whole body plethysmography

In rats assigned to the D+2 group, ventilatory parameters were measured in unrestrained animals using whole body plethysmography (WBP) prior to drug treatments, during drug treatments and immediately after each day of air or ozone exposure. Briefly, rats were placed in pre-calibrated WBP chambers (Model PLY3213; Buxco Electronics, Inc., Wilmington, NC). During the first two minutes, the rats were allowed to acclimate. Then data were averaged for a total time of 5 minutes using EMKA iox 2 software (SCIREQ, Montreal, Canada). Ventilatory parameters included breathing frequency, tidal volume (TV), minute volume (MV), peak inspiratory flow (PIF), peak expiratory flow (PEF), inspiratory time (IT), expiratory time (ET), and enhanced pause (Penh), an index of air flow limitation and surrogate measure of bronchoconstriction (Hammelmann et al., 1997).

3.2.4 Cytokine protein quantification

Immediately after exposure on day 1 (D+1), and after exposure and plethysmography on day 2 (D+2), rats were necropsied during the course of 1-2 hours. The rats were euthanized with an overdose of Fatal Plus (sodium pentobarbital, Virbac AH, Inc., Fort Worth, TX; >200 mg/kg, i.p.). Blood samples were collected from the abdominal aorta directly in vacutainer tubes. EDTA containing blood samples were used to perform complete blood count on a Beckman-Coulter AcT blood analyzer (Beckman-Coulter Inc., Fullerton, California). Additionally, blood smears were prepared and stained with Wright-Giemsa (Fisher Scientific) using a Hema-Tek 2000 slide stainer (Miles, Inc., Elkhart, IN, USA). Because this hematology analyzer provided data for only total white blood cells and lymphocytes, additional blood smear slides were used to verify the number of lymphocytes and to evaluate relative numbers of monocytes and neutrophils for the D+1 animals based on 100 white blood cells per slide.

The right lung was lavaged with $Ca^{2+}$ and $Mg^{2+}$ free PBS, pH 7.4, at 37 °C. The lavage volume was calculated based on 28 mL/kg body weight total lung capacity, and the right lung weight being 60% of total lung weight (Bass et al., 2013). Bronchoalveolar lavage fluid (BALF)

was used to determine total cell count (Z1 Coulter Counter, Coulter, Inc., Miami, FL) and cell differentials by preparing Cytospin slides (Thermo Fisher Scientific, Waltham, MA). These slides were stained with Diff-Quik (Thermo Fisher Scientific) and cell differentials were performed under light microscopy (300 cells/slide). Cell-free BALF (supernatant from whole BALF centrifuged at 1500xg) was used to measure total protein (Coomassie plus Protein Assay Kit, Pierce, Rockford, IL), albumin (DiaSorin, Stillwater, MN), and *N*-acetyl-β-D-glucosaminidase (NAG) activity (Roche Diagnostics, Indianapolis IN) using commercially available kits adapted for use on a Konelab Arena 30 clinical analyzer (Thermo Chemical Lab Systems, Espoo, Finland). Following lavage, the right caudal lobe was removed, frozen in liquid nitrogen, and stored at -80$^{o}$C for RNA extraction.

3.2.5 Lung RNA Isolation and real time-quantitative PCR

Total RNA was isolated from a uniform portion (10-20 mg) of the right caudal lung lobe using RNeasy mini kit (Qiagen, Valencia, CA). RNA was quantified using a Qubit 2.0 fluorimeter (ThermoFisher Scientific, Waltham, MA) and reverse transcribed using Qscript cDNA Supermix (Quanta Biosciences, Beverly, MA). Primers were designed using sequences annotated in NCBI and obtained from Integrated DNA Technologies, Inc (Coralville, IA) including: β-Actin (*Actb*): f-CAACTGGGACGATATGGAGAAG, r-GTTGGCCTTAGGGTTCAGAG; tumor necrosis factor (*tnf*) f-ACCTTATCTACTCCCAGGTTCT, r-GGCTGACTTTCTCCTGGTATG; Interleukin 6 (*Il6*) f-CTTCACAAGTCGGAGGCTTAAT, r-GCATCATCGCTGTTCATACAATC; Chemokine (C-X-C motif) -ligand 2 (*Cxcl*2 or *Mip*2) f- GCCTCGCTGTCTGAGTTTATC, r-GAGCTGGCCAATGCATATCT; Metallothionein-2 (*Mt2a*) f- CAGCGATCTCTCGTTGATCTC, r-GGAGGTGCATTTGCATTGTT; TSC22 domain family protein 3 (*Tsc22d3* or *Gilz*) f-CCGAATCATGAACACCGAAATG, r- GCAGAGAAGAGAAGAAGGAGATG. Quantitative PCR was performed for each gene using SYBR® Green PCR Master Mix (ThermoFisher, Waltham, MA) on the Applied Biosystems 7900HT Sequence Detection System (Foster City, CA). Relative mRNA expression was calculated using the ΔΔCt method following initial normalization to the

housekeeping gene (*Actb*) and then with the respective vehicle-air control group. The determination of lung mRNA expression was performed only for D+1 samples since it has been shown that ozone-induced increases in mRNA expression precedes the release of associated proteins and inflammation (Hollingsworth et al., 2007; Kodavanti et al., 2015).

### 3.2.6 Cytokine quantification

Serum and BALF cytokine concentrations were quantified using the V-PLEX proinflammatory panel 2 (rat) kit per manufacturer's protocol (Mesoscale Discovery Inc., Rockville, MD). The electrochemiluminescence signals for each protein were detected using Meso Scale Discovery® platform (Mesoscale Discovery Inc., Rockville, MD). In control animals in which the levels of some cytokines in BALF were below the assay detection limit, the values were substituted with the lowest quantified value for the given cytokine in the group. Measurement of BALF and plasma cytokines was restricted only to D+2 samples since it has been noted that ozone-induced inflammation is maximal at this time point (Ward et al., 2015).

### 3.2.7 Statistics

For all endpoints, data were analyzed using a two-way analysis of variance (ANOVA). PROP, MIFE and MIFE+PROP studies were treated as independent experiments. D+1 and D+2 exposure groups were also treated as independent experiments. The two independent variables were exposure (air or 0.8 ppm ozone) and pretreatment (vehicle or drug). The Holm-Sidak *post-hoc* test was used to correct for all multiple comparisons and significant differences were considered when a *P*-value of ≤ 0.05 was achieved. All data (n=6-8 animals/group) are expressed as mean ± SEM and GraphPad prism 6.07 software was used for statistical analysis. For pulmonary gene expression analysis only, outliers were identified based on the false discovery rate and discarded using the ROUT method (robust regression and outlier removal (Motulsky and Brown, 2006).

## 3.3 Results

### 3.3.1 Ozone-induced changes in ventilatory parameters are not impacted by PROP, MIFE and PROP+MIFE pretreatment

In air-exposed rats, drug pretreatments did not affect any of the ventilatory parameters at D+1 or D+2 (Fig. 3.2). In ozone-exposed vehicle-treated rats, PEF and corresponding PenH values were consistently increased, both on D+1 and D+2, indicative of labored breathing. In ozone-exposed rats, PROP pretreatment was without effect on D+1, however, on D+2, this combination led to significantly reduced frequency of breathing (Fig. 3.2A). In ozone exposed MIFE-pretreated rats, results were similar to the ozone-only group. Finally, in the ozone-exposed PROP+MIFE pretreated rats, MV was significantly reduced at D+2 (Fig. 3.2B). Notably, immediately after the second day of exposure, PenH values were further increased in all ozone-exposed rats on D+2 (D+2>D+1), and no drug-related influences were apparent (Fig 3.2D).

**Figure 3.2**

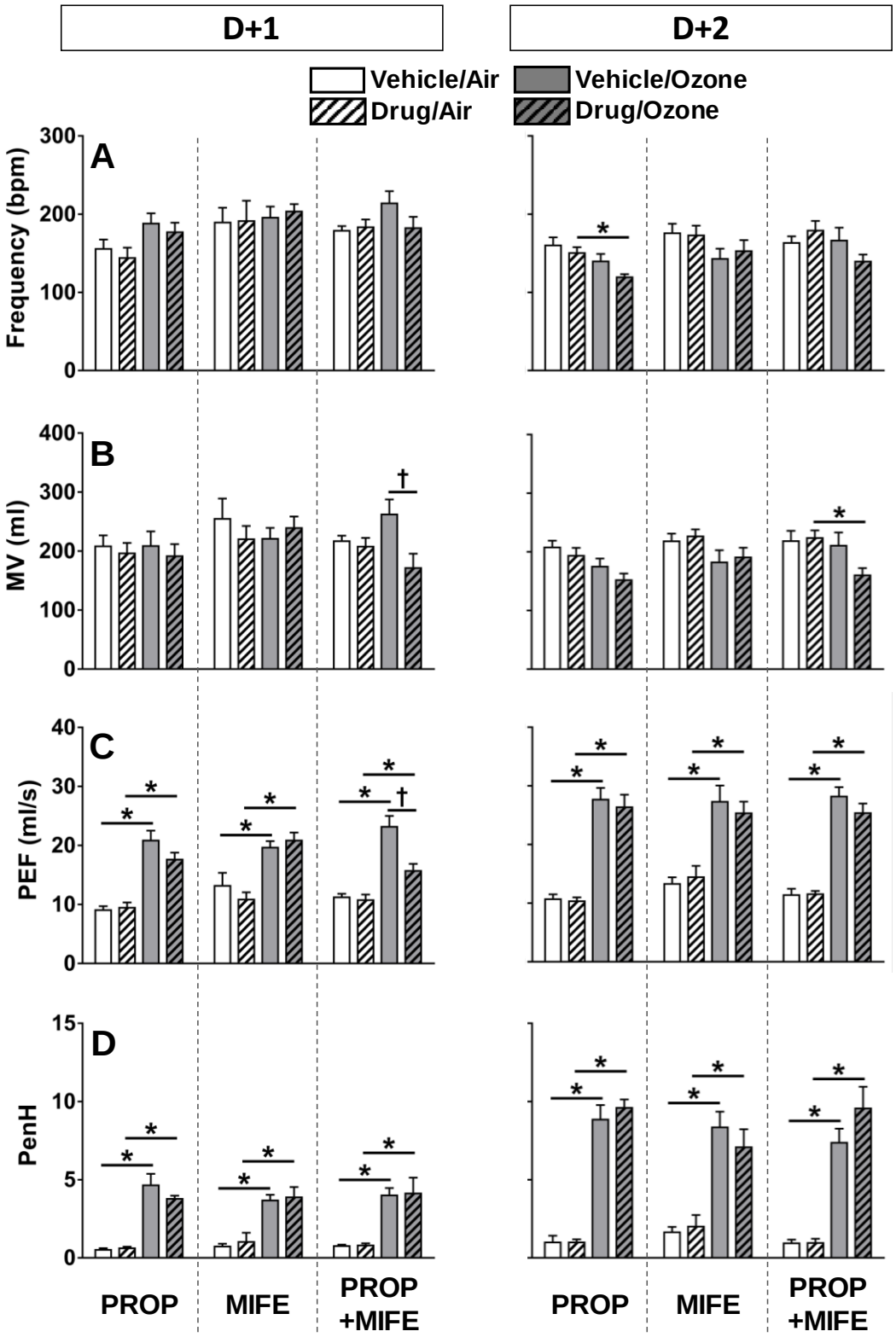

D+1
D+2
Vehicle/Air
Drug/Air
Vehicle/Ozone
Drug/Ozone
A
Frequency (bpm)
300
200
100
0
B
MV (ml)
400
300
200
100
0
C
PEF (ml/s)
40
30
20
10
0
D
PenH
15
10
5
0
PROP
MIFE
PROP +MIFE
PROP
MIFE
PROP +MIFE

**Figure 3.2 Ventilatory parameters in vehicle- or drug-pretreated rats after each day of air or ozone**. Ventilatory parameters determined after the first (D+1) and second (D+2) day of air or 0.8 ppm ozone exposure are shown (pre-air or -ozone exposure data are not shown). The breathing parameters indicate mean ± standard error of mean (SEM) of n=6-8 animals/group. Significant differences between groups ($P \leq 0.05$) are indicated by * for ozone effect and by † for drug pretreatment effect. A) Breathing frequency, B) minute volume (MV), C) peak expiratory flow (PEF), D) enhanced pause (PenH).

### 3.3.2 Ozone-induced pulmonary injury and inflammation are reduced by PROP, MIFE and/or PROP+MIFE pretreatments

Drug pretreatment in air-exposed rats did not influence any of the measures of lung injury and inflammation. Pulmonary vascular leakage, as measured by protein and albumin content in BALF, is a key event occurring after ozone exposure. Ozone-induced BALF protein (Fig. 3.3A) and albumin (Fig. 3.3B) tended to increase in all vehicle-pretreated rats at D+1. On D+2 all vehicle-pretreated ozone-exposed rats demonstrated marked increases in BALF protein and albumin that were diminished with all drug pretreatments. NAG activity in BALF, a marker of macrophage activation, was increased after both days of ozone exposure in vehicle-pretreated animals (D+2>D+1) (Fig. 3.3C). Ozone-induced increases in NAG activity on D+2 were attenuated by MIFE and MIFE+PROP but not PROP (Fig. 3.3C).

To determine the extent of ozone-induced inflammation in the lungs and effects of drug pretreatments, BALF total cells, neutrophils and lymphocytes were counted. Total cells in BALF were not changed by any of the drug pretreatments or ozone exposure except for a non-significant reduction in all ozone exposed rats in the PROP study at D+1 (Fig. 3.4A). Ozone exposure on D+1 did not increase BALF neutrophil influx substantially in any of the vehicle or drug-pretreated groups except for a small but significant increase in saline-pretreated rats in the PROP study. The ozone-induced lung neutrophilia was significant in all vehicle-pretreated rats on D+2. This ozone-induced neutrophilia was prevented by PROP and PROP+MIFE but not MIFE alone (Fig. 3.4B). Ozone exposure resulted in reduced number of lymphocytes in BALF on D+1 and D+2 in all vehicle-treated rats. There was no effect of any drug pretreatment on ozone-induced reduction in BALF lymphocytes (Fig. 3.4C).

**Figure 3.3**

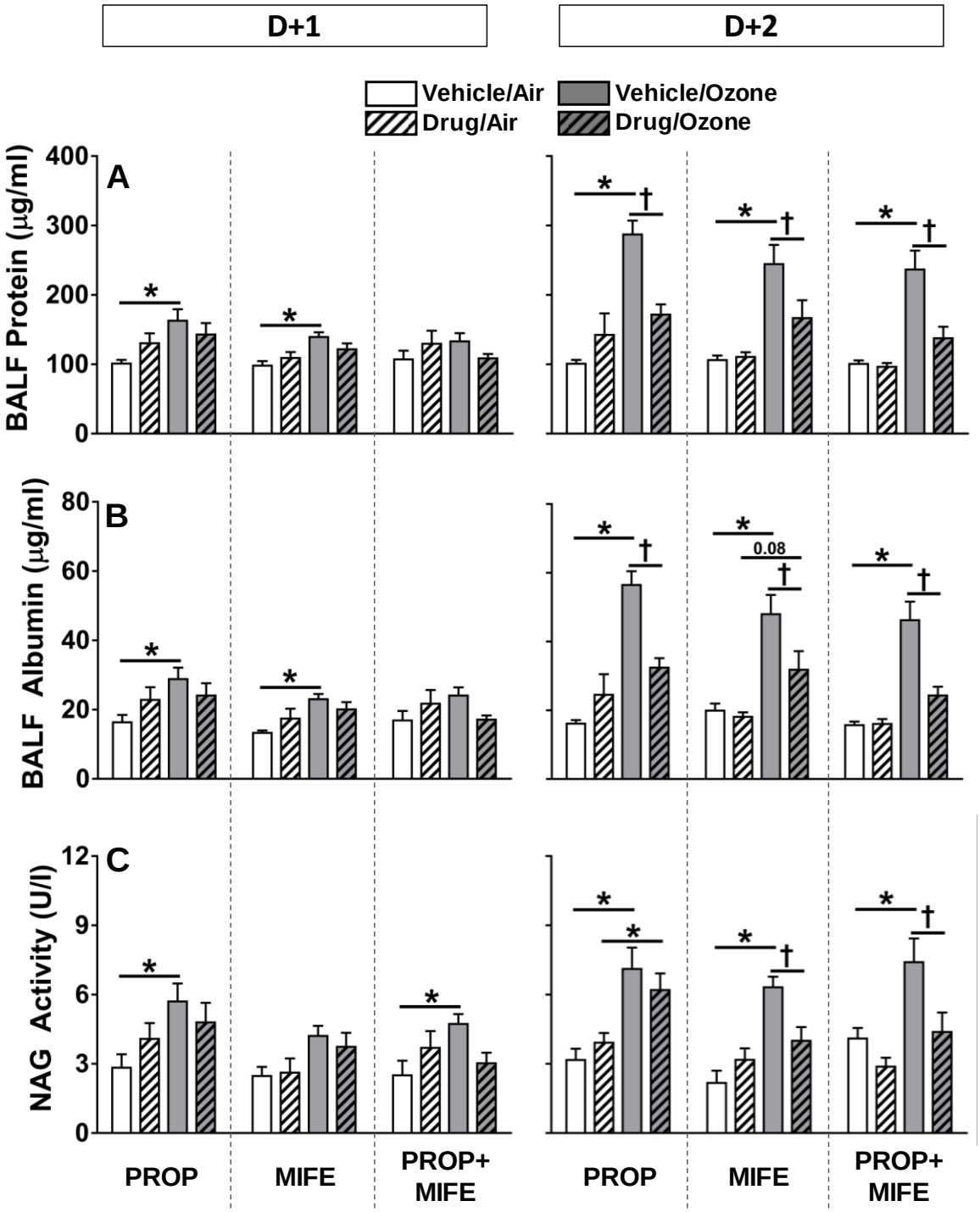

D+1
D+2
Vehicle/Air
Drug/Air
Vehicle/Ozone
Drug/Ozone
A
BALF Protein (μg/ml)
400
300
200
100
0
B
BALF Albumin (μg/ml)
80
60
40
20
0
0.08
C
NAG Activity (U/l)
12
9
6
3
0
PROP
MIFE
PROP+ MIFE
PROP
MIFE
PROP+ MIFE

**Figure 3.3 The influence of drug pretreatments on ozone-induced BALF protein leakage and N-acetyl glucosaminidase (NAG) activity.** Protein (A), albumin (B), and NAG activity (C) were determined in BALF collected immediately following exposure to air or 0.8 ppm ozone, 4 hr/day for 1 day (D+1) or 2 days (D+2). Values indicate mean ± SE of n=6-8 animals/group. Significant differences between groups ($P \leq 0.05$) are indicated by * for ozone effect in matching pretreatment groups, and by † for drug effect in matching exposure groups.

**Figure 3.4**

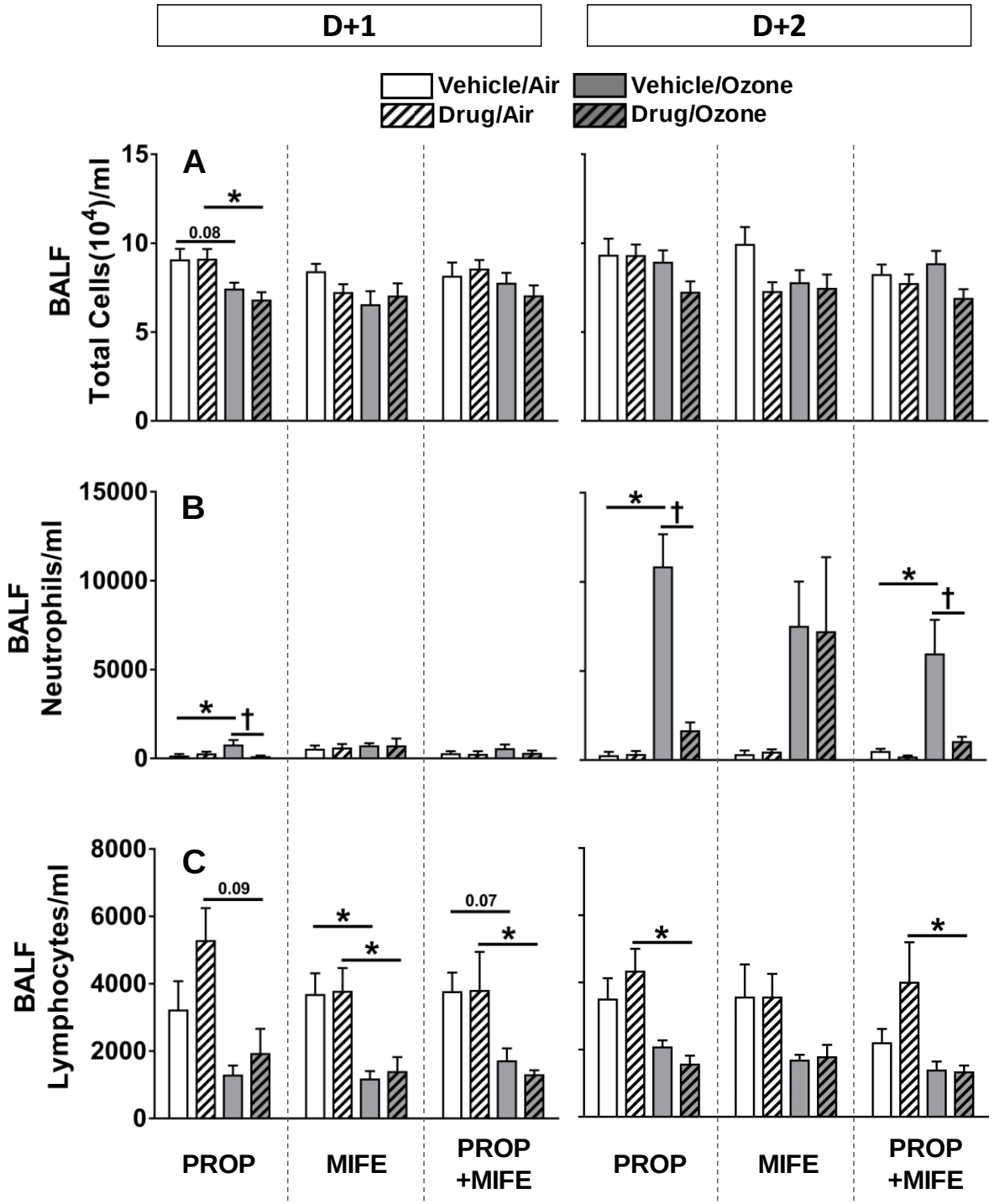

D+1
D+2
Vehicle/Air
Vehicle/Ozone
Drug/Air
Drug/Ozone
A
BALF
Total Cells($10^4$)/ml
15
10
5
0
0.08
*
B
BALF
Neutrophils/ml
15000
10000
5000
0
*
†
C
BALF
Lymphocytes/ml
8000
6000
4000
2000
0
0.09
0.07
PROP
MIFE
PROP
+MIFE
PROP
MIFE
PROP
+MIFE

**Figure 3.4 The influence of drug pretreatments on ozone-induced changes in lung inflammation as determined by BALF cell count.** Total cells (A), neutrophils (B) and lymphocytes (C) were determined in BALF collected immediately following exposure to air or ozone (0.8 ppm), 4 hr/day for 1 day (D+1) or 2 consecutive days (D+2). Values indicate mean ± SE of n=6-8 animals/group. Significant differences between groups ($P \leq 0.05$) are indicated by * for ozone effect in matching pretreatment groups, and by † for drug effect in matching exposure groups.

### 3.3.3 Ozone-induced decreases in circulating white blood cells (WBC) and lymphocytes were reversed by MIFE pretreatment

To determine the relationship between levels of immune cells in the lung and in the circulation, complete blood count and cell differentials were performed. Circulating total WBC were reduced to nearly half in all vehicle pretreated rats exposed to ozone (Fig. 3.5A). This ozone-induced decrease was prevented in rats pretreated with MIFE or PROP+MIFE but not PROP alone. This pattern was not replicated in circulating neutrophils as no specific exposure- or pretreatment-related changes were noted in the D+1 group (Fig. 3.5B). Since nearly 70-90% of circulating WBC (Cameron and Watson, 1949) in this strain of rats are lymphocytes, the changes in circulating lymphocytes reflected the changes observed in WBC after ozone exposure and drug-pretreatments (Fig. 3.5C).

**Figure 3.5**

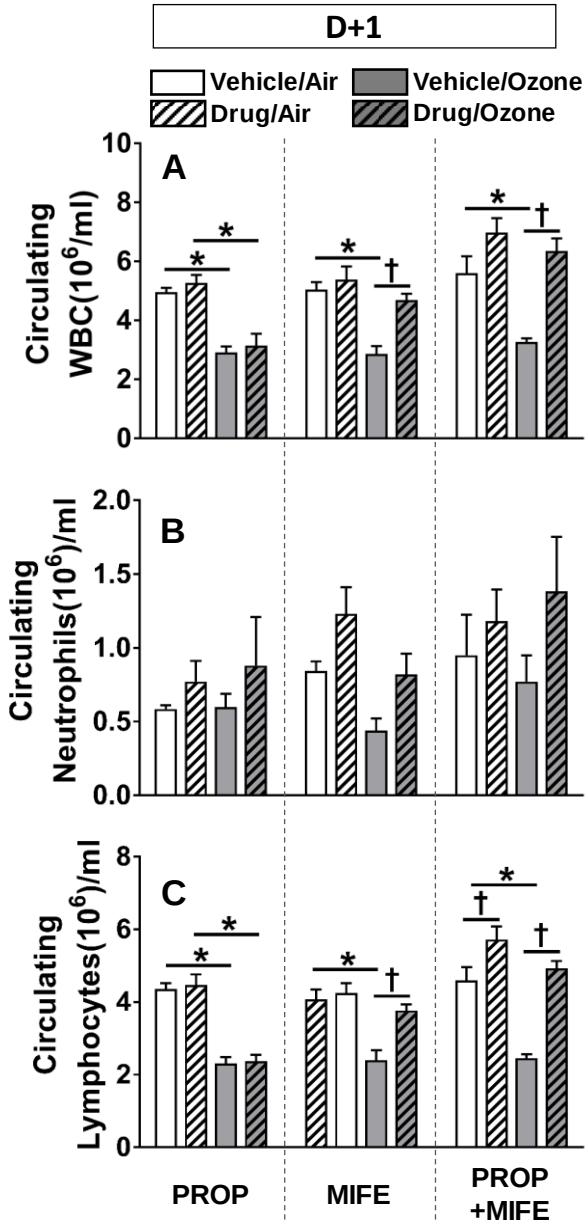



**Figure 3.5 The effects of drug pretreatments on ozone-induced changes in circulating white blood cells (WBC) in rats.** Circulating total white blood cells (A), neutrophils (B) and lymphocytes (C) were determined in rats immediately after exposure to air or 0.8 ppm ozone, 4 hr/day for 1 day (D+1). Values indicate mean ± SE of n=6-8 animals/group. Significant differences between groups ($P \leq 0.05$) are indicated by * for ozone effect in matching pretreatment groups, and by † for drug effect in matching exposure groups.

3.3.4 Ozone-induced increases in expression of pro-inflammatory mediators in the lung are diminished by PROP pretreatment

Lung mRNA (D+1) and BALF protein levels (D+2) of IL-6 and TNF-α were assessed to determine if the influence of PROP and MIFE on ozone-induced inflammatory changes occur at the transcriptional and signaling levels. Ozone exposure was associated with increases in *Il6* lung mRNA and BALF protein in all vehicle pretreated rats. Ozone's effect on *Il6* mRNA and BALF protein was significantly reduced in animals pretreated with PROP but not MIFE or PROP+MIFE (Fig. 3.6A, 3.6C). Although no consistent ozone exposure or drug pretreatment-related changes were apparent on *Tnf*$\alpha$ mRNA expression as determined on D+1, marked increases in BALF TNF-α protein were noted in all ozone-exposed vehicle-pretreated rats at D+2 (Fig. 3.6B, 3.6D). This ozone effect on TNF-$\alpha$ was slightly reduced in rats pretreated with PROP, however, there was no significant effect of MIFE or PROP+MIFE (Fig. 3.6D).

In addition to the above mentioned inflammatory cytokines, we also determined the effect of PROP and MIFE pretreatments at the D+1 time point on two lung transcripts known to be upregulated after ozone exposure: *Cxcl2* (also known as *Mip2*, a neutrophil chemoattractant) and *Mt2a* (metallothionein 2a, an acute phase reactant (Henriquez et al., 2017a; Ward et al., 2015). Ozone exposure increased expression of *Cxcl2* and *Mt2a* in vehicle pretreated rats (Fig. 3.7A, 3.7B). The ozone-induced up-regulation of *Cxcl2* was significantly decreased only by PROP and PROP+MIFE pretreatments whereas MIFE alone did not have any effect (Fig. 3.7A). Ozone-induced upregulation of *Mt2a*, a free radical scavenger released in inflammatory conditions (Kurz et al., 1991), was significantly reduced by all drug pretreatments (Fig. 3.7B). The expression of the glucocorticoid responsive gene *Tsc22d3* was also markedly increased after ozone exposure in all vehicle-pretreated rats. That ozone-induced increase was diminished by PROP, MIFE and PROP+MIFE (Fig. 3.7C).

**Figure 3.6**

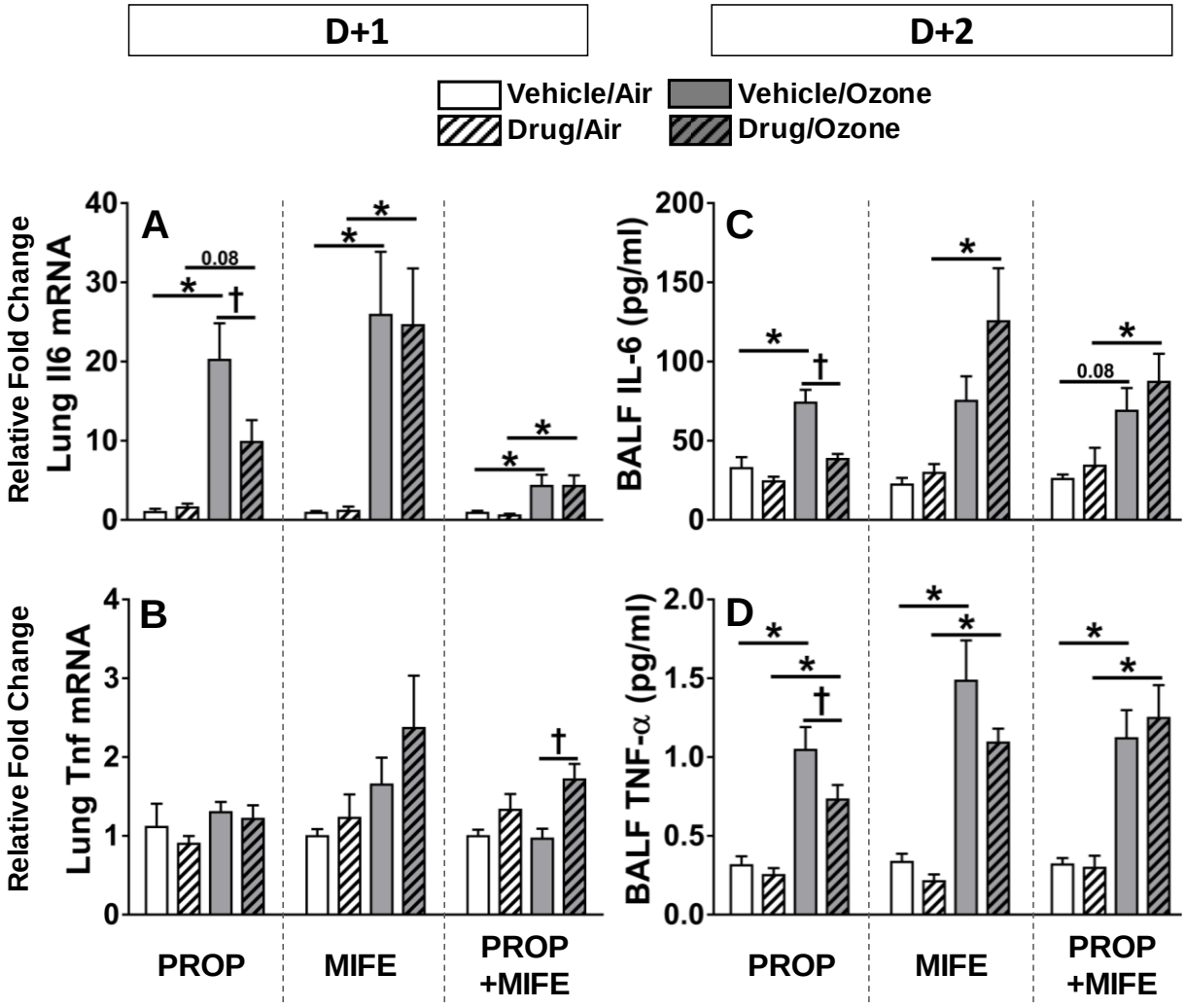



**Figure 3.6 Ozone-induced changes in *Tnf*-α and *Il6* lung mRNA and BALF proteins in rats pretreated with βAR and GR antagonists.** Lung mRNA expression of *Il6* (A) and *Tnf* (B) were determined in D+1 groups while BALF protein levels of IL-6 (C) and TNF-α (D) were assessed in D+2 groups. Values indicate mean ± SE of n=6-8 animals/group. Significant differences between groups ($P \leq 0.05$) are indicated by * for ozone effect in matching pretreatment groups, and by † for drug effect in matching exposure groups.

**Figure 3.7**

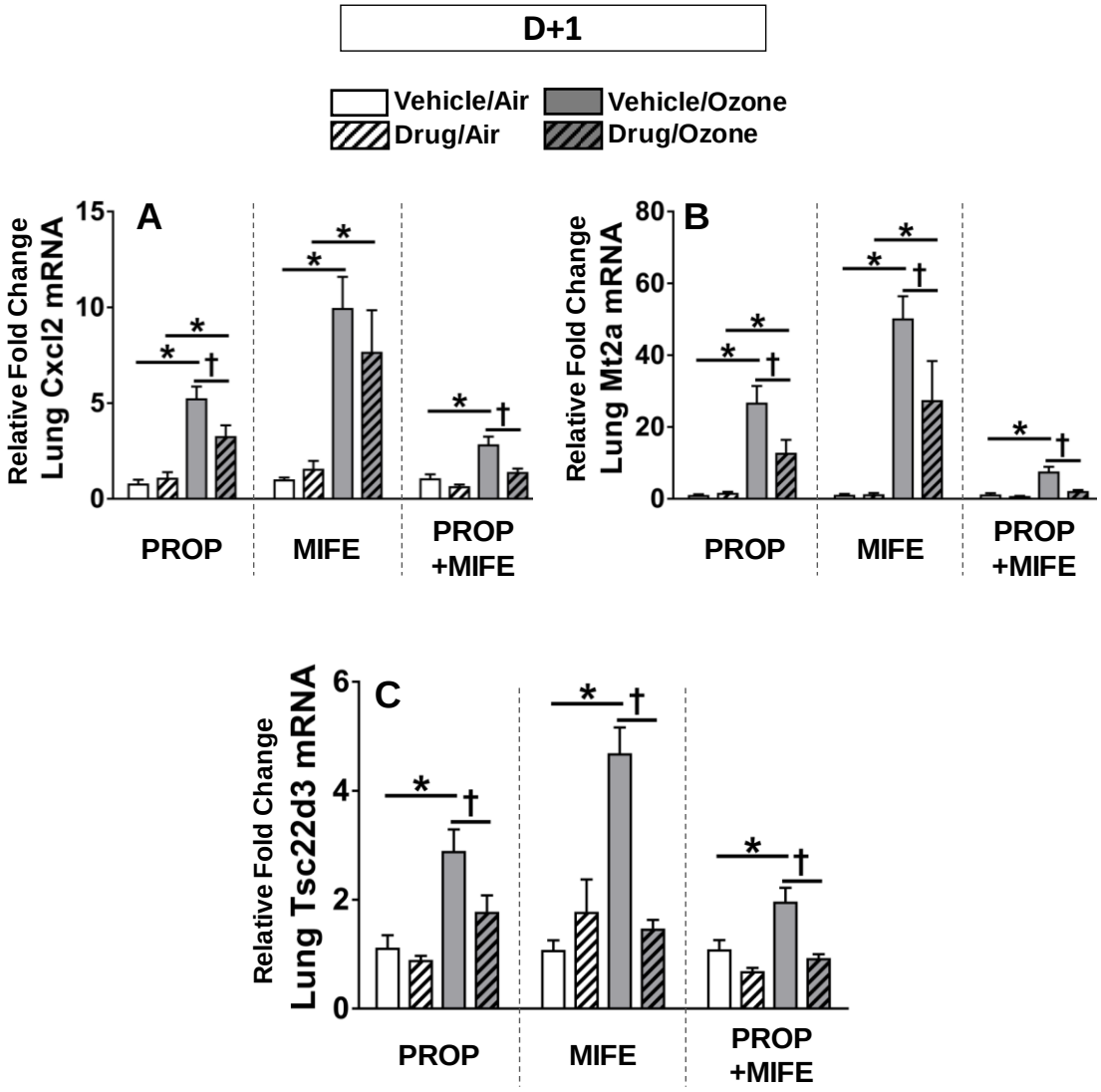



**Figure 3.7 The effect of drug pretreatments on ozone-induced increases in pulmonary *Cxcl2*, *Mt2a* and *Tsc22d3* mRNA expression.** Relative lung mRNA expression was determined in D+1 groups for *Cxcl2* (also known as *Mip2*; A), *Mt2a* (B) and *Tsc22d3* (C). Values indicate mean ± SE of n=6-8 animals/group. Significant differences between groups ($P \leq 0.05$) are indicated by * for ozone effect in matching pretreatment groups, and by † for drug effect in matching exposure groups.

## 3.4 Discussion

In this study, we blocked the activity of stress hormone receptors βAR and GR, individually or together, to better understand and define the role of stress hormones in ozone-induced pulmonary effects. The experimental design was developed to separately account for the effects likely to be mediated via epinephrine, corticosterone, or both, by pretreating rats with PROP, MIFE or PROP+MIFE, respectively (Fig. 3.8). We hypothesized that βAR and GR blockade would prevent ozone-mediated activation of downstream signaling events stimulated by increased circulating epinephrine and/or corticosterone and, as a result, the extent of ozone-induced pulmonary effects. Since only PROP+MIFE but not other individual pretreatments reduced minute volume and PEF in ozone-exposed rats, the degree of ozone-induced lung edema or neutrophilic inflammation in the PROP and/or MIFE-pretreated rats were likely not influenced by the possibility of reduction in lung ozone dose. Nevertheless, PROP, MIFE or PROP+MIFE were sufficient to attenuate ozone-induced pulmonary protein leakage; however, only PROP and PROP+MIFE reduced ozone-induced lung neutrophilic inflammation, and pro-inflammatory cytokine increases. On the other hand, only MIFE pretreatment reversed ozone-induced lymphopenia and increases in BALF NAG activity which reflects macrophage activation (Fig. 3.8). These data suggest that PROP and MIFE have distinct roles in mediating inflammatory cell-specific responses induced by ozone. It is noteworthy that pretreatment of animals with PROP, MIFE or PROP+MIFE had very little if any pulmonary and systemic effect in air-exposed animals, highlighting the role of βAR and GR as specific modulators of ozone–induced pulmonary vascular leakage and innate immune responses. Since βAR and GR have been widely manipulated in therapeutics, those receiving βAR and GR-related treatments might have altered susceptibility to high levels of ozone or other pulmonary irritants.

**Figure 3.8**

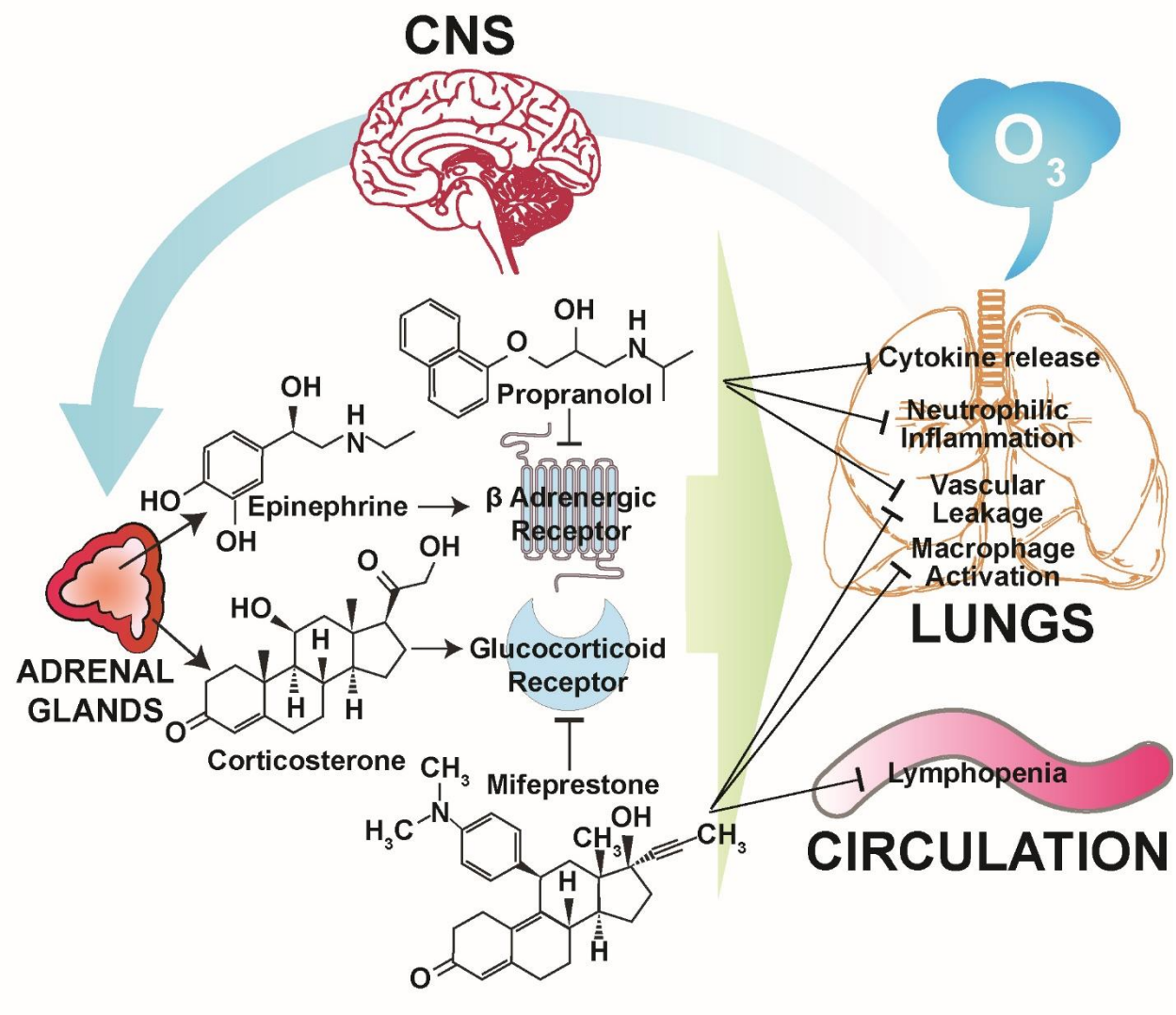



**Figure 3.8 Proposed mechanism by which βAR and GR antagonists reduce ozone-induced pulmonary protein leakage, cytokine expression, neutrophilic inflammation and lymphopenia.** This schematic is based on our earlier studies that ozone-induced lung injury and inflammation are mediated through neuroendocrine activation of stress response and involves the release of adrenal-derived stress hormones, epinephrine and corticosterone (Kodavanti, 2016). Ozone-induced effects are inhibited by pretreatment of rats with PROP and/or MIFE.

We selected PROP and MIFE to evaluate the role of βAR and GR activation in ozone-induced pulmonary injury/inflammation since these drugs are clinically used and have been validated for their desired effects (Alamo et al., 2017; Kubo et al., 2004). PROP, a non-selective βAR antagonist, blocks activity of both $\beta_1$ and $\beta_2$AR. Although epinephrine is a potent agonist of virtually all AR (Morgan and Laufgraben, 2013), in this study we focused only on the role of βAR activity since these receptors are enriched in cardiopulmonary tissues and have a major role in autonomic regulation and innate immune homeostatic balance (Bible et al., 2015; Folwarczna et al., 2011; Hegstrand and Eichelman, 1983; Kolmus et al., 2015; Sato et al., 2010). MIFE, a GR antagonist, has been widely used to antagonize the GR in psychiatric disorders (Howland et al., 2013) and adrenal hyper-production of cortisol (Morgan and Laufgraben, 2013). Several experimental studies have validated its anti-GR activity in animal models (Navarro-Zaragoza et al., 2017; Sharrett-Field et al., 2013; Wang et al., 2014).

When examining the effects of PROP and MIFE pretreatment, it is critical to determine if the observed ozone effects are influenced by the change in its effective lung dose. We have done whole body plethysmography in several of our studies to determine the likely change in ozone dosimetry (Dye et al., 2015b; Snow et al., 2016). It is noteworthy that no drug pretreatments affected ventilatory parameters in air-exposed rats. As we have observed in previous studies (Dye et al., 2015b), all ozone-exposed rats regardless of drug pretreatment demonstrated increases in PenH and PEF, measures of labored breathing, but had no effect on minute volume, except for a small decrease only in PROP+MIFE rats. Since the manipulation of both AR and GR has been shown to change breathing parameters in humans (Antonelli-Incalzi and Pedone, 2007; Hirst and Lee, 1998), the observed small changes in MV only in PROP+MIFE group may be due to the combined βAR+GR blockade. Since only this group, but not PROP or MIFE alone, showed a slight reduction minute volume after ozone exposure, it is

not likely that the lung injury and inflammation changes are impacted by the differential ozone dose with different pretreatments.

There are a number of potential mechanisms by which lung vascular permeability might be increased by ozone. An imbalance between peripheral sympathetic influence due to increased epinephrine and acute dominance of parasympathetic influence on the heart can shift the blood flow from high resistant peripheral vasculature to low resistant pulmonary vasculature, leading to pulmonary microvascular leakage (Li and Pauluhn, 2017). Our previous work has shown that ozone increases circulating epinephrine with no change in norepinephrine (Miller et al., 2015, 2016b). Thus, lung permeability changes are likely influenced by epinephrine's effect on $\beta_2$AR, which caused dilation of pulmonary vasculature, an effect that could be prevented by PROP antagonism of these receptors (Pourageaud et al., 2005). Since PROP and MIFE, each prevented ozone-induced lung microvascular leakage, it is possible that AR and GR effects are inter-related and/or centrally-mediated. Both drugs are known to cross the blood brain barrier and regulate expression of stress responses through their effects on the hypothalamus (Check et al., 2014; Neil-Dwyer et al., 1981). Interestingly, GR activation can also mediate effects of norepinephrine to regulate blood pressure (Shi et al., 2016).

Ozone-induced innate inflammatory responses in the lung involve neutrophil extravasation through enhanced trans-endothelial migration (Krishna et al., 1997). Catecholamines and glucocorticoids, which are increased by ozone inhalation (Bass et al., 2013; Miller et al., 2015; Miller et al., 2016a, 2016b) play a central role in initiating a dynamic process of egress of innate immune cells from their site of depot to the circulation and then migration to the site of inflammation, in this case the lung. It has been shown that increased circulating epinephrine and corticosterone can induce the innate neutrophil immune response (Dhabhar et al., 2012). We have shown that increases in pulmonary neutrophil extravasation is associated with ozone-induced increases in stress hormones and that adrenal demedullation and/or total adrenalectomy diminishes this inflammatory effect of ozone (Miller et al., 2016b).

Here we observed that inhibiting βAR with PROP and both βAR+GR with PROP+MIFE, mimicking systemic impacts of adrenal demedullation and total adrenalectomy, respectively, nearly inhibited neutrophil increases in the lung in its entirety. However, inhibition of only GR using MIFE was ineffective in reversing lung neutrophilic inflammation, suggesting that βAR activation might be central in neutrophil extravasation to the lung after ozone exposure. This is further supported by studies demonstrating that PROP pretreatment prevents cigarette smoke-induced lung damage (Zhou et al., 2014), and improves survival in a sepsis model (Wilson et al., 2013).

The effects of AR and GR manipulation on ozone-induced responses are immune cell specific. The lack of ozone-induced lymphopenia in MIFE- but not PROP-pretreated rats highlights the role of GR in modulating the migration, redistribution and proliferation of lymphoid cells in the circulation (Dhabhar et al., 2012). This effect of MIFE was not recapitulated in the number of BALF lymphocytes, suggesting that ozone-induced changes in circulating lymphocytes are likely influenced by GR-mediated egress from their storage site but not extravasation into the lung. The acute stress response fine-tunes immune function depending on the timing and magnitude of the stressor by enhancing innate and adaptive immune responses (Dhabhar, 2014). MIFE pretreatment has been shown to reestablish lymphocyte mediated immune responses in immunosuppressed mice (Rearte et al., 2010). Further, a MIFE-mediated reduction in glucocorticoid-induced lymphocyte apoptosis may also explain these results (Smith and Cidlowski, 2010) since glucocorticoids inhibit the release of lymphocytes into the circulation and induce apoptosis (Baschant and Tuckermann 2010; Laakko and Fraker 2002; Viegas et al., 2008).

A marked inhibition of ozone-induced increase in BALF NAG activity by MIFE might indicate a role for GR in modulating the macrophage response. It is possible that the initiation of a stress response by epinephrine might modulate the lung innate immune response and subsequent systemic corticosterone immune effects (Johnson et al., 2005; Vida et al., 2004;

Zhou et al., 2014). This assumption is supported by the observation that PROP markedly diminished ozone-induced inflammatory cytokine increases and neutrophilic inflammation in the lung, supporting a major contribution of βAR in local pulmonary modulation of the immune responses. Although the activation of the sympathetic arm of the stress axis (epinephrine and norepinephrine) has been shown to inhibit innate immune responses in humans (Kox et al., 2014), it is known that $\beta_2$AR, which are abundant in the lung, can modulate nuclear factor kappa-beta-mediated inflammatory processes. Previous studies have shown that PROP blocked the stress-induced elevation of circulating IL-6 levels (van Gool et al., 1990) and reduced air pollution-induced increases in IL-6 in mice (Chiarella et al., 2014).

*Mt2a*, known to be upregulated by ozone (Inoue et al., 2008), was also increased in vehicle-pretreated rats exposed to ozone, however, this effect was markedly reduced by both PROP and MIFE, suggesting that the neuroendocrine response is linked to ozone-induced acute phase protein expression. In humans, *Mt2a* gene transcription is activated by GR activation (Sato et al., 2013) and in a murine restrained stress model, *Mt2a* expression correlated with increased corticosterone levels (Jacob et al., 1999). This observation is in agreement with previous work from our lab showing that the removal of adrenal glands – which is the source of both epinephrine and corticosterone – reduced the ozone-induced up-regulation of *Mt2a* and many other acute phase response genes (Henriquez et al., 2017a). The increased expression of the glucocorticoid-responsive gene *Tsc22d3* after ozone exposure supports the corticosterone-mediated activation of GR in the lungs.

In conclusion, by blocking βAR and GR activity using PROP and MIFE pretreatments, respectively, we selectively minimized ozone-induced lung protein leakage, macrophage activation, neutrophilic inflammation, lymphopenia, and pulmonary inflammatory cytokine expression in rats (Fig. 3.8). These findings increase the plausibility of a significant mechanistic role for circulating stress hormones and downstream effects of βAR and GR activation in most pulmonary effects of ozone. Moreover, since βAR and GR agonists are commonly used in the

treatment of chronic inflammatory conditions of the lung, including asthma and chronic obstructive pulmonary disease, those receiving such treatments may show differential susceptibility to air pollution-induced lung effects. βAR and GR antagonists, may reduce lung effects induced by ozone and other air pollutants.

# CHAPTER 4: BETA-2 ADRENERGIC AND GLUCOCORTICOID RECEPTOR AGONISTS MODULATE OZONE-INDUCED PULMONARY PROTEIN LEAKAGE AND INFLAMMATION IN HEALTHY AND ADRENALECTOMIZED RATS

## 4.1 Introduction

Neuroendocrine stress responses involving activation of the sympathetic-adrenal-medullary (SAM) and hypothalamus-pituitary-adrenal (HPA) axes have been linked to development of asthma (Douwes et al., 2011), chronic cardiovascular diseases (An et al., 2016), obesity (Hewagalamulage et al., 2016, Hirotsu et al., 2015), and diabetes (Joseph and Golden, 2017). Furthermore, maternal stress during pregnancy has been postulated to increase risk of developing childhood asthma and later life chronic obstructive pulmonary disease (COPD) through reprograming of the HPA stress axis (Rosa et al., 2017). Furthermore, exposure to elevated levels of air pollution has been associated with increased asthma and COPD symptomatology, especially in individuals living in disadvantaged communities with additional exposure to psychosocial stressors (Clougherty and Kubzansky, 2008; Clark et al., 2015; Shmool et al., 2014; Wright, 2011). Relatedly, activation of these SAM- and HPA-mediated stress responses leads to increase the systemic release of epinephrine and corticosterone/cortisol, respectively, from adrenal glands, which in turn, produce tissue-specific homeostatic changes in metabolic and immune processes associated with the fight-or-flight response (Gorman, 2013).

We have previously shown that exposure to the reactive air pollutants, such as ozone or acrolein not only caused respiratory injury and inflammation, but exposure led to also activation of these neuroendocrine stress pathways in both healthy and diabetic rat models (Bass et al., 2013; Miller et al., 2015; Snow et al., 2017) and in humans (Miller et al., 2016a). We have also

shown that ozone exposure increased circulating epinephrine and corticosterone and produced metabolic alterations similar to a fight-or-flight response (Bass et al., 2013; Miller et al., 2015, 2016a, Snow et al., 2017). While ozone inhalation has been shown to activate sensory vagal C-fibers in the lung (Taylor-Clark et al., 2011), stress responsive regions in the central nervous system (Gackière et al., 2011), autonomic reflex mechanisms (Gordon et al., 2014), and also the release of adrenocorticotrophic hormone (Thomson et al., 2013), the precise mechanisms by which ozone inhalation activates the SAM and HPA axes are not known.

Once released to the circulation, epinephrine and corticosterone exert their tissue effects through adrenergic and glucocorticoid receptors. Adrenergic receptors (AR) are G-protein coupled receptors involved in a variety of cellular processes induced during a fight-or-flight stress response (Tank and Lee Wong, 2015; Ghanemi and Hu, 2015). There are two distinct classes of AR α and β and these subtypes are widely distributed in all organs and associated vessels, and αAR and βAR are activated by circulating epinephrine and nor-epinephrine with different affinities such that a wide array of changes are produced in tissues upon their activation (Daly and McGrath, 2011). Circulating corticosterone exerts its cellular effects by binding to glucocorticoid receptors (GR). GR are also distributed in virtually all tissues and cells in the body, and are involved in maintaining homeostasis of metabolic and immune processes (Oakley and Cidlowski, 2011). Activation of GR by glucocorticoids leads to immunosuppression through transcriptional repression of proinflammatory genes (De Bosscher et al., 2000). Hence, AR and GR are often the target of a variety of pharmacological agonists and antagonists used to treat functional abnormalities associated with cardiovascular and pulmonary diseases and associated inflammatory conditions.

We have recently demonstrated that adrenalectomy, which diminishes circulating epinephrine and corticosterone in rats, diminished ozone-induced metabolic changes and also lung injury, protein leakage and inflammation (Miller et al., 2016b; Henriquez et al., 2017a), suggesting a proinflammatory role of endogenously released epinephrine and corticosterone.

Although glucocorticoids have been shown to suppress immune response and inhibit lymphocyte function in rats, the proinflammatory effect of newly released epinephrine and corticosterone has also been reported after an acute restraint stress (Dhabhar et al., 2012) and after a treatment with exogenous epinephrine and glucocorticoids (Dhabhar et al., 2014). However, it is not well understood whether these stress hormones are involved in altered innate immune responses after ozone inhalation exposure and, if so, which pathways or potential mechanisms are mediating their effects on the inflammation induced by ozone exposure.

β Adrenergic receptors (βAR) are widely distributed in the lung. Of those ~70% are of $\beta_2$-type (β2AR) while 30 % are β1AR which are primarily activated by nor-epinephrine (Barns, 2004). β 2AR are the predominantly distributed in the airway and vascular smooth muscle, and also the epithelial cells. These receptors are activated by epinephrine to cause vasoconstriction and bronchial relaxation. Increased activity of these receptors has been linked to bronchodilation and reduction in vascular permeability (Barnes, 2004). A variety of $\beta_2$AR agonists have been developed for use as therapeutic agents to achieve bronchodilation in asthma and COPD (Rassler, 2013). Likewise, GR are also widely distributed in the lung tissues, and steroid agonists are the primary therapeutic agents used for inhibition of the chronic lung inflammation present these pulmonary diseases (Barnes, 2017). In many instances, a combination of $\beta_2$AR and GR agonists are used to reverse and suppress airway bronchoconstriction and inflammation present in asthma and COPD (Cain and Cidlowski, 2015; Waldeck 2002). Therefore, herein we hypothesized that combined treatment of rats with agonists of $\beta_2$AR, clenbuterol (CLEN) plus GR, dexamethasone (DEX), would exacerbate resultant ozone-induced vascular leakage and inflammation in controls (previously undergone a sham surgical procedure; SH), and conversely, in adrenalectomized (AD) rats, these drugs would restore ozone effects back to that observed in ozone-exposed SH rats with intact SAM and HPA axes.

## 4.2 Materials and Methods

### 4.2.1 Animals

Male, 11-12 week old Wistar Kyoto (WKY) rats, purchased from Charles River Laboratory (Raleigh, NC), were pair-housed in polycarbonate cages with beta chip bedding under controlled conditions (50-65% Relative Humidity [RH], 21°C and 12 hr light/dark cycle) in our animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Water and food (5001 Purina rat chow; Brentwood, MO) were provided *ad libitum* except where indicated. All animal procedures were approved by the US Environmental Protection Agency (US EPA), National Health and Environmental Effects Research Laboratory (NHEERL) Animal Care and Use Committee prior to the start of the experiment.

### 4.2.2 Animal surgeries and drug treatments

At 12-13 weeks of age, rats underwent either total bilateral adrenalectomy (AD) or control sham (SH) surgeries as previously described (Miller et al., 2016b). Briefly, the rats were anesthetized by i.p. injection of ketamine (25-50 mg/kg/ml in saline). Once under anesthesia, buprenophrine was injected (0.02 mg/kg/ml in saline; s.c.) for analgesia. During the surgery, additional anesthesia was induced by inhalation of vaporized isoflurane (≈3%) in a nose-only cone as needed. Veterinarians from Charles River Laboratories Inc. performed surgeries under aseptic conditions. Animals were placed in sternal recumbency. Surgeries were performed using protocols established at Charles River Inc. Except for the removal of adrenal glands, SH surgeries involved similar anesthesia and surgical approaches as AD. The recovery was assured by the observation of animal's movement while on heating pads. Once awake, animals were treated with Meloxicam (0.2 mg/mL/kg; s.c.) for analgesia. Additional analgesia was provided by administering buprenorphine (0.02 mg/mL/kg, s.c.) every 8-12 hr for 2 times. Half of the rats (D+2 groups as indicated below) were injected with temperature sensitive transponders s.c. over the dorsal abdominal surface to allow measurement of body temperature

(BDMS, Seaford, DE). SH rats received normal tap water for drinking while AD rats were provided saline (0.9% sodium chloride) after the surgery to maintain adequate fluid and electrolyte balance. Following surgery, the animals were pair housed with Enviro Dry enrichment/nesting material, provided with powdered as well as pelleted food, and allowed to recover for 4-6 days prior to any drug treatment related to this study. This approach was based on a number of adrenalectomy studies that used/recommended a 4-6 day recovery period to avoid secondary changes in the body occurring as a result of AD (Nicolaides et al., 2013; Sakakibara et al., 2014; Miller et al. 2016b).

SH and AD rats were randomized by body weight into four treatment groups (vehicle:air, vehicle:ozone, CLEN+DEX:air and CLEN+DEX:ozone) over two time periods (1-day [D+1] or 2-day [D+2] exposures). This resulted in 8 total groups with 8 rats/group. Vehicle or drug treatments began one day prior to first air or 0.8 ppm ozone exposure (4 hr/day for 1 or 2 consecutive days). Vehicle and drug treatments were administered each day just prior to inhalation exposures. Vehicle injections were comprised of saline (1 mL/kg, i.p.) followed by pharmaceutical grade corn oil (1 mL/kg, s.c.). Drug treatments included clenbuterol hydrochloride, a long acting $\beta_2$AR agonist (CLEN; 0.2 mg/mL saline/kg, i.p.) and a GR agonist, dexamethasone (DEX; 2 mg/mL corn oil/kg, s.c.). Since we were attempting to restore both the basal levels that were completely depleted by AD procedure and provide the increased levels observed during ozone-exposure in non-AD rats, these relatively high doses were selected. Notably, however, the doses are several fold higher than that used therapeutically. Nevertheless, these CLEN and DEX concentrations are within the range used in other laboratory rodent studies for the purpose of inducing bronchodilation and immunosuppression, respectively. The general experimental design is showed in Fig. 4.1.

**Figure 4.1**

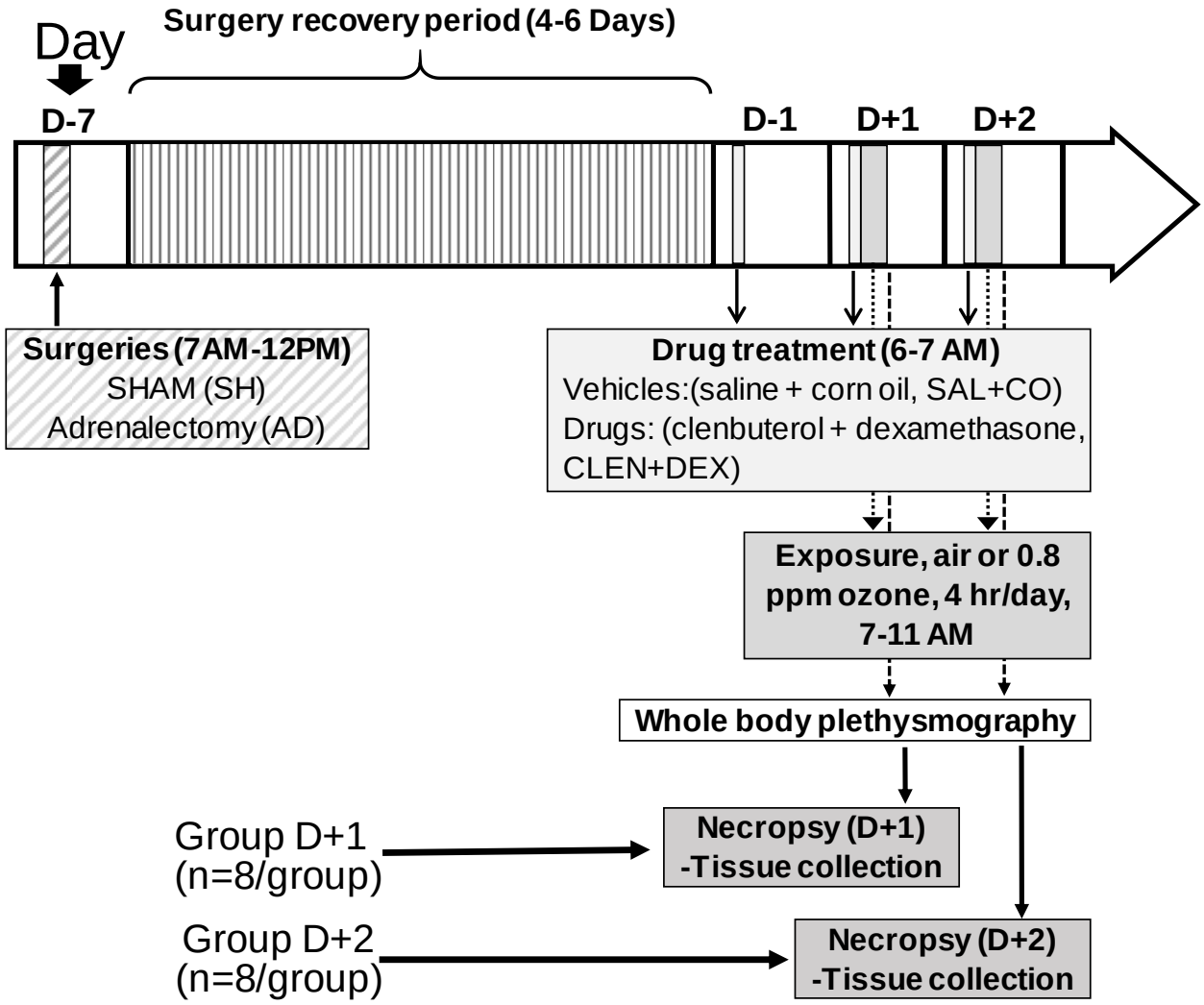



**Figure 4.1 Experimental design and time-line.** The timing for surgery, drug treatments, exposures, whole body plethysmography (WBP), and necropsies are indicated by arrows. Animals assigned to 1-day or 2-day air or ozone exposure (4 hr/day) are referred to as groups D+1 and D+2, respectively. Animals belonging to D+2 groups were subjected to WBP immediately after the first and second day of exposure. Necropsy and tissue collection were performed immediately after exposure on D+1 groups (within 2 hours) or immediately after exposure and WBP for D+2 groups (within 2.5 hours). SH, sham surgery; AD, adrenalectomy; SAL, saline; CO, corn oil; CLEN, clenbuterol; DEX, dexamethasone.

4.2.3 Ozone exposure

Ozone was generated using a silent arc discharge generator (OREC, Phoenix, AZ) from oxygen and transported to Rochester style “Hinners” chambers using mass flow controllers (Coastal Instruments Inc., Burgaw, NC). Ozone concentration was controlled and recorded by photometric analyzers (API Model 400, Teledyne, San Diego, CA). Mean chamber temperature, humidity and air flow were recorded hourly (control chamber: 71.05±0.32 $^{o}$F, 54.08±0.67 and 256.1±0.57 liters/min; ozone chamber: 73.10±0.14 $^{o}$F, 50.40±0.44 and 261.2±0.43 liters/min, respectively). Rats were exposed to air or 0.8 ppm ozone, 4 hr/day for either 1 day (D+1) or 2 consecutive days (D+2). The actual daily mean chamber concentration of ozone was 0.800±0.04 ppm (mean ± SD).

4.2.4 In life assessment

Body weights were measured prior to and daily after the surgery for all rats, including each day post exposure. Subcutaneous temperature was assessed immediately before and after each ozone exposure in the D+2 groups (n=4/group). Whole body plethysmography (WBP) was performed immediately following each day of exposure in the D+2 groups to detect altered in tidal breathing patterns (n=8/group). Rats were acclimated to the WPB chambers for two days prior to the first ozone exposure. Immediately post-exposure to air or ozone, on a rotational basis, rats were placed in rat-sized WBP chambers and, after a 2 min acclimation period, ventilatory parameters were recorded for an additional 5 minutes. Measurements were averaged every 10 seconds for the 5 min period using EMKA iox 2 software (SCIREQ, Montreal, Canada). All traces were visually inspected to ensure that valid breath patterns were not excluded. Ventilatory parameters obtained included: breathing frequency, tidal volume, minute volume, peak inspiratory flow (PIF), peak expiratory flow (PEF), inspiratory time (Ti), expiratory time (Te), relaxation time (RT) and enhanced pause (PenH).

4.2.5 Necropsy, blood collection, complete blood counts and assessment of circulating stress hormones

An overdose of fatal plus (sodium pentobarbital, Virbac AH, Inc., Fort Worth, TX; >200 mg/kg, i.p.) was used to euthanize rats. For D+1 groups, rats were necropsied immediately (within 1-2 hr) after their first exposure while for D+2 groups, rats were necropsied after their second exposure and WBP data acquisition (within 1.5-2.5 hr). Blood samples from the abdominal aorta were collected in vaccutainers tubes. Complete blood counts were performed using EDTA blood samples on a Beckman-Coulter AcT blood analyzer (Beckman-Coulter Inc., Fullerton, CA), which provides a relative measure of total white blood cells (WBC) and lymphocytes. Blood smears were also obtained and then stained with Diff-quick to enumerate relative neutrophil and monocyte numbers. The remaining EDTA blood samples were centrifuged at 3500 × g for 10 min and resulting plasma samples were stored at -80°C until analysis. Serum was collected after centrifugation of blood samples collected in serum separator tubes at 3500 × g for 10 min and stored at −80°C until use. Epinephrine levels in plasma samples were quantified using a kit obtained from Rocky Mountain Diagnostics following the manufacturer's protocol (Colorado Springs, CO). Corticosterone levels were quantified in serum employing an immunoassay kit and following manufacturer's directions (Arbor Assays, Ann Arbor, MI).

4.2.6 Bronchoalveolar lavage and cell counts

Bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea. The left lung was tied and the right lung was lavaged 3 times using the same aliquot of $Ca^{2+}$ and $Mg^{2+}$ free PBS, pH 7.4, 37°C, at 28 mL/kg body weight for total lung capacity with the right lung weight being 60% of the total lung weight. Whole BALF was diluted to 10 mL using isotone and spiked with 0.2 mL saponin to lyse cells. Nucleated cells were counted using a Z1 Coulter Counter (Coulter Inc., Miami, FL). Whole BALF was also used to prepare cytospin slides, which were stained with Diff-quick and cell differentials were determined under light microscopy (300

cells/slide, one slide/ animal). The remaining BALF samples were centrifuged (1500 x g for 5 min) and cell free BALF aliquots were analyzed for lung injury markers. The lavaged caudal lobe from the right lung was removed, blotted, frozen in liquid nitrogen and stored at -80$^{o}$C for RNA extraction. We have previously noted that ~10% of male WKY rats exhibit spontaneous cardiac hypertrophy and associated pulmonary complications (Shannahan et al., 2010). The data obtained from those with cardiac hypertrophy (i.e. heart weights 20% above average at necropsy) were removed from further analysis to minimize the occurrence of underlying cardiac confounders that would impact the endpoints of interest.

4.2.7 Assessment of BALF protein leakage markers, inflammatory cytokines

BALF total protein was assessed using Coomassie Plus Protein Reagent from Thermo Fisher Diagnostics (Rockford, IL) and albumin standards from Sigma-Aldrich (St. Louis, MO). BALF albumin levels were determined using a kit from Sekisui Diagnostics (Lexington, MA) while N-acetylglucosaminidase (NAG) activity was assessed using reagents and controls from Sigma-Aldrich Diagnostics (St. Louis, MO). These assays were modified for use on the Konelab Arena 30 clinical analyzer (Thermo Chemical Lab Systems, Espoo, Finland). Cell free BALF samples were used to quantify cytokine proteins using the V-PLEX proinflammatory panel 2 (rat) kit following the manufacturer's protocol (Meso Scale Discovery, Gaithersburg, MD). The electrochemiluminescence signals for each protein were detected using Meso Scale Discovery® platform (Mesoscale Discovery Inc., Rockville, MD). For some cytokines, the BALF levels in control animals were below the limit of detection. Those values were imputed with the lowest quantified value for a given cytokine.

4.2.8 Flow cytometry of WBC

For flow cytometry analyses of blood samples, the general procedures were performed following previously published methods (DeWitt et al., 2015). Briefly, fresh aortic blood samples collected in EDTA blood tubes from the D+1 animals were treated with RBC lysis buffer (Affymetrix eBioscience, Santa Clara, CA) and then washed twice with HBSS without $Ca^{2+}$ and

$Mg^{2+}$ (Thermo Fisher, Waltham, MA). The cells were suspended in staining buffer containing HBSS with 1% bovine serum albumin (BSA) and 0.1% sodium azide (Sigma-Aldrich, St. Louis, MO). WBC concentrations were determined using a Z1 Coulter Counter (Coulter, Inc., Miami, FL) and adjusted to 1 x $10^6$ cells/ml. Cells were washed once with PBS and incubated for 30 min at room temperature with LIVE/DEAD$^{TM}$ fixable violet dead cell stain (Thermo Fisher, Waltham, MA) to determine viable cells. After incubation, cells were washed three times with staining buffer and then incubated with mouse anti-rat CD32 (BD Pharmingen, San Jose, CA) to block FC receptor-mediated nonspecific antibody binding as per manufacturer's instructions. Cells were then washed three times with staining buffer and labeled for 30 min with the following monoclonal antibodies: APC-Cy7 mouse anti-rat CD45, PE mouse anti-rat RP1, PE-Cy7 mouse anti-rat CD4, FITC mouse anti-rat CD8a (BD Pharmingen, San Jose, CA). Cells labeled with fluorochrome-conjugated isotype control antibodies (APC-Cy7 mouse anti-rat IgG,κ; PE mouse anti-rat IgG2a,κ; PE-Cy7 mouse anti-rat IgG2a,κ; FITC mouse anti-rat IgG1 (BD Pharmingen, San Jose, CA)) were used as negative controls. Additional cell samples, including unstained cells and fluorescence minus one (FMO) controls were utilized to aid in identifying and ensuring accurate gating of negative and positive cell populations. After staining, cells were washed three times with staining buffer, fixed with 0.05% formaldehyde in PBS and kept in the dark at 4$^{o}$C (no longer than one day) until FACS analysis. Data were collected on a LSR II flow cytometer (BD Biosciences, Mississauga, Canada) using FACS Diva software (BD Biosciences, Mississauga, Canada). The quantification of the data was performed using FlowJo software (TreeStar, Inc., Ashland, OR). Live cells were gated based on CD45 expression for the analysis of subpopulations $CD4^-/CD8a^-$, $CD4^-/CD8a^+$, $CD4^+/CD8a^-$, $CD4^+/CD8a^+$, and $RP1^+$. Due to the low number of leukocytes available, a minimum of 1000 events per sample were counted.

4.2.9 Lung RNA isolation and real time-quantitative PCR

An uniform portion of frozen caudal lung lobe was separated and weighed (20-30 mg) for RNA extraction using the RNeasy mini kit (Qiagen, Valencia, CA). RNA was quantified using

Qubit 2.0 fluorimeter (Thermo Fisher, Waltham, MA). Qscript cDNA Supermix (Quanta Biosciences, Beverly, MA) was used to synthesize cDNA by reverse transcription. Primers were designed using Rattus Norvegicus sequences annotated using NCBI and obtained from Integrated DNA Technologies, Inc. (Coralville, IA; Table 4.1). SYBR® Green PCR Master Mix (Thermo Fisher, Waltham, MA) was used to perform quantitative PCR using the Applied Biosystems 7900HT Sequence Detection System (Foster City, CA). mRNA expression was expressed in relative units employing the ΔΔCt method using β-actin as the housekeeping gene and the vehicle-air treated SH group as control. The determination of relative lung mRNA expression was restricted to D+1 groups since ozone-induced changes in RNA expression peak rapidly one day after ozone exposure (Kodavanti et al., 2015; Hollingsworth et al., 2007). *Il6*, *Tnf*, *Cxcl2* and *Il4* were selected as pro-inflammatory endpoints, which have shown consistent up-regulation after ozone exposure in previous publications (Henriquez et al., 2017a). *Mt2a* was selected as a marker of oxidative stress, which consistently increases after ozone exposure in the lungs (Inoue et al., 2008). Furthermore, expression of *Tsc22d3*, also known as *Gilz* a validated glucocorticoid responsive gene (Pepin et al., 2015) and *Adrb2,* the gene coding for $\beta_2$AR, were measured to evaluate the role of our exposures and treatments on the lungs.

**Table 4.1**

| Gene | Forward | Reverse |
|---|---|---|
| **β-Actin (*Actb*)** | 5'-CAACTGGGACGATATGGAGAAG-3' | 5'-GTTGGCCTTAGGGTTCAGAG-3' |
| **Tumor necrosis factor (*Tnf*)** | 5'-ACCTTATCTACTCCCAGGTTCT-3' | 5'-GGCTGACTTTCTCCTGGTATG-3' |
| **Interleukin 6 (*Il6*)** | 5'-CTTCACAAGTCGGAGGCTTAAT-3' | 5'-GCATCATCGCTGTTCATACAATC-3' |
| **Interleukin 4 (*Il4*)** | 5'-GTCACCCTGTTCTGCTTTCT-3' | 5'-GACCTGGTTCAAAGTGTTGATG-3' |
| **Chemokine (C-X-C motif)-ligand 2 (*Cxcl2* or *Mip2*)** | 5'-GCCTCGCTGTCTGAGTTTAT-3' | 5'-GAGCTGGCCAATGCATATCT-3' |
| **Metallothionein-2 (*Mt2a*)** | 5'-CAGCGATCTCTCGTTGATCTC-3' | 5'-GGAGGTGCATTTGCATTGTT-3' |
| **TSC22 domain family protein 3 (*Tsc22d3* or *Gilz*)** | 5'-CCGAATCATGAACACCGAAATG-3' | 5'-GCAGAGAAGAGAAGAAGGAGATG-3' |
| **Beta-2 adrenergic receptor (*Adrb2*)** | 5'-CTCCTTAACTGGTTGGGCTATG-3' | 5'-CCTGGAAGGCAATCCTGAAA-3' |

**Table 4.1 Forward and reverse primer sequences designed for each gene used in PCR.**

4.2.10 Statistics

For each endpoint, data were log transformed when normal distribution and homoscedasticity were not satisfied using Shapiro-Wilk and Levene's tests, respectively. Data were analyzed using a two-way analysis of variance (ANOVA). The effects of adrenalectomy were determined by analyzing vehicles and CLEN+DEX treated groups separately using independent two-way ANOVAs while drug treatment effects were determined by analyzing SH and AD groups separately as independent factors using two-way ANOVA. This strategy was used separately for D+1 and D+2 experiments. Tukey's test was used to correct for multiple comparisons. Significant differences were considered when $p \leq 0.05$. For all bar graphs, data (n=6-8 animals/group) are expressed as mean ± SEM. GraphPad prism 7.03 and Statext 2.7 software were used for statistical analysis. For gene expression analysis, outliers were identified and discarded using the ROUT method (robust regression and outlier removal, Motulsky and Brown 2006) prior to statistical analysis.

## 4.3 Results

### 4.3.1 Ozone exposure, adrenalectomy and CLEN+DEX treatments change body weight and subcutaneous temperature.

To examine the general physiological effects of ozone exposure, AD and CLEN+DEX treatment, body weights and subcutaneous temperatures were monitored after each day of exposure in the D+2 groups. Although the changes in body weights were relatively small, all AD rats had reduced body weight gain after surgery relative to SH rats (up to 12% on D+2). Slight reductions were also noted in ozone-exposed rats after each day of exposure (up to 10% on D+2) and/or in CLEN+DEX-treated SH rats, with the greatest reduction occurring in ozone-exposed CLEN+DEX-treated AD rats (up to 22% on D+2) (Fig. 4.2A).

Acute ozone exposure has been shown to cause hypothermia in animals as determined by reduction in core body temperature (Gordon et al., 2015). We noted that subcutaneous temperature was reduced by approximately 3°C in all ozone-exposed SH and AD rats when determined immediately following exposure for both days. Neither AD nor CLEN+DEX treatment changed subcutaneous temperature in air-exposed rats or modified the ozone-related reductions in body temperature (Fig. 4.2B).

**Figure 4.2**

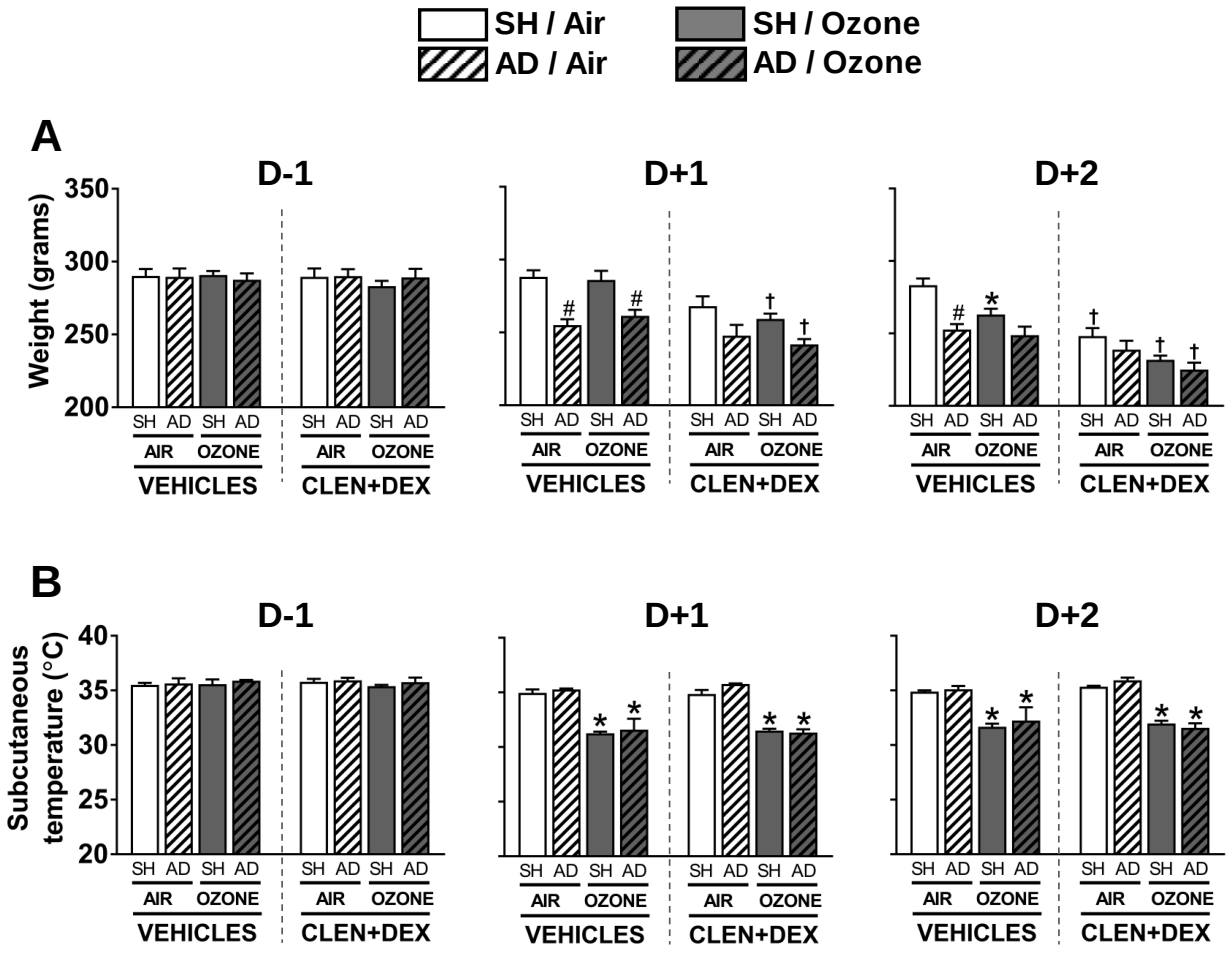



**Figure 4.2 Body weight and temperature changes induced by ozone exposure in vehicle- and CLEN+DEX-treated SH and AD rats.** Body weights (A) of rats were recorded before surgery (D-1) and immediately after each day of exposure to air or ozone in the D+2 animals (n=8/group). Subcutaneous temperatures (B) were measured using a receiver which acquired signals through subcutaneously injected transponders immediately before and after each day exposure to air or ozone (0.8ppm) for the D+2 groups (n=4/group). Bar graphs show mean ± SEM. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to matching air-exposed rats, # for AD effect when compared to matching SH rats, and † for CLEN+DEX effect when compared to matching vehicle-treated rats.

4.3.2 Circulating stress hormones are changed after ozone exposure in SH and AD rats treated with vehicle and CLEN+DEX

Based on the expected variability in levels of stress hormones due to several variable influencing their levels (precise time of blood collection, stress levels in animals during euthanasia injection), we have seen variable degree of response to ozone in our past studies in male WKY rats (Miller et al., 2015, Miller et al., 2016b; Miller et al., 2016c). In this study, although not significant, ozone exposure tended to increase circulating epinephrine levels at D+1 and D+2 ($p=0.13$, each day using single group comparison) in SH rats (Fig. 4.3A and B). The trend for ozone-induced increase circulating corticosterone on D+1 in vehicle-treated SH rats was not significant either ($p=0.24$ using single group comparison) (Fig. 4.3C). However, as expected, AD essentially eliminated detectable plasma epinephrine and corticosterone levels D+1 and D+2 regardless of exposure and drug treatment (Fig. 4.3). CLEN+DEX treatment by itself markedly reduced circulating corticosterone in SH rats (Fig. 4.3C and D) whereas CLEN+DEX treatment had no influence on epinephrine levels regardless of the day of exposure (Fig. 4.3A and B).

**Figure 4.3**

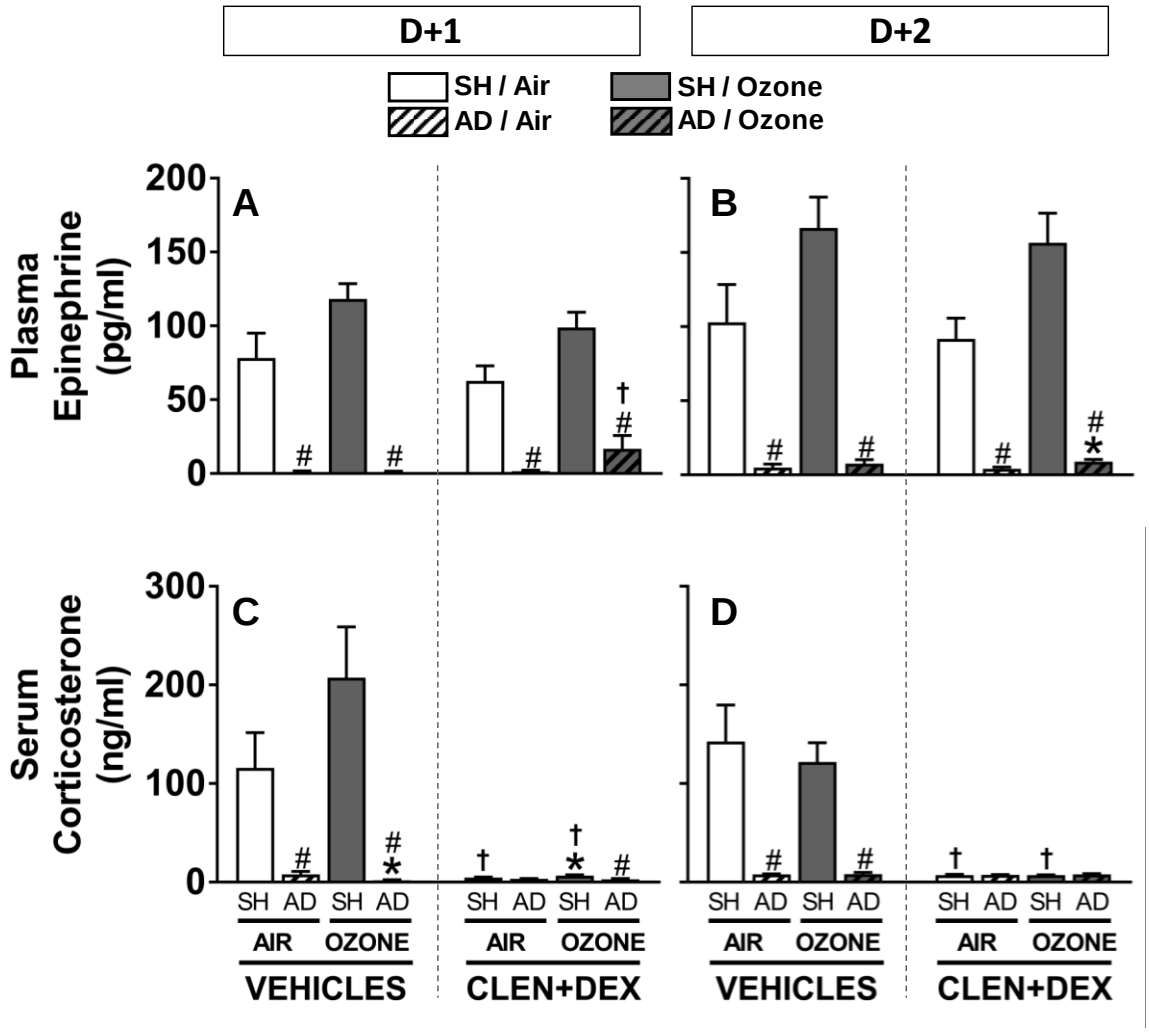



**Figure 4.3 Ozone-induced changes in circulating stress hormones in vehicle- and CLEN+DEX-treated SH and AD rats.** Circulating stress hormones, epinephrine (A-B) and corticosterone (C-D), were measured in samples collected immediately after air or ozone (0.8ppm) exposure (4 hr/day) in D+1 and D+2 groups. Bar graphs show mean ± SEM of n=6-8 /group. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to corresponding air-exposed rats, # for AD effect when compared to corresponding SH rats, and † for CLEN+DEX effect when compared to corresponding vehicle-treated rats.

4.3.3 Ozone-induced changes in ventilatory parameters in SH and AD rats with and without CLEN+DEX treatment

Examining first the ventilatory changes related to AD alone, we observed minor reductions (~20%) in breathing frequency (Fig. 4.4A) and corresponding increases in the success rate (% Sr) of acceptable breaths obtained during the 5-min. data acquisition period following D+1 air exposures (% Sr increased ~50%; data not shown). These changes are consistent with the generally sedate behavior of the AD rats in that they were less likely to engage in activities such as sniffing and grooming which typically reduce the relative number of acceptable breaths acquired. By comparison, the AD rats receiving CLEN+DEX failed to exhibit slowing of breathing frequency. These trends were likewise apparent following the D+2 air exposures. No other ventilatory changes were observed for the AD-only or AD rats treated with CLEN+DEX.

Examining next the effects of ozone-exposure in SH (vehicle-treated) rats, we detected characteristic changes in spontaneous breathing patterns including: (1) intermittent slowing of the respiratory rate and (2) periods during which breaths had a shorter inspiratory time relative to expiratory time (e.g., reduced Ti/Te ratios). If exposure to an irritant gas like ozone results in development of bronchoconstriction, such alterations would allow disproportionately more time for exhalation of potentially trapped air within the deep lung. Limitations in airflow becomes increasingly problematic during expiration owing to dynamic narrowing of airway caliber as the lung size decreases. Other ventilatory changes observed in these rats included: (3) corresponding increases in peak expiratory flow rates (relative to inspiratory flow rates) (see PEF/PIF ratios) (Fig. 4.4G); (4) increases in pause [(Te-Rt) – 1] (Fig. 4.4I), and (5) corresponding increases in the product of the PEF/PIF ratio and pause, namely the so-called enhanced pause, or PenH parameter (Fig. 4.4K). Of note, all of these ozone-related ventilatory changes were further worsened in SH rats treated with CLEN-DEX (Fig. 4.4). More specifically, frequency and the Ti/Te ratio decreased significantly, whereas the PEF/PIF ratio, Pause and

PenH were significantly increased. However, as shown in the Fig. 4.4E insert of representative "breath traces" (i.e., WPB chamber flow rate fluctuations), we observed that in the SH ozone-exposed rats receiving CLEN+DEX, the PEF/PIF ratios were increased in part due to higher PEF values, and in part due to truncated PIF values as these rats often exhibited a biphasic inspiration pattern, thus blunting their PIF values.

Lastly, we compared the superimposed effects of ozone-exposure and CLEN+DEX treatment in the previously AD rats. These rats appeared to require higher breathing frequencies, longer Ti (to Te) ratios, but had equivalently increased PEF/PIF ratios, again in part due to a biphasic inspiration (data not shown). By D+2 of ozone and CLEN-DEX treatment, these rats showed significantly reduced Pause and corresponding PenH values. Results suggest a mixed ventilatory pattern of worsening inspiratory effects in combination with reduced airflow obstruction during exhalation.

**Figure 4.4**

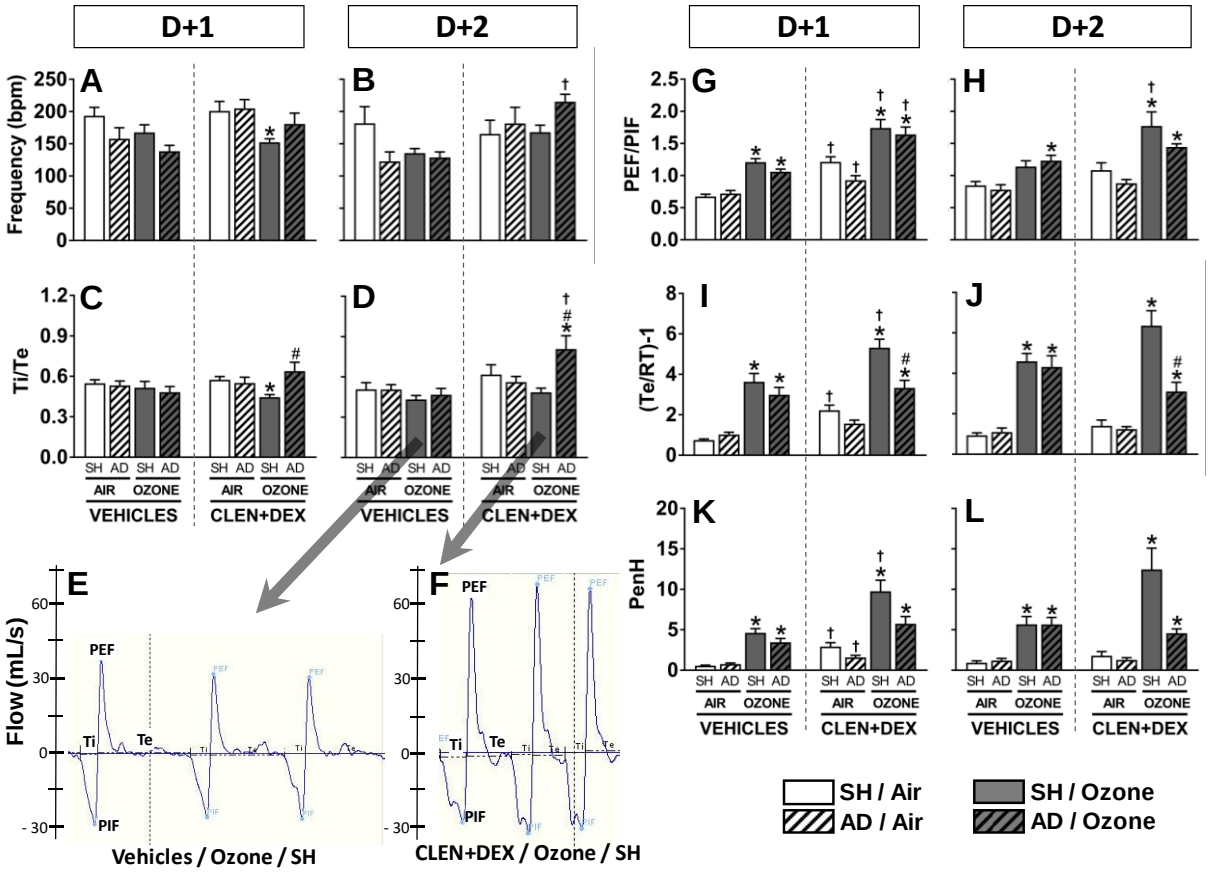



**Figure 4.4 Ventilatory parameters in rats are modulated by ozone, AD and CLEN+DEX.**

Breathing frequency in breaths per minute (bpm; A-B), Ti/Te ratio (C-D), pause [ (Te/RT)-1] (E-F), PEF/PIF (G-H), and PenH [(Te/RT)-1 x (PEF/PIF)] (I-J) were assessed immediately following air or ozone (0.8 ppm) exposure (4 hr/day) on both days for the D+2 groups. Bar graphs show mean ± SEM of n=6-8 /group. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to matching air-exposed rats, # for AD effect when compared to matching SH rats, and † for CLEN+DEX effect when compared to matching vehicle-treated rats.

4.3.4 Ozone-induced vascular leakage and macrophage activation are reduced by AD and exacerbated by CLEN+DEX treatment

BALF protein and albumin were assessed to determine microvascular leakage in the lung. A slight increase in BALF protein was noted in air-exposed vehicle-treated AD rats on both days. In vehicle-treated SH rats, ozone exposure was associated with significant increases in BALF protein, which was progressive over two days. However, ozone-induced protein increase at D+2 was not evident in vehicle-treated AD rats (Fig. 4.5A and B). Regardless of the exposure or surgery status, CLEN+DEX treatment alone was associated with extensive protein leakage. Ozone exposure of CLEN+DEX-treated SH rats induced greater increases in BALF protein, at both D+1 and D+2. AD moderately reduced the ozone-induced increase of BALF protein in CLEN+DEX-treated rats at D+2 (Fig. 4.5A and B). Changes in BALF albumin generally followed a similar pattern of changes as BALF protein, except the increase in albumin was greatest in ozone-exposed AD rats treated with CLEN+DEX at D+2 (Fig. 4.5C and D).

As we have noted in previous publications (Henriquez et al., 2017b), ozone exposure was also associated with BALF NAG activity increases in vehicle-treated SH rats at D+2. AD diminished this ozone effect at D+2 (Fig. 4.5F). Regardless of the exposure and surgery condition, CLEN+DEX treatment significantly increased BALF NAG activity at both time points. Ozone exposure further increased NAG activity in CLEN+DEX-treated rats at D+1 and D+2 while AD blunted this ozone-induced response (Fig. 4.5E and F).

**Figure 4.5**

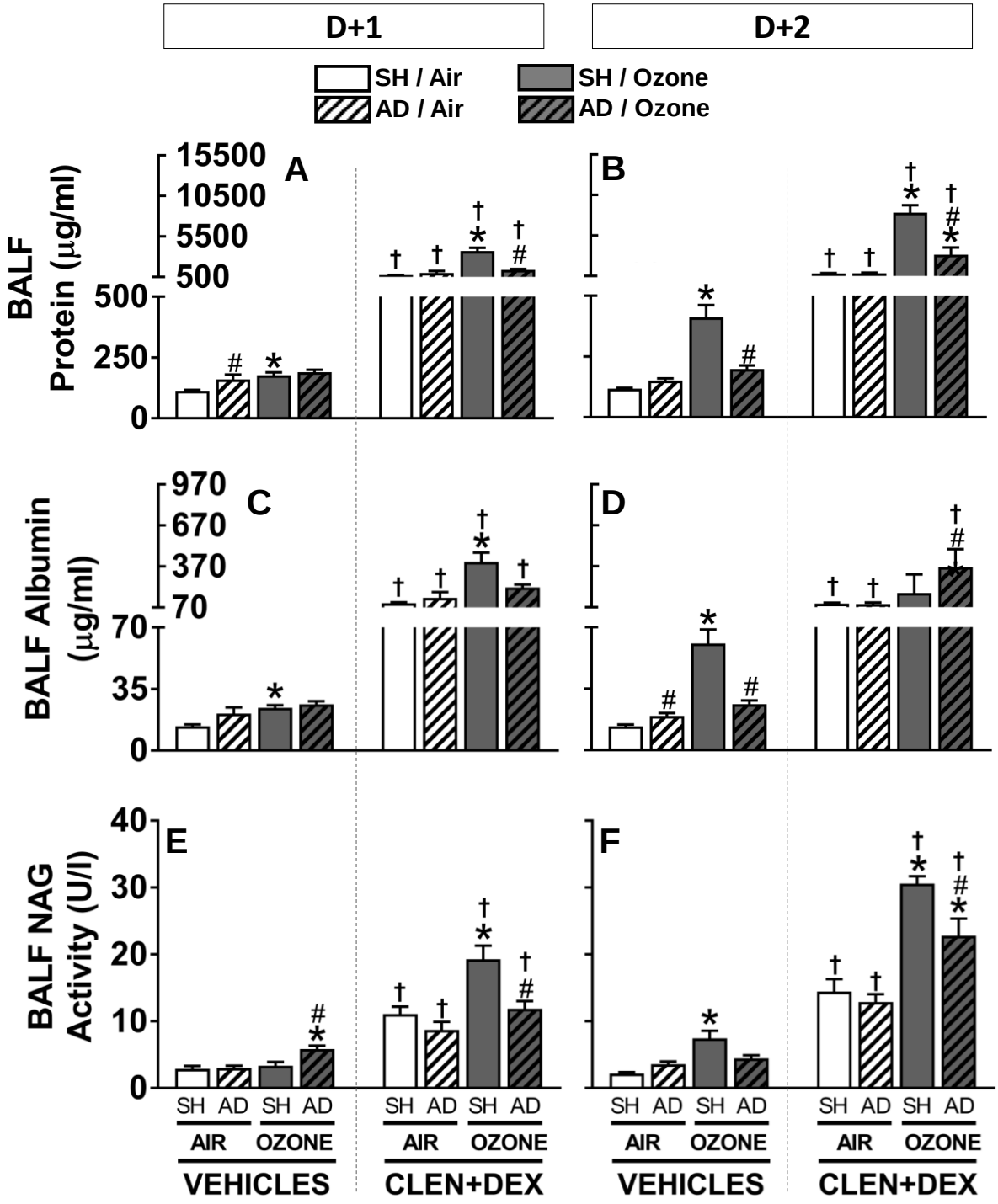

D+1
D+2
SH / Air
AD / Air
SH / Ozone
AD / Ozone
BALF Protein (μg/ml)
15500
10500
5500
500
500
250
0
A
B
BALF Albumin (μg/ml)
970
670
370
70
70
35
0
C
D
BALF NAG Activity (U/l)
40
30
20
10
0
E
F
SH AD SH AD
AIR OZONE
VEHICLES
CLEN+DEX

**Figure 4.5 Ozone-induced pulmonary vascular leakage and macrophage activation are modulated by AD and CLEN+DEX.** BALF protein (A-B), albumin (C-D), and NAG activity (E-F) were assessed in rats 1-2.5 hr following air or ozone (0.8 ppm) exposure (4 hr/day) for D+1 and D+2 groups. Bar graphs show mean ± SEM of n=6-8 /group. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to corresponding air-exposed rats, # for AD effect when compared to corresponding SH rats, and † for CLEN+DEX effect when compared to corresponding vehicle-treated rats.

4.3.5 Ozone-induced pulmonary inflammation is reduced by AD and restored by CLEN+DEX

Ozone exposure decreased BALF alveolar macrophages but AD reversed this effect in vehicle-treated SH rats at D+1 (Fig. 4.6A). CLEN+DEX pretreatment increased alveolar macrophages in ozone- but not air-exposed SH rats at both time points. This CLEN+DEX effect was significantly smaller in AD rats exposed to ozone relative to SH rats (Fig. 4.6A and B). Ozone exposure increased BALF neutrophils at D+2 in vehicle-treated SH rats. This ozone effect was diminished in vehicle-treated AD rats (Fig. 4.6D). Treatment with CLEN+DEX, in general, caused small increases in BALF neutrophils in air-exposed SH and AD rats at D+2, with the effect being exacerbated in ozone-exposed SH and AD rats at D+2 (AD>SH) (Fig. 4.6C and D). BALF lymphocytes were not impacted significantly by AD in vehicle-treated air- or ozone-exposed rats at any time point whereas CLEN+DEX pretreatment increased BALF lymphocytes in ozone-exposed SH and AD rats at D+2 (Fig. 4.6E and F). AD increased BALF eosinophils in air- exposed vehicle-treated rats at both time points. Ozone led to increased eosinophils in vehicle-treated SH rats at D+2. CLEN+DEX treatment, in general, increased eosinophils in all rats; this effect was greatest in ozone-exposed SH rats at both times (Fig. 4.6G and H).

**Figure 4.6**

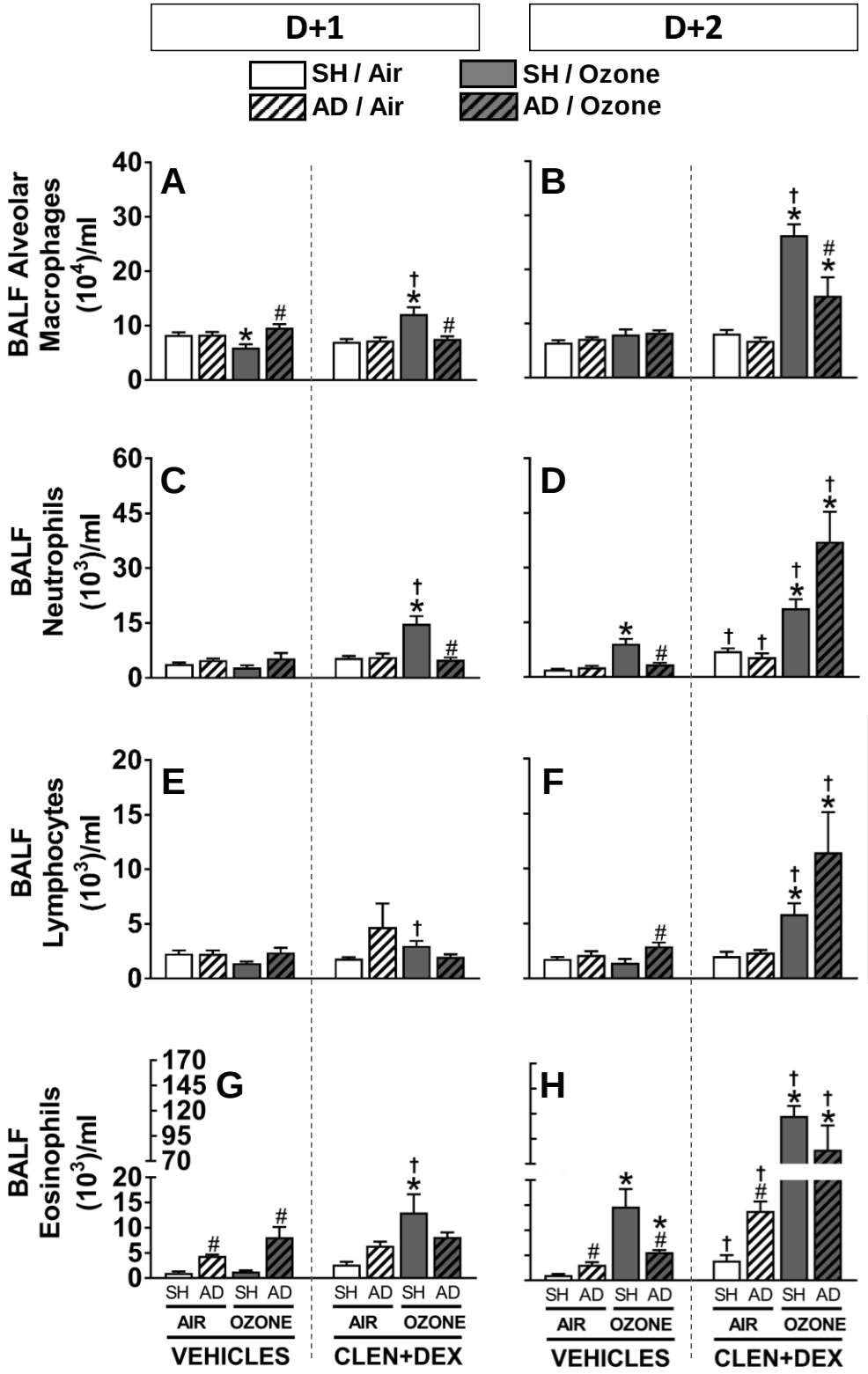

D+1
D+2
SH / Air
SH / Ozone
AD / Air
AD / Ozone
BALF Alveolar Macrophages ($10^4$)/ml
A
B
BALF Neutrophils ($10^3$)/ml
C
D
BALF Lymphocytes ($10^3$)/ml
E
F
BALF Eosinophils ($10^3$)/ml
G
H
SH AD SH AD
AIR OZONE
VEHICLES
CLEN+DEX

**Figure 4.6 Ozone-induced lung inflammation is modulated by AD and CLEN+DEX.** BALF macrophages (A-B), neutrophils (C-D), lymphocytes (E-F), and eosinophils (G-H) were calculated based on cell differentials and total cell counts in rats 1-2.5 hr following air or ozone (0.8 ppm) exposure (4 hr/day) for D+1 and D+2 groups. Bar graphs show mean ± SEM of n=6-8 /group. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to corresponding air-exposed rats, # for AD effect when compared to corresponding SH rats, and † for CLEN+DEX effect when compared to corresponding vehicle-treated rats.

4.3.6 Ozone-induced reduction of circulating WBC and lymphocytes is modulated by AD and CLEN+DEX treatment

In vehicle-treated SH rats, ozone exposure decreased circulating WBC at D+1; however, this ozone effect was not apparent in vehicle-treated AD rats. CLEN+DEX treatment tended to decrease WBC in all rats at D+1 with a maximum drop occurring in ozone-exposed AD rats (Fig. 4.7A). This CLEN+DEX effect was not apparent on D+2 (Fig. 4.7B). A small drop in circulating lymphocytes was noted in air-exposed vehicle-treated AD rats at D+1. Ozone exposure in vehicle-treated SH rats markedly decreased circulating lymphocytes at D+1 and D+2. This ozone effect was not observed in vehicle-treated AD rats. CLEN+DEX treatment was associated with remarkable reduction of circulating lymphocytes in all animals regardless of surgery or exposure condition at both time points (Fig. 4.7C and D). Circulating neutrophils were not affected by AD or ozone in vehicle-treated rats, however, CLEN+DEX caused significant increases in circulating neutrophils in all rats regardless of surgery or exposure (Fig. 4.7E and F).

In order to further examine the types of lymphocytes impacted by ozone and CLEN+DEX at the D+1 time point, subpopulations of lymphocytes were quantified as a percentage of total leukocytes ($CD45^+$) and then normalized using total $CD45^+$ leukocyte count. Generally, $CD4^-$ $CD8a^-$ and $CD4^+CD8a^-$ made up the substantial portion of WBC (60-95%, Fig. 4.8A and C). Ozone exposure, as noted above, decreased overall leukocyte counts ($CD4^-CD8a^-$), and tended to decrease all cells positive for lymphocyte markers such as $CD4^-CD8a^+$ (Fig. 4.8B), $CD4^+CD8a^-$ (Fig. 4.8C), and $CD4^+CD8a^+$ (Fig. 4.8D) in vehicle-treated SH rats; however, this trend was not observed in AD rats. CLEN+DEX treatment significantly decreased all T-lymphocyte subpopulations regardless of surgery or exposure status except for $CD4^-CD8^-$ leukocytes (Fig. 4.8).

**Figure 4.7**

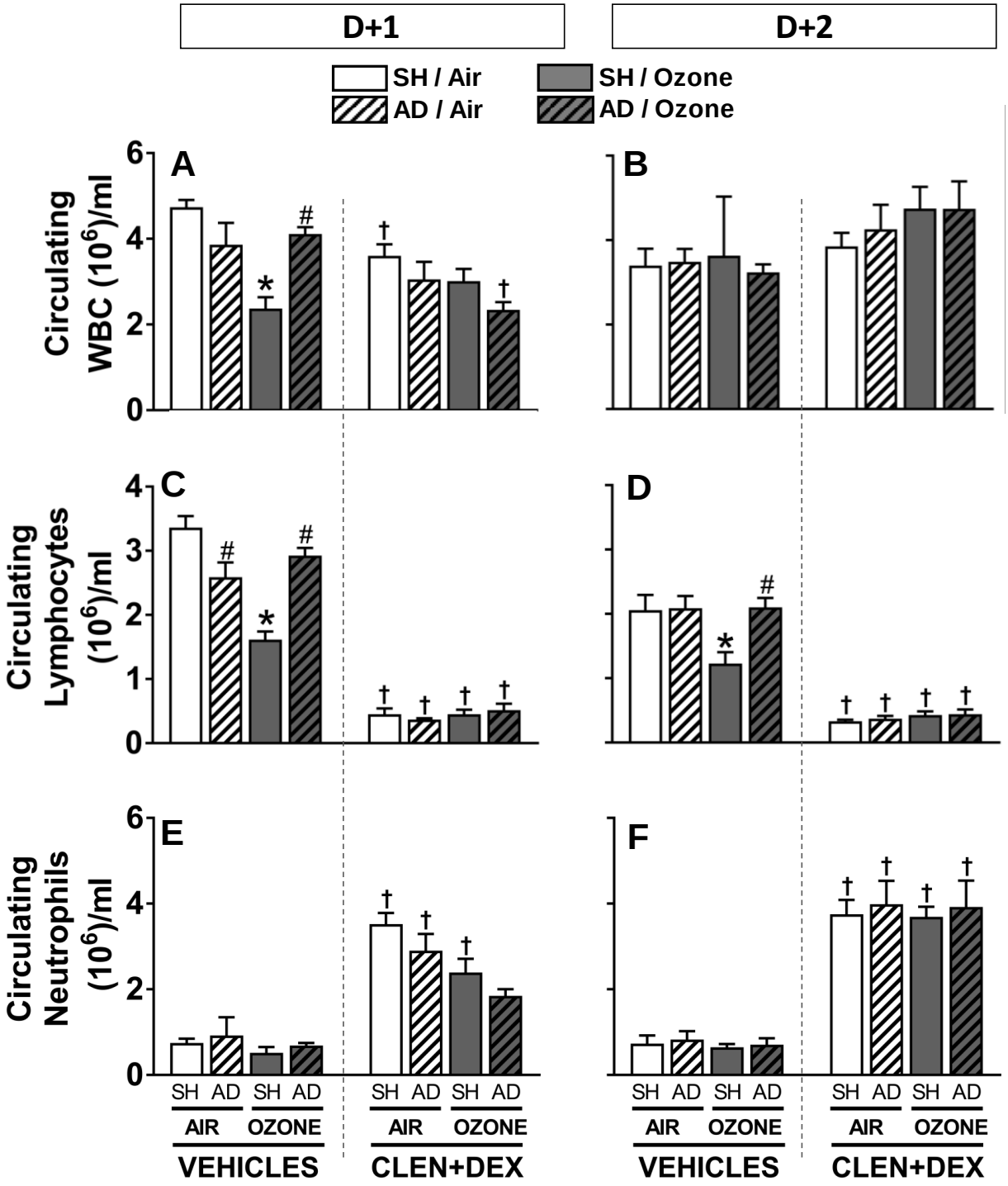

D+1
D+2
SH / Air
AD / Air
SH / Ozone
AD / Ozone
A
B
C
D
E
F
Circulating WBC ($10^6$)/ml
Circulating Lymphocytes ($10^6$)/ml
Circulating Neutrophils ($10^6$)/ml
SH AD SH AD
AIR OZONE
VEHICLES
CLEN+DEX

**Figure 4.7 Ozone-induced changes in circulating WBC and lymphocytes are modulated by AD and CLEN+DEX.** Circulating WBC (A-B), lymphocytes (C-D) and neutrophils (E-F) were assessed following air or ozone (0.8 ppm) exposure (4 hr/day) for D+1 and D+2 groups. Bar graphs show mean ± SEM of n=6-8 /group. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to corresponding air-exposed rats, # for AD effect when compared to corresponding SH rats, and † for CLEN+DEX effect when compared to corresponding vehicle-treated rats.

**Figure 4.8**

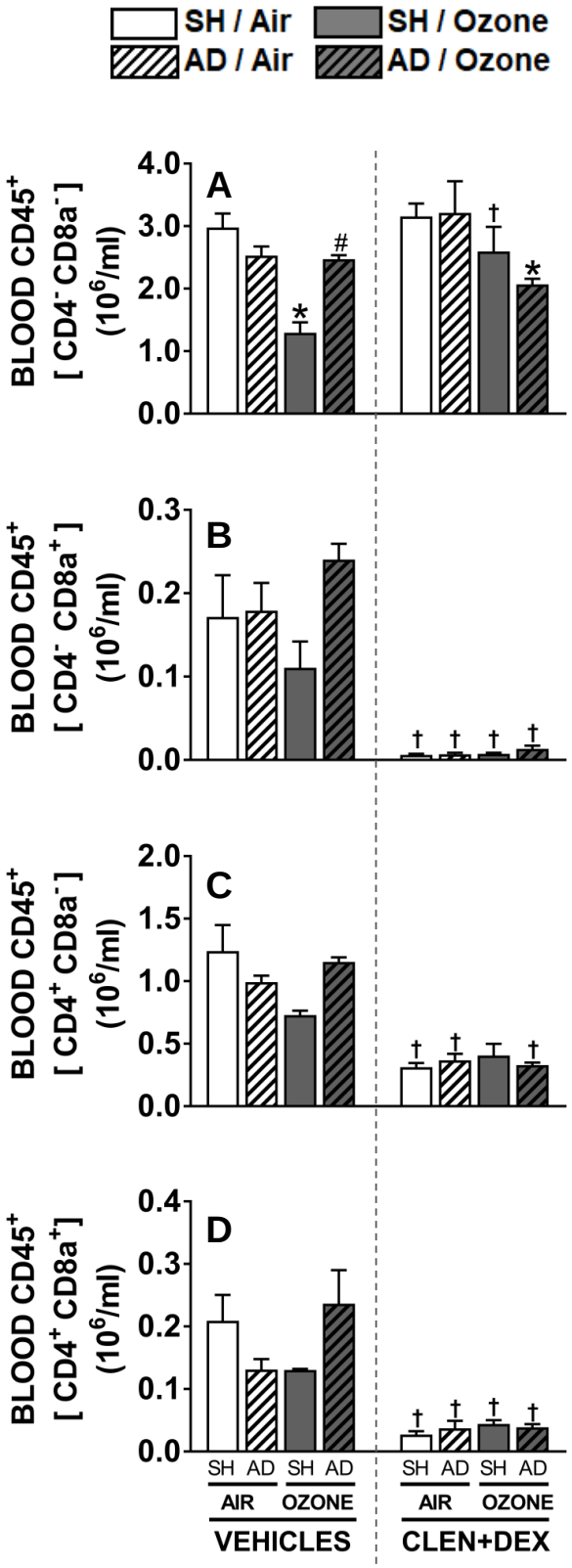

SH / Air
SH / Ozone
AD / Air
AD / Ozone
BLOOD CD45+ [ CD4- CD8a- ] (10^6/ml)
A
BLOOD CD45+ [ CD4- CD8a+ ] (10^6/ml)
B
BLOOD CD45+ [ CD4+ CD8a- ] (10^6/ml)
C
BLOOD CD45+ [ CD4+ CD8a+ ] (10^6/ml)
D
SH AD SH AD
AIR OZONE
VEHICLES
SH AD SH AD
AIR OZONE
CLEN+DEX

**Figure 4.8 Flow cytometry assessment of circulating leukocyte subpopulations after ozone exposure in SH and AD rats treated with vehicle or CLEN+DEX.** Percentage of circulating leukocyte subpopulations $CD4^{-}CD8a^{-}$ (A), $CD4^{-}CD8a^{+}$ (B), $CD4^{+}CD8a^{-}$ (C), and $CD4^{+}CD8a^{+}$ (D) were determined following air or ozone (0.8 ppm) exposure (4 hr/day) for D+1 groups. Cells positive for $CD4^{-}CD8a^{+}$, $CD4^{+}CD8a^{-}$, and $CD4^{+}CD8a^{+}$ were considered T-lymphocyte subpopulations. Relative numbers were determined based on $CD45^{+}$ cells (total leukocytes). Bar graphs show mean ± SEM of n=6-8 /group. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to corresponding air-exposed rats, # for AD effect when compared to corresponding SH rats, and † for CLEN+DEX effect when compared to corresponding vehicle-treated rats.

4.3.7 Ozone-induced effects on BALF cytokines in SH and AD rats treated with vehicles or CLEN+DEX

Proinflammatory cytokines were assessed to determine the role of AD and CLEN+DEX on ozone-mediated lung inflammation. BALF IL-6 levels were increased in all vehicle-treated SH and AD rats exposed to ozone relative to air at both time points. CLEN+DEX treatment resulted in highly exacerbated IL-6 increases in SH rats exposed to ozone at D+1 and D+2. This interactive effect of ozone and CLEN+DEX was less remarkable in AD rats (Fig. 4.9A and B). Ozone exposure increased BALF levels of TNF-α at D+1 in both SH and AD vehicle-treated rats. CLEN+DEX-treatment did not influence ozone-induced increases in BALF TNF-α at D+1. However, on D+2, CLEN+DEX treatment was associated with marked increases in BALF TNF-α in ozone-exposed rats regardless of surgery (Fig. 4.9C and D). BALF IL-4 levels were generally low in D+1 samples and below the detection limit in most D+2 samples with no apparent ozone effect in vehicle-treated rats. CLEN+DEX treatment increased IL-4 levels in ozone-exposed SH, but not AD rats, at D+1 (Fig. 4.9E and F).

**Figure 4.9**

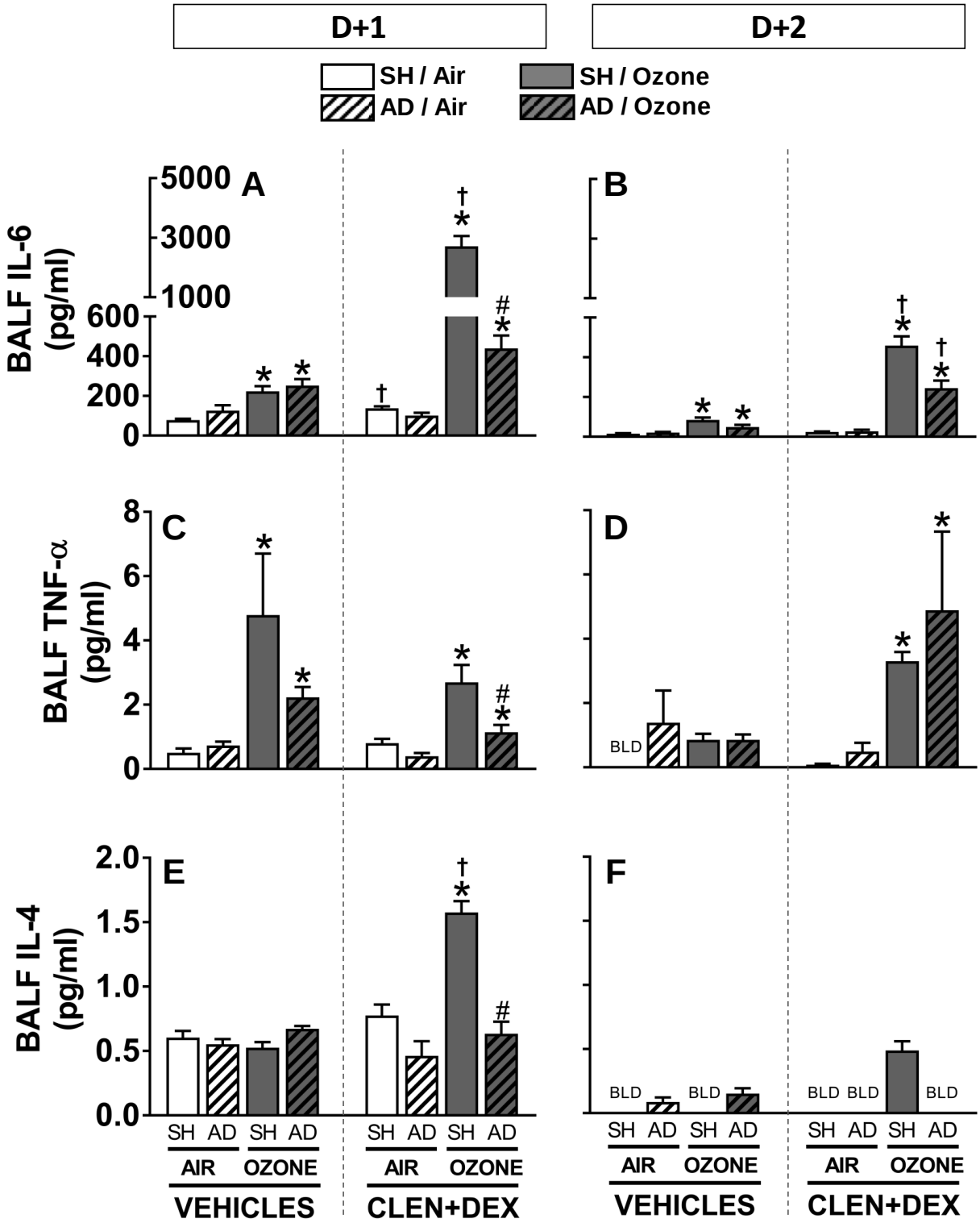

D+1
D+2
SH / Air
SH / Ozone
AD / Air
AD / Ozone
BALF IL-6 (pg/ml)
5000
3000
1000
600
400
200
0
A
B
BALF TNF-α (pg/ml)
8
6
4
2
0
C
D
BLD
BALF IL-4 (pg/ml)
2.0
1.5
1.0
0.5
0.0
E
F
BLD
SH AD SH AD
AIR OZONE
VEHICLES
CLEN+DEX

**Figure 4.9 Ozone-induced changes in BALF cytokine levels are influenced by AD and/or CLEN+DEX treatment.** BALF IL-6 (A-B), TNF-α (C-D) and IL-4 (E-F) proteins were quantified in samples collected 1-2.5 hr following air or ozone (0.8 ppm) exposure (4 hr/day) for D+1 and D+2 groups. Bar graphs show mean ± SEM of n=6-8 /group. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to corresponding air-exposed rats, # for AD effect when compared to corresponding SH rats, and † for CLEN+DEX effect when compared to corresponding vehicle-treated rats. BLD: below the limit of detection.

4.3.8 Ozone-induced pulmonary cytokine mRNA changes in SH and AD rats treated with vehicle or CLEN+DEX

Lung expression of genes involved in inflammatory processes, acute phase response and those responsive to AR and GR signaling was assessed in all samples from D+1 groups to determine if stress hormone receptors are involved in transcriptional regulation of genes known to be induced by ozone (Henriquez et al., 2017a). Changes in *Il6* mRNA expression were similar to those observed for IL-6 BALF protein at D+1 and D+2. AD in vehicle-treated air-exposed rats increased lung *Il6* expression. However, ozone exposure up-regulated *Il6* expression only in vehicle-treated SH but not AD rats. CLEN+DEX treatment further exacerbated *Il6* expression in ozone-exposed SH and AD rats (SH>AD; Fig. 4.10A). Even though BALF protein was increased, *Tnf* gene expression was not changed by ozone exposure or CLEN+DEX treatment in SH rats. However, CLEN+DEX treatment down-regulated *Tnf* expression in both air- and ozone-exposed AD rats (Fig. 4.10B). *Il4* expression tended to increase in vehicle-treated SH rats exposed to ozone but this effect was not evident in AD rats. CLEN+DEX treatment in SH but not AD rats increased *Il4*, especially in air-exposed SH rats (Fig. 4.10C). In vehicle-treated rats, *Cxcl2* expression was up-regulated after ozone exposure and AD effectively inhibited this effect. CLEN+DEX treatment increased *Cxcl2* expression in all rats exposed to air or ozone; however, these increases were smaller in AD rats relative to SH rats (Fig. 4.10D). Lung *Mt2a* (an acute phase response gene) expression in vehicle-treated rats followed a similar pattern of change as observed with *Cxcl2* in regards to ozone and AD effects (Fig. 4.10E). CLEN+DEX in all animals increased Mt2a expression to approximately 50-200 fold (SH:ozone>SH:air>AD:ozone>AD:air). In SH vehicle-treated rats, ozone tended to increase *Ardb2,* the gene expressing the β2AR protein; however, its expression was decreased in all vehicle-treated AD rats. CLEN+DEX significantly induced Ardb2 expression in the air-exposed vehicle-treated rats while this effect was less pronounced in ozone-exposed SH rats and air- or ozone-exposed AD rats (Fig. 4.10F). Expression of *Tsc22d3*, a GR responsive gene, tended to

increase after ozone exposure in the lungs of vehicle-treated SH rats; however, AD effectively down-regulated *Tsc22d3* expression in air- and ozone-exposed vehicle-treated rats. CLEN+DEX treatment significantly increased the expression of *Tsc22d3* for all groups except for ozone-exposed SH rats (Fig. 4.10G).

**Figure 4.10**

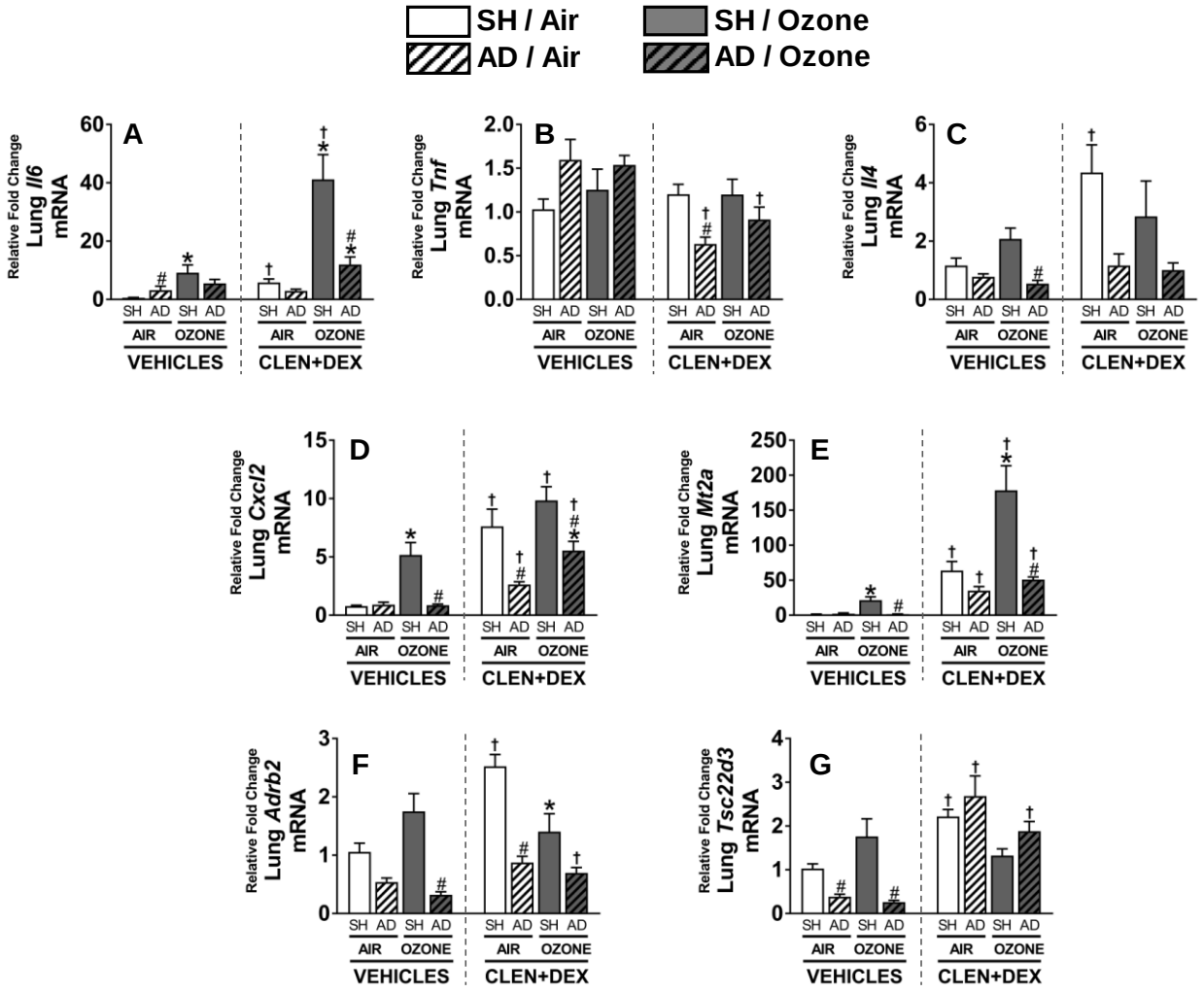



**Figure 4.10 Ozone-induced changes in lung inflammatory gene expression, and the effects of AD and CLEN+DEX.** Relative lung gene expressions for *Il6* (A), *Tnf* (B), *Il4* (C), *Cxcl2* (D), *Mt2a* (E), *Adrb2* (F) and *Tsc22d3* (G) were determined in tissues collected 1-2.5 hr following air or ozone (0.8 ppm) exposure (4 hr/day) for D+1 groups. Significant differences between groups (p value ≤ 0.05) are indicated by * for ozone effect when compared to corresponding air-exposed rats, # for AD effect when compared to corresponding SH rats, and † for CLEN+DEX effect when compared to corresponding vehicle-treated rats.

## 4.4 Discussion

We have previously shown that neuroendocrine activation leading to increased circulating stress hormones was necessary for mediating ozone-induced lung injury and inflammation since AD rats were protected from these ozone effects (Miller et al., 2016b; Henriquez et al., 2017a). Because AD is invasive and also eliminates circulating mineralocorticoids along with stress hormones, one cannot rule out their contribution in diminution of ozone-induced lung effects. The goal of this study was to evaluate if agonists of stress hormone receptors $\beta_2$AR and GR were able to restore ozone-induced lung injury, inflammation and innate immune cell trafficking in AD rats, and exacerbate these effects in SH rats. Here, we reconfirmed that the pulmonary and systemic effects of ozone inhalation, characterized by vascular leakage, neutrophilic inflammation, cytokine release in the lungs and peripheral vascular lymphopenia, were significantly diminished by AD (Miller et al. 2016b). The treatment with a combination of $\beta_2$AR and GR agonists (CLEN+DEX) was able to restore these ozone effects in AD rats, and further exacerbate ozone-induced lung protein leakage, inflammation and lymphopenia in SH rats. It was also noted that CLEN+DEX itself caused injury and cytokine increases in the lung. Although a variety of $\beta_2$AR and GR agonists have been widely used for the treatment of chronic lung diseases (Fireman, 1995; Barnes, 2011), $\beta_2$AR agonists have been shown to exacerbate lung inflammation in asthmatics (Mcivor et al., 1998, Boulet et al., 2001) and epidemiological studies have indicated exacerbation of lung inflammation in asthmatics during increased air pollution episodes (Qian et al., 2009). Even though high concentrations of agonists were used, our study provides a potential causal mechanistic link between activation of stress hormone receptors in mediation of air pollution health effects, and how these effects might be exacerbated in those receiving asthma therapy.

Hypothermia following air pollution exposure has been postulated to be a protective autonomic mechanism in rodents (Gordon et al., 2014), likely reducing the pollutant dosimetry and/or impact on the lung (Terrien et al., 2011, Gorr 2017). In this study, we too observed a

hypothermic response to ozone in SH rats via assessment of subcutaneous temperature, a measurement known to reflect ozone-induced effects on core body temperature (Gordon et al., 2014). Interestingly, although AD reversed most ozone effects on the lung and periphery, hypothermia was not reversed, suggesting this autonomic response is likely regulated upstream and independent of neuroendocrine stress axes activation. It is also likely that this response might be regulated by nor-epinephrine action, especially producing local effects at sympathetic nerve endings, since adrenalectomy did not influence the levels of nor-epinephrine (Miller et al., 2016b).

$\beta_2$AR and GR agonists are widely used for the treatment of chronic lung diseases such as asthma and COPD to cause bronchodilation and inhibition of inflammation through immunosuppression, respectively. Since ozone-induced lung protein leakage and inflammation are also associated with increased levels of endogenous $\beta_2$AR and GR agonists, it is likely that air pollution effects are exacerbated in those receiving this therapy. A variety of agonists and antagonists of different formulations are available and are given to patients as a singular therapy or as part of a combination. CLEN, a specific $\beta_2$AR agonist, although not prescribed in the US, is used in other counties with a recommended dose of 0.02-0.06 mg/day for bronchodilation, and much higher doses of up to 0.12 mg/day for inducing weight loss (Drug Enforcement Administration, 2013). Likewise, DEX is a widely used steroid in humans, in veterinary practice and employed extensively in research. At the recommended repeated adult dose levels of 0.75 to 9 mg every 6-12 hr for an average 70 kg person it is anti-inflammatory, whereas for patients with adrenal insufficiency, doses of up to 0.15 mg/kg/day every 6-12 hr are given (http://reference.medscape.com/drug/decadron-dexamethasone-intensol-dexamethasone-342741- accessed 9-20-17). In this study we justify using higher levels than what is used therapeutically in humans to assure a sufficient coverage of expected change due to adrenalectomy-mediated depletion and ozone-induced increases.

As we previously demonstrated (Miller et al., 2016b), AD nearly completely eliminated stress hormones, corticosterone and epinephrine, from the circulation. Importantly, corticosterone, but not epinephrine, was significantly diminished by CLEN+DEX treatment. Since the HPA axis is regulated by a negative feedback inhibition controlled by glucocorticoids themselves (Keller-Wood 2015), it is likely that this is due to the known DEX-dependent inhibition of corticosterone synthesis and release (Kolebinov et al., 1975). The increased expression of the glucocorticoid responsive gene *Tsc22d3*, which has been shown to increase following ozone exposure in multiple organs (Thomson et al., 2013, 2016), upregulation of the adrenergic receptor β2 gene (*Adrb2),* together with increased trend of circulating epinephrine and corticosterone in SH rats following ozone exposure supports the conclusion that $\beta_2$AR and GR are involved in mediating ozone lung effects.

Since inhaled $\beta_2$AR and GR agonists are widely used as bronchodilators and immunosuppressant, respectively (Cazzola et al., 2012, Brusselle and Bracke 2014, Yayan and Rasche 2016), we wanted to determine if ozone-induced ventilatory changes were influenced by systemic CLEN+DEX and AD. While CLEN+DEX treatment in air-exposed animals had some effect and increased pause, PEF/PIF and PenH, ozone effects in general were exacerbated by the drug treatment when considering these endpoints even though measurements were taken post-exposure. It is important to note that the biphasic inspiration trace observed in CLEN+DEX treated animals might have influenced ventilation and changes in Ti/Te, pause and PEF/PIF. Since CLEN+DEX in all air- and ozone-exposed rats was associated with lung protein leakage and increased NAG activity but not increases in alveolar macrophages, it is likely that ozone-induced changes in ventilatory parameters in CLEN+DEX-treated rats were perhaps linked to macrophage influx and release of inflammatory cytokines as discussed below.

As reported earlier (Miller et al., 2016b), AD prevented the ozone-induced vascular protein leakage in the lungs. Conversely, CLEN+DEX treatment in air-exposed animals was enough to induce marked vascular leakage, indicating a crucial role for $\beta_2$AR and GR in this

response. CLEN treatment has been shown to decrease pulmonary vascular resistance (PVR) in horses (Dodam et al., 1993). Likewise, salbutamol, a different $\beta_2$AR agonist, significantly decreased PVR in humans (Spiekerkoetter et al., 2002). $\beta_2$AR agonist treatment is also associated with acute pulmonary edema in females (Eliat et al., 2002) while epinephrine-induced pulmonary edema has been characterized in rats (Hao et al., 2001). In addition, lung epithelial permeability is increased by $\beta_2$AR agonists (Unwalla et al., 2015, Unwalla et al., 2012). Collectively, this suggests that CLEN may decrease PVR, which can enhance blood flow in the pulmonary vasculature, and increase pulmonary edema and epithelial permeability, leading to increased levels of BALF protein and albumin in CLEN+DEX-treated rats. In the SH rats, these effects are likely mediated by the increased epinephrine released following ozone exposure. Conversely, increased glucocorticoid activity has been implicated in decreased permeability of lung epithelium (Kielgast et al., 2016, Matheson et al., 2004) and alleviation of pulmonary edema (Matthay 2014). However, since our protocol included both CLEN+DEX, it is not clear how DEX might have influenced the effects of CLEN.

The changes in lavageable macrophages after ozone exposure are likely influenced by the temporality of the assessment (Kumarathasan et al., 2015; Laskin et al., 1998). Increased adhesion of activated alveolar macrophages might reduce the efficiency of recovery during lavage procedure (Gordon et al., 2016, Bhalla et al., 1996). While the decrease in alveolar macrophages after ozone exposure on D+1 was prevented by AD, it is noteworthy that CLEN+DEX treatment increased alveolar macrophages but only in ozone-exposed rats (SH>AD). The duration and the degree of stress hormone increases and the dose of drugs might influence this disparity between ozone and CLEN+DEX. Broug-Holub et al. (1998) demonstrated that physical stress in rats leads to enhanced macrophage activity that is reversed by parasympathetic but not sympathetic blockade while McGovern et al. (1996) found that ozone and β adrenergic inhibition cooperatively inhibit macrophage activity. Furthermore, exercise-dependent suppression of macrophage antiviral function was dependent on stress

hormones (catecholamines) and reversed by AD, suggesting their role in modulating macrophage function (Kohut et al., 1998).

Neutrophilia is one of the hallmarks of ozone exposure (Hollingsworth et al., 2007, Alexis et al., 2010; Kodavanti et al., 2015) and acute stress scenarios directly modulate neutrophil recruitment, migration and mobilization through the action of stress hormones (Dhabhar et al., 2012).Consistent with our previous publications (Miller et al., 2016b; Henriquez et al., 2017a), ozone exposure increased the accumulation of neutrophils in BALF while stress hormone absence (by AD) was associated with decreased BALF neutrophils in ozone-exposed rats. Interestingly, ozone-induced increases in BALF neutrophil numbers is 2-3-fold higher in SH treated with CLEN+DEX. These results suggest the combination of ozone along with CLEN+DEX produce exacerbated lung neutrophilic inflammation.

Effects of ozone inhalation are not limited to the respiratory system. Our results support the previous observation that ozone exposure reduces circulating WBC, especially lymphocytes, while increasing circulating epinephrine and corticosterone in rats (Miller et al., 2016b; Henriquez et al., 2017b). Since CLEN+DEX also produces a substantial reduction in circulating lymphocytes (likely due to the immunosuppressive dose of DEX) while increasing BALF neutrophils, it is likely that these effects of ozone are modulated by stress hormones in a dynamic manner (Dhabhar et al., 2012). Although the separation between circulating T-lymphocytes and B-lymphocytes could not be assessed since CD3, a pan marker of lymphocytes, was not included, it was apparent that T-lymphocyte subpopulations $CD4^{-}CD8a^{+}$ (cytotoxic), $CD4^{+}CD8a^{-}$ (Helper) and $CD4^{+}CD8a^{+}$ were decreased by CLEN+DEX and to some extent by ozone. This demonstrates that T-lymphocytes are sensitive to stress hormone levels and their receptor activation. It is well known that glucocorticoids induce thymic atrophy (Roggero et al., 2006, Pazirandeh et al., 2002) and apoptosis in circulating T-cells (Cidlowski et al., 1996), which is consistently observed in different models of stress (Szabo et al., 2017). The pattern of ozone-induced decrease and AD-mediated recovery of circulating leukocyte cell

subpopulations suggest that endogenous levels of stress hormones are sufficient to dynamically regulate the relative size of cytotoxic and helper T-cell pools.

We have previously reported that global lung gene expression changes observed after ozone exposure are diminished in AD rats (Henriquez et al., 2017a), suggesting that diminution of circulating stress hormones reduce ozone effects at the transcriptome level. In this study, we sought to determine the specific influence of activating $\beta_2$AR and GR in AD rats on inflammatory and acute phase markers. We noted that ozone exposure was associated with increases in proteins and mRNA for inflammatory markers, including acute phase response genes, and, as observed earlier (Henriquez et al., 2017a), this response was inhibited in AD rats. Moreover, we noted that CLEN+DEX itself was sufficient to induce inflammatory genes to much higher levels than ozone, such that no further increases were discernible by ozone. These changes confirm that activation of $\beta_2$AR and GR modulates lung transcriptional changes, which likely influence the inflammatory response to ozone.

The concentration of ozone used in this study (0.8 ppm) is much higher than what is likely encountered environmentally. However, this level in resting rats can be comparable to exercising humans exposed to 0.2 ppm ozone (Hatch et al., 2014), and it has been recently shown that ambient ozone concentrations can rise up to 0.2 ppm under certain circumstances in the US (EPA, 2017).

There are a number of limitations to our study. The dynamic nature of ozone-induced changes was not examined to determine how the recovery or progression of injury might occur after ozone exposure, especially in the case of AD. Also, the CLEN+DEX doses used in this study, although within the range of those used by many research publications (Jonasson et al., 2013; Sato et al., 2008; Ryan et al., 2010; Kopitar, 1969; Huang et al., 2014; Sadarani and Majumdar 2015; Sun et al., 2009), were much higher than human therapeutic doses, and the interaction of CLEN and DEX with air pollutants at therapeutic levels need to be carefully examined.

In conclusion, our results suggest that both endogenous and exogenous levels of epinephrine and glucocorticoids dynamically regulate ozone-induced pulmonary and extra-pulmonary responses. Ozone-induced lung vascular protein leakage, inflammation and peripheral lymphopenia were diminished in AD rats, and agonists of $\beta_2$AR and GR restored this phenotype implicating their role (Fig. 4.11). Moreover, these agonists by themselves were sufficient to induce lung vascular leakage and inflammation producing ozone-like lung injury and lymphopenia (Fig. 4.11). These data provide a potential mechanistic link for the epidemiological evidence that air pollution effects are exacerbated in those socioeconomically disadvantaged communities with high levels of psychosocial stresses and high levels of circulating stress hormones (Douwes et al., 2011). Furthermore, this study may suggest that the pulmonary effects of air pollutants might be exacerbated in asthmatics or COPD patients receiving chronic bronchodilator treatment with or without immunosuppressant steroids, since air pollutants increase levels of circulating stress hormones.

**Figure 4.11**

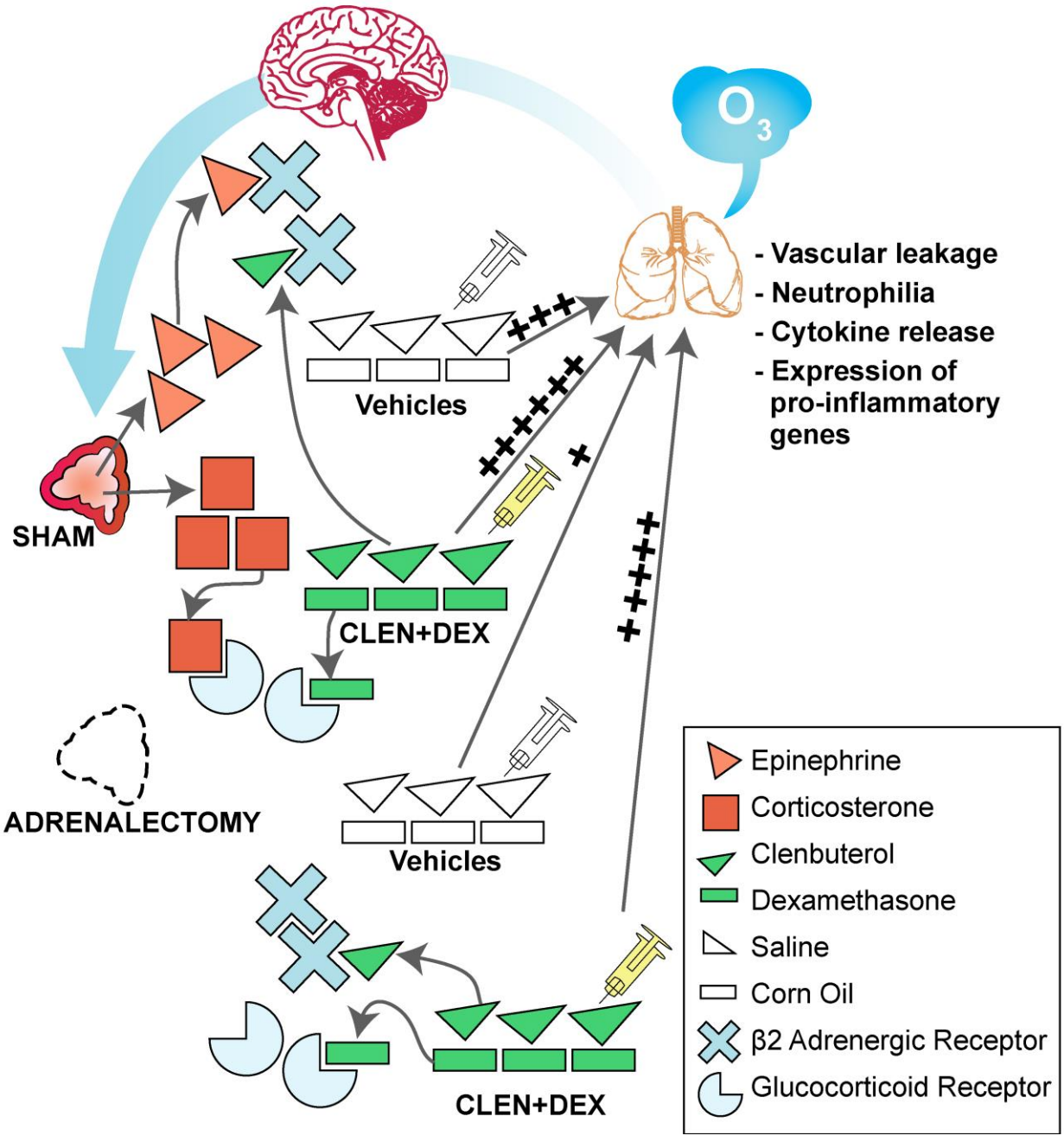

O3
- Vascular leakage
- Neutrophilia
- Cytokine release
- Expression of pro-inflammatory genes
Vehicles
SHAM
CLEN+DEX
ADRENALECTOMY
Vehicles
CLEN+DEX
Epinephrine
Corticosterone
Clenbuterol
Dexamethasone
Saline
Corn Oil
β2 Adrenergic Receptor
Glucocorticoid Receptor

**Figure 4.11 Potential mechanisms involved in ozone-induced lung injury and inflammation in SH and AD rats treated with CLEN+DEX.** It is well established that normal rats (SH) when exposed to ozone (0.8 ppm, 4 hr/day) develop lung injury and inflammation (indicated by +++). This data summary shows that all effects of ozone are reduced or prevented by adrenalectomy (AD) as a result of lack of circulating stress hormones, epinephrine and corticosterone (indicated by +). Epinephrine and corticosterone are known to exert organ-specific effects through adrenergic (AR) and glucocorticoid (GR) receptors, respectively. Lung tissue primarily expresses βAR. When SH and AD rats are treated with agonists of $\beta_2$AR and GR (CLEN+DEX), the ozone effects are exacerbated/restored (indicated by ++++++ or +++++) in SH/AD rats, respectively. It is noteworthy that the CLEN+DEX treatment in air-exposed SH rats were sufficient to induce lung injury/inflammation while drug supplementation in ozone-exposed sham rats exacerbated lung injury/inflammation.

# CHAPTER 5: CONCLUSIONS AND PERSPECTIVES

## 5.1 Air pollution and neuroendocrine stress response

Air pollution is a “silent killer” and millions of people die prematurely every year as a consequence (Jerret et al., 2015). Epidemiological studies have shown that psychosocial stresses exacerbate responses triggered by air pollution exposure (Clougherty and Kubzansky, 2009). Even maternal stress has been associated with increased offspring susceptibility to air pollution. Stress, a non-specific host response to an external threat produced in all living beings, has been linked to major human ailments. Neuroendocrine stress response is produced centrally after receiving sensory danger signals from external environment or internal changes in the body, which causes HPA and SAM activation. The processes initiated in the hypothalamus and other areas of CNS mediate the release of stress hormones into the circulation, which prepare the host to act, face or escape any threat. This neuroendocrine activation, also referred to as a “fight or flight response”, is widely conserved in mammals. Some of the hallmark changes associated with stress response include the redirection of blood flow to muscles, the mobilization of energy sources to the site of stress, hypothermia or hyperthermia (depending on the type of stress and host) and the stimulation of an immune response to counter stress. Although this stress response has been linked to air pollution exposure in epidemiology, no earlier studies have examined the contribution of this response in mediating pulmonary and systemic health effects of air pollutants.

Systemic health effects of air pollutants and the mechanisms by which peripheral organs are affected have been at the forefront of air pollution research since there is a great interest in understanding how virtually all chronic diseases of non-pulmonary and pulmonary origin are

associated with exposure to air pollution. It is believed that the complex milieu of circulating factors, often referred to as “molecular shrapnel”, is involved in mediating systemic effects; however, the origin and the causes of change in these circulating factors are not understood. The current mechanistic paradigm implicates that the release of bioactive components such as cytokines, oxidation by-products and vasoactive substances released from the pollutant-injured lung tissue into the circulation induce systemic and distant organ effects. However, our laboratory has recently shown an alternative mechanism involving neuroendocrine activation causing widespread systemic multi organ as well as pulmonary effects of inhaled pollutants, which challenges the current mechanism paradigm. We have shown that ozone and acrolein-induced extra-pulmonary effects are similar to those produced during a “fight or flight response”. Based on the neuroendocrine activation, and the characteristic of air pollution response, it is believed that sensory input from lung to brain, rather than spillover of bioactive substances into the circulation mediates changes in the circulating milieu and peripheral effects (Kodavanti 2016). The activation of neuroendocrine system by air pollutants and the release of stress hormones in the circulation can affect peripheral organs through their action on stress hormone receptors.

## 5.2 Ozone exposure and stress hormones: possible mechanisms of lung CNS communication

We have shown that through activation of neuroendocrine stress axes such as SAM and HPA, ozone exposure induces a rapid release of circulating stress hormones, epinephrine and corticosterone, which through their organ specific effects cause the release of hundreds of metabolites, acute phase proteins and more importantly stress hormones into the circulation (Bass et al., 2013; Miller et al., 2015, 2016a; 2016b; 2016c). More importantly, our studies led us to identify the two fundamental survival processes implicated in the fight-or-flight response (i.e energy mobilization and immune surveillance) to be induced by exposure to ozone, which explains how multi-organ effects are produced. Furthermore, our intervention studies demonstrate that circulating stress hormones are necessary for producing a vast number ozone effects including lung injury and innate immune response (Miller et al., 2016b).

Ozone is an irritant which activates vagal C fibers in the airways. These afferent C fibers originate from jugular and nodose ganglion, and relay sensory input from the lung to the NTS and surrounding areas of the CNS to produce autonomic respiratory reflexes and cardiovascular responses (Taylor-Clark and Undem, 2010). This lung-CNS communication axis might explain how ozone exposure activates stress responsive regions in the brain and would explain the activation of neuroendocrine stress responses (Gackière et al., 2011). Neuronal firing in stress responsive areas in the brain mediates SAM and HPA axes activation, which upregulate the release of catecholamines and corticosteroids, respectively, capable of mediating metabolic and immune responses in the body. However, the precise mechanisms by which the lung communicates with the brain in inducing SAM and HPA after air pollution exposure remains to be examined. Based on our findings that not only systemic but also lung effects of ozone are diminished in adrenalectomized rats (Miller et al., 2016b), our main goal was to examine how stress hormones that are released from the adrenal glands in response to ozone exposure elicit pulmonary injury and inflammation. We hypothesized that the CNS-mediated release of stress

hormones, epinephrine and corticosterone, through their action on AR and GR, respectively, modulate ozone-induced vascular leakage and inflammation in the lungs. We sought to examine how immune responses are regulated under air pollutant stress through stress hormone-mediated action on their cellular receptors.

## 5.3 AR and GR activation by stress hormones is necessary to induce pulmonary injury and inflammation after ozone exposure

In the first specific aim (Henriquez et al., 2017a), we characterized the role of circulating stress hormones in lung transcriptional processes induced by ozone using global mRNAseq in rats that have undergone ADREX or DEMED. In this study, we demonstrated that lung transcriptional response to ozone was nearly abolished in DEMED and ADREX rats, implying that the effect of circulating stress hormones after ozone exposure was occurring at the transcriptional level in the lung. Using IPA we found that ADREX and DEMED attenuated the changes in genes involved in pathways classically up-regulated by ozone exposure, such as oxidative stress and pro-inflammatory PI3K/AKT signaling. In addition, when ozone-induced gene expression changes were compared against a known database from other chemicals using Ingenuity comparison tools, the signature of global gene expression changes in ozone-exposed SHAM rats resembled those induced by glucocorticoids (methylprednisolone and dexamethasone) and cAMP-increasing substances such as forskolin (an effect induced by AR activation). These findings indicated that ozone-induced transcriptional changes are similar to those induced by drugs activating AR and GR. Further assessment of cytokine proteins and mRNA suggested a tendency of ozone to produce Th2 immune responses, and to up-regulate the expression of glucocorticoid responsive genes in lung tissue were diminished by ADREX.

Although this study provided significant insights into the potential contribution of epinephrine and corticosterone in mediating ozone lung effects, both DEMED and ADREX are invasive procedures. Furthermore, adrenal glands also produce mineralocorticoids and sex hormones in addition to corticosteroids and catecholamines, thus, one cannot rule out the contribution of these factors in ozone-induced lung effects. Therefore, the goal of the second study was to further assess the specific roles of each stress hormone in mediating ozone-induced lung injury and inflammation using a targeted pharmacological approach *in vivo*. Conducting this study was important in determining the role of stress hormones for two reasons:

1) it allowed us to avoid the side-effects of adrenalectomy surgery and suppression of mineralocorticoids/sex hormones, and 2) we were able to specifically isolate the role of each stress hormone separately by treating rats to AR and GR blocker individually or in combination. With DEMED and ADREX, we were able to block the effect of only epinephrine or both epinephrine and corticosterone together, respectively, but not corticosterone by itself. In this specific aim, we examined the contribution of AR and GR in mediating ozone effects by pretreating rats with βAR antagonist, PROP, and GR antagonist, MIFE, individually or in combination. We hypothesized that the epinephrine and corticosterone released into the circulation following exposure would bind to pulmonary βAR and GR, respectively, and collectively mediate ozone-induced lung injury and trafficking of immune cells to the lung.

The results from this specific aim (Henriquez et al., 2017b) demonstrated that inhibition of both stress hormone receptors with PROP and MIFE led to overall decreases in all ozone-induced lung effects and, therefore, it was concluded that the circulating stress hormones epinephrine and corticosterone are required for ozone to induce lung injury and inflammation. Ozone-induced lung vascular leakage was prevented by PROP, MIFE, and PROP+MIFE treatments, suggesting that corticosterone and epinephrine are contributing individually to ozone-induced lung vascular leakage. However, ozone-induced pulmonary neutrophilia was prevented primarily by PROP whereas lymphopenia was prevented primarily by MIFE, illustrating the specific involvement of βAR in mediating neutrophil extravasation to the lung and GR in mediating egress of lymphocytes in the circulation. The attenuation of ozone-induced increases in inflammatory mediator mRNA and proteins by PROP but not MIFE implicated the role of epinephrine and βAR in local effects of ozone in the lung. The use of the strategy to isolate the ozone effects mediated by epinephrine and corticosterone emphasized the role of HPA and SAM axes as independent arms of neuroendocrine stress response activation, individually controlling each effect observed following ozone inhalation.

Asthma and chronic lung diseases, often linked to environmental exposures, are some of the leading causes of human morbidity and mortality worldwide (Soriano et al., 2017). Bronchodilators and immune suppressants are the two major therapeutics used in the treatment of these lung diseases, as well as for cardiovascular and chronic inflammatory conditions. More importantly, these are the therapeutics that mimic stress hormones and exert their effects through binding to AR and GR receptors. This implies that normal functioning of neuroendocrine stress axes is critically important in diseases, and that these axes are therapeutically manipulated for combating chronic diseases. In addition, it has been reported epidemiologically that individuals receiving asthma mediation have exacerbated air pollution effects (Gent et al., 2003). The activation of AR and GR receptors by increased air pollution exposure might coincide with the use of these receptor agonists in lung disease patients to exacerbate pulmonary injury and inflammation.

In the third aim, to demonstrate the direct contribution of stress hormone receptors activation in ozone-induced effects, we carried out a “gain of function” experiment (Henriquez et al., 2017c, in review). We hypothesized that the treatment of adrenalectomized rats with $\beta_2$AR and GR agonists will reverse the protective effect of AD and restore ozone-induced lung injury and inflammation. The re-gain of ozone-sensitivity in AD rats, by providing drugs mimicking the activity of stress hormones, would unequivocally demonstrate the causal role of epinephrine and corticosterone as bioactive circulating factors mediating ozone-induced lung injury. Further, we postulated that this intervention would allow us to examine if air pollution effects are exacerbated in those receiving dual bronchodilator and immunosuppressant therapy. A combination of widely used bronchodilator (clenbuterol) and steroid (dexamethasone) was used (CLEN+DEX) to examine their influence on ozone-induced lung injury and innate immune response in AD and SH rats. This study allowed us to reconfirm our earlier findings that ozone-induced lung injury and inflammation, increased cytokine expression, as well as lymphopenia are diminished by depleting circulating stress hormones through AD. More importantly, by

pharmacologically activating $\beta_2$AR, which are predominantly located in lung bronchial smooth muscle cells, and GR using CLEN+DEX, we not only restored the ozone injury phenotype in AD rats but further exacerbated ozone effects in healthy SH rats. These findings support the conclusion that those receiving bronchodilators and combinational therapy are likely to experience increased air pollution adverse effects through a common mechanism. Subsequent studies in our lab that used therapeutic doses of CLEN+DEX provides further support to this hypothesis, and raises concern for exacerbated air pollution effects in asthmatics and COPD patients receiving therapy.

In these three preceding core chapters, by using different experimental strategies, we conclude that air pollutants activate neuroendocrine stress axes, and through the release of epinephrine and corticosterone into the circulation, mediate ozone-induced lung injury and inflammation. The contribution of circulating stress hormones in mediating pulmonary effects of air pollution was not investigated in previous air pollution studies. Further, using pharmacological and surgical interventions, we show that ozone-induced effects in the lung are mediated through $\beta$AR and GR receptors. Since $\beta$AR and GR agonists are widely used in treatment of chronic lung diseases, our results show a potential common mechanism where these drugs might exacerbate acute air pollution effects. Finally, our findings bring forth a new dimension to air pollution health effects research and show that ozone pulmonary injury and innate immune effects are mediated by circulating stress hormones.

## 5.4 Challenging a classic paradigm

The main results of this dissertation challenge the current mechanistic paradigm explaining how ozone mediates pulmonary and systemic health effects. It is believed that spillover of bioactive substances from the lung to circulation may serve to transport signals to the periphery and induce effects in distant organs. We provide a new mechanism whereby inhaled pollutants through CNS activation of neuroendocrine stress axes contribute to not only effects in the peripheral organs, but also in the lung. Since neuroendocrine activation and resulting effects are observed rapidly after air pollution exposure, prior to induction of lung vascular leakage and inflammation, the systemic and pulmonary changes are a direct consequence of CNS activation. Using surgical and pharmacological interventions, we confirm the central role of AR and GR receptors, which were previously unrecognized in air pollution studies (Fig. 5.1).

There is a body of evidence justifying the role of lung-CNS communication as a mechanism explaining how inhaled irritants trigger autonomic responses and reflex bronchoconstriction such as cough, sneeze or apnea, and in the case of ozone, cardiovascular depression or hypothermia. However, the neuroendocrine stress axes activation was not investigated prior to our studies. Although we did not explore how the lung communicates with the brain following ozone exposure, follow-up investigations could test if cervical vagotomy or desensitization of C-fibers using capsaicin will prevent ozone-induced release of stress hormones and further downstream effects. Likewise, blockade of pain receptors with long-lasting anesthesia could be used to examine the role of pain receptors in stimulation of neuroendocrine stress axes. A number of studies, including our own unpublished findings, have used TRPA and TRPV receptor blockers, but the results have not provided consistent findings delineating their role in stimulation of vagus nerves and central stress responsive regions. Animal models of gene knock outs lacking critical genes involved in SAM or HPA axes activation can also be used to explore in more detail the ozone-induced neuroendocrine stress

response. For instance, knock out mice deficient for corticotrophin releasing hormone gene in hypothalamus (Jobe and Ikegami, 1998), specific AR (Chiarella et al., 2014) and enzymes involved in the biosynthesis of catecholamines and glucocorticoids (Alves et al., 2016) are available and could serve to investigate how ozone and stress cooperatively interact to induce pulmonary and extra-pulmonary alterations.

In addition, the use of humans with diseases involving altered glucocorticoid and catecholamine production or metabolism could serve as models for evaluation of stress hormone modulation of ozone-induced responses. For example, post-traumatic stress disorder (PTSD) or adrenal gland disorders, such as Addison's disease or Cushing's syndrome where cortisol levels are abnormally low and high, respectively, could represent human models to investigate the role of stress hormones on air pollution-induced health effects. Perhaps these models could also be used to identify subpopulations with exaggerated or reduced ozone-induced responses. The use of accepted clinical tests which modulate circulating glucocorticoid levels, such as dexamethasone suppression test or ACTH stimulation test, in conjunction with ozone exposures could be useful to establish the role of HPA axis activation in ozone-induced lung injury and inflammation in humans. Furthermore, although our results clearly support the role of stress hormones in ozone-induced pulmonary responses, we believe that other pathways might interactively influence ozone effects. For example, the role of serotonin might shed some light on how environmental stressors, such as ozone and psychosocial stressors, interact to exacerbate disease phenotype. An interesting example is the therapeutic use of ACTH in the treatment of pulmonary sarcoidosis and granuloma (Baughman et al., 2016), which suggests potential interaction of stress axes and inflammatory processes at different levels.

Long-acting bronchodilator monotherapy has been discouraged for treatment of asthma since it increases the risk of asthma-related death (Rottenkolber et al., 2015). Gent et al. (2003) found that levels of ozone below the current NAAQS were significantly associated with exacerbation of respiratory symptoms only in asthmatic children taking maintenance medication

such as long-acting β-agonist (LABA). In a separate experiment, we found that CLEN by itself at doses higher than what was used in the third aim had no effect on lung injury and inflammation in air-exposed animals; however, this treatment highly exacerbated ozone-induced lung injury (data not shown). Interestingly, in our third specific aim, we found that the combination of $\beta_2$AR and GR agonists (CLEN+DEX) effectively exacerbated ozone-induced responses in SH rats. Although the drug doses were carefully selected based on previous publications, these doses were higher than the therapeutic doses used for lung disease. Thus, a follow-up investigation of how CLEN, DEX, and/or other agonists at therapeutic doses might exacerbate air pollutant-induced pulmonary injury and inflammation will be important in determining a causal link between potential drug/air pollution interactions observed in epidemiology.

Another area of research worth further analysis is the exploration of strategies to assess how minimizing stress responses could alleviate or attenuate air pollution-induced adverse health effects. In our second publication, we found that βAR and GR antagonists significantly reduced acute lung injury and inflammation induced by ozone exposure. Since PTSD and other psychosocial stressors are associated with increased circulating stress hormones, the use of therapeutics that reduce circulating stress hormones or inhibit AR and GR might be considered for mitigation of air pollution health effects. Also, the inclusion of other compounds known to modulate stress responses, such as oxytocin which plays a key role as a suppressor of HPA axis activation (Crockford et al., 2017), could be used to evaluate interventions aimed to reduce the health effects of air pollution inhalation and stress.

**Figure 5.1**

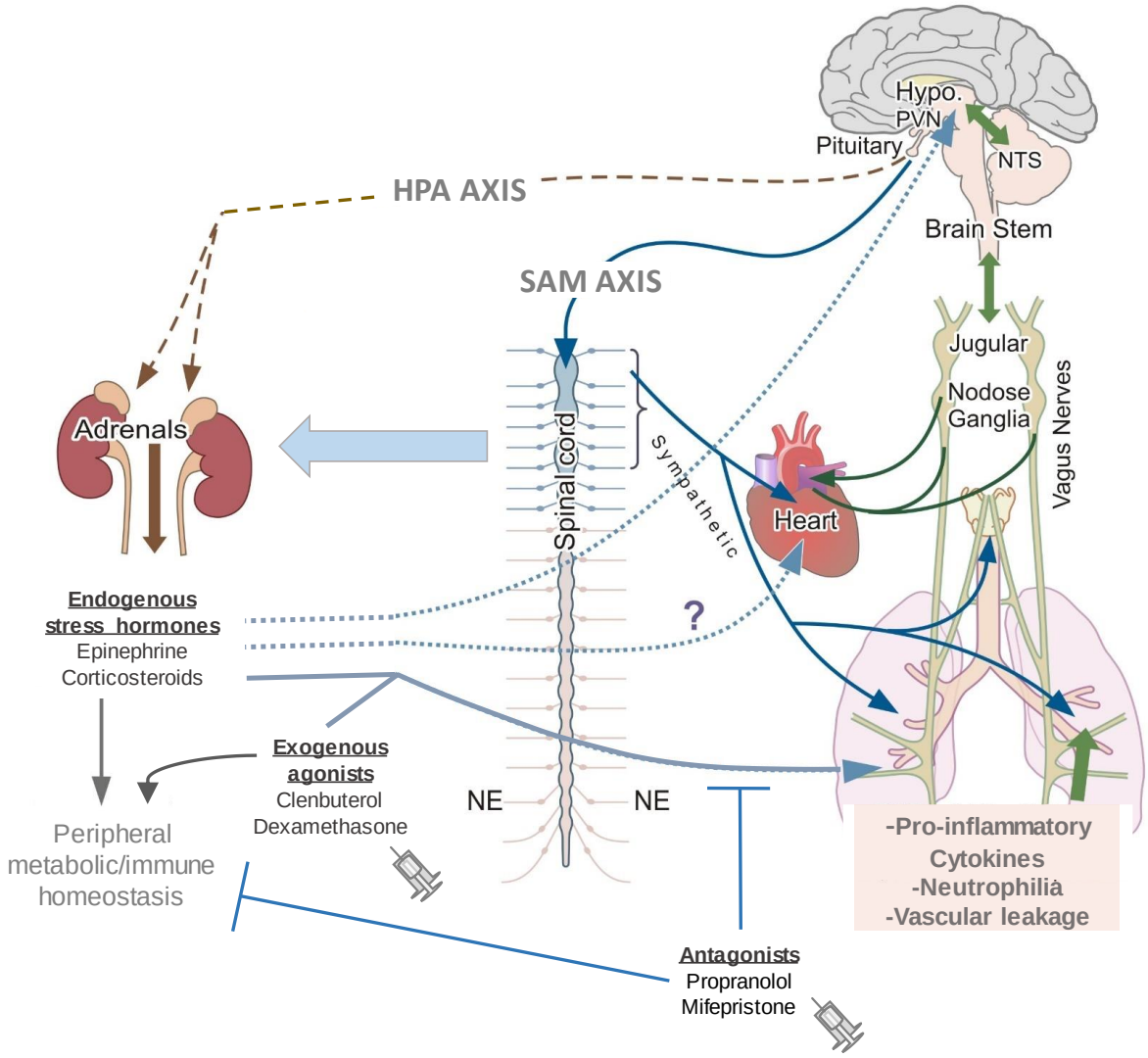



**Figure 5.1 Proposed mechanism of hormonal stress mediated pulmonary effects of ozone inhalation.** Hypo: Hypothalamus, HPA Hypothalamic-Pituitary-Adrenal: SAM: Sympathetic-Adrenal-Medullary, NE: Norepinephrine, PVN: Paraventricular Nucleus, NTS: Nucleus Tractus Solitarius. Adapted from Kodavanti, 2016.

## 5.5 Additional hypotheses

In this study, we also highlight the role of adrenal glands as a modulator of ozone-induced responses in the lung. Recently, the role of “inter-organ communication” (Droujinine and Perrimon, 2013) has gained attention. Although our paradigm involving stress hormones as important mediators of ozone-induced responses were limited to the lungs and circulation, results not included in this dissertation showed wide-spread, drug intervention-dependent metabolic modulation of ozone-induced changes. We will follow-up these studies by analyzing other relevant metabolic markers in achieved tissues such as the liver, muscle, adipose, and pancreas. Remarkably, the lung-liver axis and lung-kidney axis has been researched in the context of pulmonary infection, thus indicating that pulmonary responses are influencing or being influenced by changes occurring in other organs (Hilliard et al., 2015; Kaczmarczyk, 1994).

Finally, although we propose neuroendocrine stress response as a controlling mechanism governing pulmonary and extra-pulmonary effects induced by ozone, we cannot rule out the contribution of other novel mediators. For example, Fei et al. (2017) and Huffman et al. (2006) demonstrated that progesterone and thyroid hormone act as modulators of ozone-induced responses. Ozone-induced changes in circulating metabolites and acute phase response proteins are extensive, and the research in our laboratory includes examination of other pathways (i.e. metabolic). Undoubtedly, the extension of these findings to evaluate the action of other air pollutants seems promising. For example, acrolein, another gaseous airway irritant, has been shown to induce similar activation of this neuroendocrine stress response (Snow et al., 2017).

## 5.6 Significance and Impact

Despite significant air quality improvement during the past few decades, the associations between relatively low levels of air pollutants and detrimental health effects are still apparent (Pinault et al., 2016; Shi et al., 2016). Ozone, one of the six criteria air pollutants, is ubiquitously distributed in the atmosphere and expected to increase as a result of climate change. While this homeostatic neuroendocrine stress response can be induced by a variety of physical and psychosocial stresses, this project provides mechanistic insights on how environmental stressors can contribute to a variety of diseases through impairment of the dynamic orchestration of stress hormone-mediated immune and metabolic processes in multiple organs. Accordingly, the recognition of interactive effects between ozone exposure and non-chemical stressors will help to elucidate the mechanisms of disease susceptibility and the role of neuroendocrine stress pathways.

The impairment of the neuroendocrine system has been linked to a variety of stress-related chronic disease conditions including cardiovascular, metabolic, neurological and developmental. The contribution of environmental pollutants and psychosocial stressors as independent activators of neuroendocrine stress response opens the field for the characterization and evaluation of how shared mechanisms can cooperatively contribute to exacerbate air pollution health effects in socially disadvantaged and psychologically vulnerable populations. Thus, the assessment of psychosocial stress as an aggravating factor worsening population's responses to environmental pollutants might be an important area of future research effort. It will also be critical to understand how SAM and HPA axes interact with the serotonergic system and lead to changes in circulating steroidal and adrenergic hormones that mediate downstream immune and metabolic changes. Furthermore, since developmental programming of the HPA axis has been implicated in susceptibility of children to chronic diseases, it will be important to understand how environmental or psychosocial stressors during

critical windows of development in pregnancy might impair children's HPA programming and predispose them to exacerbated air pollution health effects.

The understanding of how endogenous stress hormones, exogenous drugs, and stress hormone receptors, which are widely targeted in the treatment of chronic pulmonary conditions such as asthma and COPD, modulate pollutant-induced lung responses will have important translational impacts, and will provide insights into the questioned effectiveness of current therapeutic approaches for pulmonary inflammatory conditions. The role of the neurohormonal stress response has not been fully integrated into the environmental toxicology field, nor in currently available adverse outcome pathways (AOP). Since stress response is a common outcome of a variety of environmental and social risk factors, this project has major implications in harmonizing the risk assessment strategy for different stressors, and it can serve as a common AOP for many environmental exposures to improve *in vitro* screening strategies for better *in vivo* predictions. Currently, efforts are under way at the EPA to incorporate neuroendocrine SAM and HPA axes activation in future AOP paradigms. Thus, the research findings from this dissertation will serve to alter the direction of the current environmental toxicology field to emphasize the consideration of a systems biology approach when examining air pollution adverse health effects.

## APPENDIX

### Supplementary Table 2.1A

| Gene Name | Gene Symbol | SHAM O3/Air | p-value | DEMED O3/Air | p-value | ADREX O3/ Air | p-value |
|---|---|---|---|---|---|---|---|
| fatty acyl CoA reductase 2 | Far2 | 4.75 | **0.00** | 2.11 | **0.00** | 1.30 | 0.30 |
| apolipoprotein L domain containing 1 | Apold1 | 4.30 | **0.00** | 1.97 | **0.00** | 1.30 | 0.31 |
| C-type lectin domain family 12, member B | Clec12b | 4.18 | **0.00** | 2.78 | **0.00** | 2.37 | **0.00** |
| peroxisomal biogenesis factor 11 alpha | Pex11a | 4.08 | **0.00** | 1.63 | **0.00** | 1.36 | 0.10 |
| solute carrier family 30 member 3 | Slc30a3 | 4.06 | **0.00** | 1.77 | **0.00** | 1.36 | 0.24 |
| cyclin-dependent kinase inhibitor 1A | Cdkn1a | 3.90 | **0.00** | 1.87 | **0.01** | 1.41 | 0.26 |
| salt-inducible kinase 1 | Sik1 | 3.80 | **0.00** | 1.37 | 0.11 | 1.36 | 0.18 |
| potassium channel, voltage gated eag related subfamily H, member 1 | Kcnh1 | 3.66 | 0.00 | 1.76 | 0.05 | 1.26 | 0.55 |
| transmembrane protein 252 | Tmem252 | 3.55 | **0.00** | 1.71 | **0.02** | 1.28 | 0.44 |
| metallothionein 2A | Mt2A | 3.55 | **0.00** | 1.85 | **0.03** | 1.56 | 0.16 |
| interleukin 1 alpha | Il1a | 3.54 | **0.00** | 1.79 | **0.01** | 1.67 | 0.06 |
| thyrotropin releasing hormone | Trh | 3.54 | **0.00** | 1.83 | **0.03** | 1.64 | 0.12 |
| LON peptidase N-terminal domain and ring finger 3 | Lonrf3 | 3.53 | **0.00** | 2.24 | **0.00** | 2.37 | **0.00** |
| FK506 binding protein 5 | Fkbp5 | 3.50 | **0.00** | 2.09 | **0.01** | 1.38 | 0.37 |
| ERBB receptor feedback inhibitor 1 | Errfi1 | 3.45 | **0.00** | 1.39 | 0.11 | 1.54 | 0.05 |
| adenosine A1 receptor | Adora1 | 3.40 | **0.00** | 1.88 | **0.02** | 1.36 | 0.38 |
| galanin/GMAP prepropeptide | Gal | 3.38 | **0.00** | 1.86 | ~1.00 | 1.11 | 0.81 |
| sphingosine kinase 1 | Sphk1 | 3.34 | **0.00** | 2.03 | **0.00** | 1.27 | 0.47 |
| cytokine like 1 | Cytl1 | 3.28 | **0.00** | 1.13 | 0.70 | 0.97 | 0.93 |
| polypeptide N-acetylgalactosaminyltransferase 10 | Galnt10 | 3.28 | **0.00** | 1.67 | **0.01** | 1.32 | 0.31 |
| interferon-related developmental regulator 1 | Ifrd1 | 3.13 | **0.00** | 1.56 | **0.00** | 1.41 | **0.03** |
| potassium calcium-activated channel subfamily M regulatory beta subunit 4 | Kcnmb4 | 3.12 | **0.00** | 1.75 | **0.03** | 1.87 | **0.03** |
| adhesion G protein-coupled receptor G2 | Adgrg2 | 3.11 | **0.00** | 1.58 | **0.02** | 1.09 | 0.80 |
| solute carrier family 25 member 33 | Slc25a33 | 3.11 | **0.00** | 1.31 | 0.37 | 1.56 | 0.14 |
| dual specificity phosphatase 10 | Dusp10 | 3.08 | **0.00** | 1.23 | 0.27 | 1.15 | 0.55 |
| semaphorin 7A, GPI membrane anchor | Sema7a | 3.07 | **0.00** | 1.45 | 0.08 | 1.46 | 0.12 |
| TNF alpha induced protein 6 | Tnfaip6 | 3.05 | **0.00** | 1.83 | **0.02** | 1.03 | 0.95 |
| regulator of G-protein signaling 16 | Rgs16 | 3.02 | **0.00** | 2.35 | **0.00** | 1.45 | 0.26 |
| interleukin 24 | Il24 | 3.01 | **0.00** | 2.10 | **0.01** | 2.51 | **0.00** |
| aspartoacylase | Aspa | 2.99 | **0.00** | 0.85 | 0.43 | 0.93 | 0.82 |
| early growth response 1 | Egr1 | 2.98 | **0.00** | 1.86 | **0.01** | 1.36 | 0.33 |
| small integral membrane protein 3 | Smim3 | 2.96 | **0.00** | 1.47 | **0.01** | 1.54 | **0.01** |
| interleukin 6 | Il6 | 2.94 | **0.00** | 1.56 | ~1.00 | 1.52 | 0.16 |
| cilia and flagella associated protein 53 | Cfap53 | 2.91 | **0.00** | 1.06 | 0.86 | 1.19 | 0.57 |
| sphingomyelin synthase 1 | Sgms1 | 2.89 | **0.00** | 1.38 | **0.02** | 1.22 | 0.25 |
| potassium voltage-gated channel interacting protein 3 | Kcnip3 | 2.89 | **0.00** | 1.48 | 0.17 | 1.15 | 0.74 |
| glycerol-3-phosphate acyltransferase 3 | Gpat3 | 2.89 | **0.00** | 1.37 | **0.05** | 1.30 | 0.19 |
| interleukin 1 receptor, type II | Il1r2 | 2.89 | **0.00** | 1.05 | 0.91 | 0.72 | 0.36 |
| tumor necrosis factor receptor superfamily, member 21 | Tnfrsf21 | 2.88 | **0.00** | 1.11 | 0.65 | 1.24 | 0.31 |
| ADAM metallopeptidase with thrombospondin type 1 motif, 9 | Adamts9 | 2.88 | **0.00** | 1.36 | 0.14 | 1.03 | 0.94 |

Values indicate the ratio of each gene expression in ozone-exposed rats to air-exposed rats for a given surgery condition. Bold values indicate significant change (adjusted $p<0.05$).

**Supplementary Table 2.1A:** Relative fold change (ozone/air) for the forty most up-regulated genes after ozone exposure in SHAM rats and their relative expression changes in DEMED and ADREX rats (1-day time point).

**Supplementary Table 2.1B**

| Gene Name | Gene Symbol | SHAM O3/Air | p-value | DEMED O3/Air | p-value | ADREX O3/ Air | p-value |
|---|---|---|---|---|---|---|---|
| G0/G1switch 2 | G0s2 | 0.26 | **0.00** | 0.56 | **0.00** | 0.57 | **0.01** |
| solute carrier family 16, member 9 | Slc16a9 | 0.27 | **0.00** | 0.64 | **0.01** | 0.82 | 0.40 |
| cilia and flagella associated protein 99 | Cfap99 | 0.29 | **0.00** | 0.83 | 0.60 | 0.77 | 0.47 |
| adhesion G protein-coupled receptor E5 | Adgre5 | 0.30 | **0.00** | 0.56 | **0.00** | 0.62 | **0.00** |
| phytanoyl-CoA 2-hydroxylase interacting protein-like | Phyhipl | 0.31 | **0.00** | 0.41 | **0.00** | 0.76 | 0.23 |
| Rho GTPase activating protein 4 | Arhgap4 | 0.32 | **0.00** | 0.63 | **0.01** | 0.55 | **0.00** |
| rhotekin 2 | Rtkn2 | 0.33 | **0.00** | 0.46 | **0.00** | 0.72 | 0.11 |
| B-cell scaffold protein with ankyrin repeats 1 | Bank1 | 0.33 | **0.00** | 0.67 | **0.01** | 1.12 | 0.63 |
| CD19 molecule | Cd19 | 0.33 | **0.00** | 0.97 | 0.94 | 0.82 | 0.57 |
| ArfGAP with coiled-coil, ankyrin repeat and PH domains 1 | Acap1 | 0.34 | **0.00** | 0.89 | 0.73 | 0.82 | 0.56 |
| leucine rich repeat containing 9 | Lrrc9 | 0.35 | **0.00** | 0.61 | 0.05 | 0.88 | 0.76 |
| leucine rich repeat neuronal 3 | Lrrn3 | 0.35 | **0.00** | 0.67 | **0.00** | 0.81 | 0.27 |
| proline rich Gla (G-carboxyglutamic acid) 3 (transmembrane) | Prrg3 | 0.35 | **0.00** | 0.58 | **0.01** | 0.72 | 0.19 |
| CD22 molecule | Cd22 | 0.36 | **0.00** | 0.67 | 0.15 | 0.66 | 0.16 |
| ATPase phospholipid transporting 10B (putative) | Atp10b | 0.36 | **0.00** | 0.55 | **0.00** | 0.76 | 0.27 |
| coiled-coil and C2 domain containing 2A | Cc2d2a | 0.36 | **0.00** | 0.90 | 0.52 | 0.83 | 0.30 |
| family with sequence similarity 65, member B | Fam65b | 0.37 | **0.00** | 0.96 | 0.89 | 0.75 | 0.21 |
| coiled-coil domain containing 142 | Ccdc142 | 0.37 | **0.00** | 0.76 | 0.40 | 0.63 | 0.14 |
| spondin 2 | Spon2 | 0.37 | **0.00** | 0.45 | **0.00** | 0.77 | 0.31 |
| receptor accessory protein 4 | Reep4 | 0.37 | **0.00** | 0.85 | 0.36 | 0.69 | **0.03** |
| dynein light chain LC8-type 1 | Dynll1 | 0.37 | **0.00** | 0.69 | **0.02** | 0.57 | **0.00** |
| coiled-coil domain containing 40 | Ccdc40 | 0.37 | **0.00** | 0.92 | 0.84 | 0.79 | 0.48 |
| leucine rich repeat containing 23 | Lrrc23 | 0.37 | **0.00** | 0.66 | 0.13 | 0.77 | 0.44 |
| coiled-coil domain containing 114 | Ccdc114 | 0.38 | **0.00** | 0.74 | 0.37 | 0.71 | 0.33 |
| adaptor-related protein complex 3, mu 2 subunit | Ap3m2 | 0.38 | **0.00** | 0.85 | 0.64 | 0.90 | 0.79 |
| similar to GLE1-like, RNA export mediator isoform 1 | RGD1565693 | 0.38 | **0.00** | 0.75 | 0.31 | 0.94 | 0.90 |
| metastasis suppressor 1-like | Mtss1l | 0.38 | **0.00** | 0.79 | 0.06 | 0.83 | 0.25 |
| dystrophin related protein 2 | Drp2 | 0.38 | **0.00** | 0.62 | **0.02** | 0.83 | 0.53 |
| bone morphogenetic protein 7 | Bmp7 | 0.38 | **0.00** | 0.80 | 0.57 | 0.84 | 0.69 |
| dispatched RND transporter family member 1 | Disp1 | 0.39 | **0.00** | 0.62 | **0.00** | 0.75 | 0.09 |
| interferon regulatory factor 4 | Irf4 | 0.39 | **0.00** | 1.05 | 0.85 | 0.82 | 0.40 |
| F-box and leucine-rich repeat protein 19 | Fbxl19 | 0.39 | **0.00** | 0.83 | 0.37 | 0.76 | 0.19 |
| coiled-coil alpha-helical rod protein 1 | Cchcr1 | 0.39 | **0.00** | 0.88 | 0.69 | 0.79 | 0.41 |
| similar to hypothetical protein MGC29761 | RGD1306233 | 0.39 | **0.00** | 0.79 | 0.51 | 0.67 | 0.24 |
| cholinergic receptor, muscarinic 2 | Chrm2 | 0.39 | **0.00** | 0.79 | 0.51 | 1.32 | 0.44 |
| mesoderm induction early response 1, family member 2 | Mier2 | 0.39 | **0.00** | 0.79 | 0.42 | 0.69 | 0.20 |
| ferredoxin reductase | Fdxr | 0.39 | **0.00** | 0.88 | 0.75 | 0.86 | 0.72 |
| transient receptor potential cation channel, subfamily M, member 6 | Trpm6 | 0.39 | **0.00** | 0.83 | 0.32 | 0.98 | 0.96 |
| mutated in colorectal cancers | Mcc | 0.39 | **0.00** | 0.65 | 0.05 | 0.70 | 0.19 |
| tubulin tyrosine ligase like 12 | Ttll12 | 0.39 | **0.00** | 0.68 | **0.03** | 0.78 | 0.25 |

**Supplementary Table 2.1B:** Relative fold change (ozone/air) for the forty most down-regulated genes after ozone exposure in SHAM rats and their relative expression changes in DEMED and ADREX rats (1-day time point).

## Supplementary Table 2.2

| A | 1-D | | | | | |
|---|---|---|---|---|---|---|
| Comparison | # of genes changed by $O_3$ | | Gene name | GO Biological Process | FE | p-value |
| | TOTAL | UP/DOWN | | | | |
| SHARED BETWEEN SHAM AND DEMED | 193 (140+ 53) | 112↑ | Adamts1,Adamts4,Adgrg2,Adora1,Aff1,Ampd3,Apold1,Aqp3,Areg,Arid5a,Bmp2, Bnip3,Casc4,Cblb,Ccdc59,Cdkn1a,Chrna7,Cirbp,Clec12b,Cry1,Csde1,Cubn, Depdc7,Disp2,Dusp1,Egr1,Ehd3,Emc7,Enc1,Esm1,Exnef,F3,Far2,Fgd4,Fkbp5, Fmo3,Fyn,Galnt10,Gclc,Gem,Higd1b,Hmg1l1,Hsd3b7,Hyal3,Ifrd1,Il1a,Il1r1,Il24, Il4r,Ipmk,Junb,Kcnh1,Kcnmb4,LOC102550391,LOC102552920,LOC103689920, LOC500475,LOC685680,Lonrf3,Lox,Maff,Malt1,Mcm8,Moxd1,Ms4a4a,Mt1,Mt2A, Mthfd2,Myc,Osmr,Pdss1,Pex11a,Pgrmc1,Plek2,Plk2,Qrich1,Rbm3, RGD1308117,RGD1560010,Rgs16,Rps27a—ps1,Rps6ka5,Rundc3b,Sdcbp2, Selp,Sema6b,Serpine1,Slc24a2,Slc25a16,Slc30a3,Slc7a5,Slco1a2,Sphk1, Srfbp1,Tfpi2,Tgfa,Timp3,Tmem178a,Tmem252,Tnfaip6,Tob1,Trh,Trmt6, Tspan11,Wee1,Wnk2,Ybx1,Ybx1—ps3,Zbed3,Zc3h12a,Zdhhc20,Zfp867 | interleukin-1-mediated signaling pathway | 62.91 | 0.0064 |
| | | | | ovulation | 35.52 | 0.0039 |
| | | | | response to temperature stimulus | 11.01 | 0.0003 |
| | | | | response to mechanical stimulus | 7.7 | 0.0088 |
| | | | | response to hypoxia | 6.21 | 0.0194 |
| | | | | response to inorganic substance | 5.73 | 0.0001 |
| | | 81↓ | Adgre5,Arhgap4,Arl15,Arnt2,Arsi,Arvcf,Atp10b,Atp13a4,Atp2b4,Banp,Bbs12, Brms1l,Ccdc124,Cdkl5,Commd1,Cpn1,Ctc1,Cyp2s1,Dach1,Dcps,Disp1, Drp2,Efcc1,Eif3e,Enkur,Fam3b,Fgf11,Fth1,G0s2,Gimap4,Gucy2g,Hs3st3b1, Hspa12a,Hsph1,Hykk,Ift140,Ikbke,Il33,Ints3,Klrk1,LOC100360575, LOC103690005,Lrrc9,Maob,Maz,Msh3,Ncs1,Otub2,P4ha1,Pcyox1l,Pdgfb, Phospho1,Phyhipl,Pla2g16,Prrg3,Radil,Rdm1,RGD1565616,RGD1566359, Rtkn2,Sdf2l1,Sgpp2,Slc16a9,Slc25a35,Slc5a1,Slx4,Sncaip,Sord,Spon2, Sstr4,Ssu72,Stbd1,Stxbp4,Taco1,Tbc1d10c,Tdrkh,Telo2,Timm8b,Tmem147, Tppp3,Xkrx | | | |
| SHARED BETWEEN SHAM AND ADREX | 180 (127+ 53) | 83↑ | Adamts1,Aff1,Ampd3,Arid5a,Arl5b,Bnip3,Btg2,Chka,Cirbp,Clec12b,Clec14a, Csde1,Csrnp1,Cxcl1,Dact2,Dnajb4,Dusp1,Edil3,Erbb3,Esm1,F2rl1,Fam76b, Gas2,Gclc,Glcci1,Hopx,Hsd3b7,Hyal3,Igf2,Il24,Il4r,Ipmk,Kbtbd11,Kcnmb4, Krt80,LOC102550391,LOC102552920,LOC103690007,LOC500475, LOC685699,Lonrf3,Lrtm2,Lyve1,Malt1,Mettl9,Moxd1,Mtmr10,Nphp3,Ogn, Paqr3,Pctp,Pdcl3,Pgrmc1,Plek2,Rbm3,Rcn2,RGD1308117,RGD1566368, Rnf39,Scgb1a1,Slc18a2,Slc25a16,Slc30a4,Slc7a5,Slco1a2,Smim3,Spry1, Spryd7,Sstr1,Timp3,Tm2d1,Tmem82,Tmprss4,Tob1,Trim2,Tspan11,Tsx, Wnt5b,Yipf4,Zbed3,Zc3h12a,Zfp503,Zfp800 | negative regulation of cellular protein metabolic process | 4.64 | 0.00708 |
| | | | | single-multicellular organism process | 2.06 | 0.0371 |
| | | 97↓ | Aasdh,Actc1,Adcy4,Adgre5,Amz1,Apbb1ip,Apip,Arhgap4,Arvcf,Atp2b4,Banp, Bin2,C5ar2,Cd180,Cd300e,Cd300lb,Cd4,Cd84,Cd8a,Cdk10,Cklf,Clec4a1, Creld2,Csf1r,Csf2ra,Csf3r,Dfnb31,Dok2,Dynll1,Efemp2,Evi2a,Ewsr1, Fam189b,Fmnl1,Ftsj1,G0s2,Gimap4,Hsph1,Ikbke,Ints3,Itgam,Itgb7,Jak3, I7Rn6,LOC100360575,LOC100364435,Lxn,Mical1,Mrgprf,Mrpl1,Ms4a7, Myo1f,Naip6,Nkd2,Nprl3,Oas3,P4ha1,Pigv,Pld4,Polr3h,Prf1,Ptpn6,Rarg, Rdm1,Rfx2,RGD1310127,RGD1564149,RGD1565222,Rpl35,Rps11, RT1—DOb,RT1—N2,RT1—T24—3,S100a10,Siglec8,Slc25a43,Slc26a6, Slpi,Slx4,Spn,Ssu72,Stip1,Stk36,Stxbp4,Syk,Tada3,Tceb2,Telo2,Tma7, Tmem229b,Tppp3,Trpm2,Tyrobp,Uap1l1,Ubfd1,Vezt,Zfyve19 | immune effector process | 7.17 | 0.0158 |
| | | | | defense response | 5.07 | 0.0001 |
| | | | | immune system process | 4 | 0.0000 |
| SHARED BETWEEN DEMED AND ADREX | 86 (53+ 33) | 53↑ | Acyp2,Adamts1,Aff1,Ampd3,Arid5a,Bnip3,Caps2,Cirbp,Cldn8,Clec12b, Csde1,Dnajb13,Dusp1,Dusp8,Esm1,Fam122a,Fv1,Gclc,Gstk1,Hsd3b7, Htr2c,Hyal3,Il24,Il4r,Ipmk,Kcnmb4,LOC102550391,LOC102552920, LOC500475,Lonrf3,Malt1,Mcmdc2,Moxd1,Nipa2,Npr3,Pgrmc1,Plek2, Ppan,Ptx3,Rbm3,RGD1308117,RT1—M6—1,Shisa4,Slc25a16,Slc7a11, Slc7a5,Slco1a2,Timp3,Tob1,Tspan11,Zbed3,Zc3h12a,Zc3h6 | | | |
| | | 33↓ | Abi3,Adgre5,Arhgap4,Arvcf,Atp2b4,Banp,Bscl2,Dnaja4,Ergic3,G0s2,Gimap4, Hsph1,Ikbke,Ints3,LOC100360575,LOC100911825,LOC102550385, LOC680121,Mrpl18,Myeov2,Nthl1,P4ha1,Pla2g15,Rdm1,Sdr9c7,Sfpq, Slc15a4,Slx4,Ssu72,Stxbp4,Telo2,Tppp3,Zfp846 | | | |
| SHARED BETWEEN SHAM, DEMED AND ADREX | 53 | 35↑ | Adamts1,Aff1,Ampd3,Arid5a,Bnip3,Cirbp,Clec12b,Csde1,Dusp1,Esm1,Gclc, Hsd3b7,Hyal3,Il24,Il4r,Ipmk,Kcnmb4,LOC102550391,LOC102552920, LOC500475,Lonrf3,Malt1,Moxd1,Pgrmc1,Plek2,Rbm3,RGD1308117,Slc25a16, Slc7a5,Slco1a2,Timp3,Tob1,Tspan11,Zbed3,Zc3h12a | organic cyclic compound catabolic process | 14.08 | 0.0322 |
| | | | | negative regulation of cellular protein metabolic process | 6.67 | 0.0434 |
| | | 18↓ | Adgre5,Arhgap4,Arvcf,Atp2b4,Banp,G0s2,Gimap4,Hsph1,Ikbke,Ints3, LOC100360575, P4ha1,Rdm1,Slx4,Ssu72,Stxbp4,Telo2,Tppp3 | | | |

| B | 1-D | | | | | |
|---|---|---|---|---|---|---|
| Comparison | # of genes changed by $O_3$ | | Gene name | GO Biological Process | FE | p-value |
| | TOTAL | UP/DOWN | | | | |
| UNIQUE TO ADREX | 250 | 53↑ | Aatk, Acox1, Aldh6a1, Ankrd39, Avpr1a, Carf, Ccnj, Cds1, Clec1a, Dnajc30, F11, Fam163a, Fut4, Gpr146, Gsta3, Hmgb3, Hmgcs1, Klf5, Klhl15, LOC100359687, LOC100909481, LOC100910178, LOC102549852, LOC498368, Lrp11, Ly6i, Mrps34, Mylip, Nqo1, Nr1d2, Pak6, Pcdhga4, Phlda1, Pkd2l2, Ppfia4, Rap2b, Rdh10, Rfk, Sema3d, Serpinb10, Spata1, Sptbn2, Stk33, Svbp, Syt6, Tfcp2l1, Trim63, Ttpa, Ube2q2, Yme1l1, Zbtb22, Zfp383, Znf750 | | | |
| | | 197↓ | Abcc3,Adat1,Adgre1,Agpat1,Alox5ap,Anapc13,Apobec1,Apol3,Arl5c,Atox1, Atp1a3,Atp5d,Atp5o,Bcdin3d,Bcl2a1,Bri3,C1qb,C1qc,C1s,C3,C3ar1,C6,Car9, Ccdc80,Ccl11,Ccl4,Cd300a,Cd300le,Cd68,Cdc5l,Cdk5rap1,Cetn2,Cfp,Clec4a3, Clic1,Clu,Cma1,Cmah,Cmya5,Col5a3,Copz2,Cox6b1,Cpa3,Cpxm1,Crip1,Cst7, Cthrc1,Ddx39a,Dhrs4,Dhrs9,Eefsec,Eno2,Enpp3,Erp29,Fam109b,Fam162a, Fancg,Fau,Fcer1a,Fcer1g,Fcgr1a,Fcgr2b,Fcrl2,Fgr,Flrt2,Fn1,Fndc5,Fntb,Folr2, Fpr3,Galm,Gas1,Gatb,Gpx1,Hck,Hprt1,Icoslg,Idh2,Igfbp3,Igfbp6,Ighg,Igip,Ikzf3, Il13ra2,Il1rl1,Itga11,Itgal,Itgb2,Jmjd6,Khdrbs3,Krt75,Lacc1,Lilrb3b,Lilrb4, LOC100360057,LOC100360679,LOC100361854,LOC100363268,LOC100363537, LOC100911851,LOC297756,LOC498555,LOC679748,LOC684797, LOC687780,Lrrc17,Lvrn,Manf,Mapk12,Mcpt1,Mcpt8l2,Med11,Mettl3,Mien1,Mkks, Mmp9,Mnda,Mob3a,Mrgprx2,Myo1g,Ncf2,Nckap1l,Ndufb4,Ndufs8,Nexn,Nfam1, Nos3,Nprl2,Nudt5,Olr1584,Pfdn1,Pgap2,Phax,Pigp,Pla2g7,Plac8,Plek,Plod2, Plxna3,Prdx5,Prss12,Ptpro,Qrfpr,Rbm14,Retnla,Retnlg,Rfx1,RGD1559482, RGD1559972,RGD1560831,RGD1561778,RGD1562402,RGD1564698, RGD1564883,Rnase4,Rogdi,Rpl13a,Rpl17,Rpl27a,Rpl31l4,Rpl34—ps1,Rpl35a, Rpl36a—ps2,Rpl41,Rpl6,Rps24,Rps3,S1pr5,Samsn1,Scn3a,Serpinf1,Sh3bgrl3, Shmt2,Siva1,Slamf8,Slc37a2,Stoml2,Taldo1,Tbxas1,Tf,Tgfbi,Thbs2,Themis2, Thy1,Timp1,Tmc6,Tmem175,Tmsb10,Tpm4,Tpsb2,Traf5,Uba52,Ucp2,Wdr34, Wdr76,Zbtb40,Zfp9 | positive regulation of type IIa hypersensitivity | 96.41 | 0.0404 |
| | | | | positive regulation of acute inflammatory response to antigenic stimulus | 51.42 | 0.0116 |
| | | | | response to lipoprotein particle | 23.8 | 0.0228 |
| | | | | mast cell activation | 22.95 | 0.0271 |
| | | | | regulation of myeloid leukocyte mediated immunity | 18 | 0.0014 |
| | | | | immunoglobulin mediated immune response | 15.43 | 0.0001 |
| | | | | | | |
| UNIQUE TO DEMED | 235 | 145↑ | Acot1,Amhr2,Anln1,Arhgap11a,Arhgap19,Aspm,Aspn,Atad2,Atad5,B3galt2,Birc3, Blm,Bora,Brip1,Btn2a2,Bub1,Camk4,Casc5,Ccl2,Ccna2,Ccne2,Cdc25c,Cdc45, Cdk1,Cenpa,Cenpf,Cenpi,Cenpw,Cep55,Chaf1a,Cit,Ckap2,Ckap2l,Cracr2a, Cstf2,Ddias,Depdc1,Diaph3,Dlgap5,Dpm2,Dtl,E2f7,Ect2,Efcab2,Esco2, Fam111a,Fanca,Fancb,Fanci,Fbxo5,Fign1,Fmo4,G2e3,Gas2l3,Gen1,Gtf2a2, Hjurp,Hmgb2l1,Hspb7,Hunk,Incenp,Iqgap3,Itga2b,Kif11,Kif15,Kif18b,Kif20a, Kif20b,Kif23,Kif2c,Kntc1,Kpna2,Lcorl,Lmnb1,LOC100359539,LOC100910017, LOC100911337,LOC100911660,LOC100911766,LOC682206,LOC691113, Lrrfip1,Mad2l1,Mastl,Mcm10,Mfap3,Mgat2,Micalcl,Mis18bp1,Mki67,Mms22l, Mybph,Mycn,Ncapd3,Ncapg,Ncapg2,Ncaph,Ndc80,Neil3,Nod2,Nsl1,Nusap1, Pask,Pbk,Pcdh17,Pcdhb20,Peg12,Per2,Per3,Phgdh,Plk1,Plk4,Pole,Pole2, Ppef1,Ptger4,Pttg1,Rab43,Racgap1,Rad51,RGD1305807,Rgs14,Ror2,Rslcan18, Rsrc2,Sgol1,Shcbp1,Spag5,Stil,Tacc3,Tmem200a,Top2a,Tpx2,Trdmt1,Trim59, Ttk,Txk,Ube2c,Uhrf1,Vom2r44,Xxylt1,Zbtb25,Zfp367,Zfp446,Zfp707 | regulation of ubiquitin protein ligase activity | 53.93 | 0.0096 |
| | | | | centromere complex assembly | 50.55 | 0.0006 |
| | | | | mitotic cytokinesis | 40.44 | 2E-08 |
| | | | | mitotic spindle assembly | 38.06 | 0.0376 |
| | | | | attachment of spindle microtubules to kinetochore | 38.06 | 0.0376 |
| | | | | regulation of mitotic sister chromatid separation | 35.51 | 6E-08 |
| | | 90↓ | Acoxl,Adcy1,Alkbh2,Arl3,Asb8,Atp6v0b,Atp6v0e2,Bmyc,Bst1,Cbx1,Commd6, Coq10a,Cxcl17,Cxcr1,Cyp2b1,Cyp39a1,Ddah2,Defb3,Dhx34,Ebpl,Fam63a,Flot1, Fmo1,Fmo5,Gabarap,Gjb1,Gng8,Gpatch11,Gpbp1l1,Gpt,Gpx4,Grin3b,Hnrnpm, Klf2,Kmt2c,LOC100909441,LOC100910957,LOC102549158,LOC102553099, Map7d3,Mfsd8,Myg1,Nek11,Nrg1,Olfm1,Parp2,Pdpn,Plp2,Pltp,Pmvk,Pnpla6, Pop5,Prdm6,Prom2,Rab38,Rfx8,RGD1304587,RGD1559962,RGD1564463, Rufy1,S100a6,Scimp,Sec11c,Sigirr,Slc39a2,Slc39a4,Slc44a3,Slc52a3,Slc9a2, Smad6,Snx1,Sox18,St7l,Tango6,Tapbp,Tmem54,Tnfrsf8,Trem3,Ttc32,Uckl1, Uqcc2,Wbscr17,Wdr86,Wipf2,Zbtb7b,Zdhhc1,Zdhhc23,Zfp2,Zfp523,Zscan29 | | | |

| C | 2-D | | | | | |
|---|---|---|---|---|---|---|
| Comparison | # of genes changed by $O_3$ | | Gene name | GO Biological Process | FE | P-value |
| | TOTAL | UP/DOWN | | | | |
| SHARED BETWEEN SHAM AND ADREX | 26 | 6↑ | Hmgcs2,Lonrf3,Pot1b,RGD1563888,Rnf39,Zc3h6 | | | |
| | | 19↓ | Asb8,Ddx58,Dmbt1,Herc6,Ifi44,Irgm,Itga6,LOC100911104,LOC102550385, Lztr1,MGC108823,Oas2,Rbm14,Rsad2,Sfpq,Tapbp,Trappc1,Usp18,Zcchc9 | | | |
| | | 1↑↓or↓↑ | 42614 | | | |

| D | 2-D | | | | | |
|---|---|---|---|---|---|---|
| Comparison | # of genes changed by $O_3$ | | Gene name | GO Biological Process | FE | P-value |
| | TOTAL | UP/DOWN | | | | |
| UNIQUE TO ADREX | 74 | 36↑ | Acot8,Akap7,Apoe,Atp5l,Brd2,Ccnl1,Csnk1g2,Dazap1,Eri3,Fads2,Fbxo6, Gigyf1,Gltscr2,Iffo1,Kdm6b,Ldlrap1,LOC100362027,LOC100362987,Mettl7a, Nmnat3,Ostf1,Pink1,Pusl1,Ranbp3l,Rpl26—ps2,Rpl6—ps1,Rps16,Rps29, RT1—CE6,Serpinb10,Sla,Snhg11,Svbp,Svep1,Tbc1d16,Uba52 | | | |
| | | 38↓ | Bag5,Bak1,Cbx5,Cenpn,Copz1,Cystm1,Dnajc11,Drg1,Ficd,Ggact,Kcne2,Klf2, Lmf1,LOC100361008,LOC314140,Ndufc1,Pigc,Plp2,Ppp1r26,Prc1,Rad1,Rbx1, Rcsd1,RGD1302996,RGD1310769,RGD1560341,Rgmb,S100a6,Smim8,Stk32a, Sumf2,Thbs3,Tmem206,Tmem59,Txndc9,Vdac3,Wfdc2,Zbtb7b | | | |

**Supplementary Table 2.2:** Significantly changed genes by ozone in DEMED and ADREX rats that were shared or unique at day 1 (1-D; A and B, respectively) and at day 2 (2-D; C and D, respectively). Values indicate the number of genes changed by ozone with their respective direction (up ↑ or down ↓ regulation, also genes which changed the direction of regulation are indicated as ↑↓ or ↓↑). Each group of genes was assessed for the determination of overrepresented biological processes. Both fold enrichment (FE) and p-values are listed. Shared and unique groups of genes in the figure 1 Venn diagram were analyzed to determine likely link to specific function or pathway. A Panther overrepresentation test was performed for each group of genes and the outcome and significance are provided in the supplementary materials table 2 A, B, C, and D. Gene names were upload to the webpage of Panther classification system (http://www.pantherdb.org/). The annotation database used was GO Ontology (released 2017-02-28) with *Rattus Novergicus* database reference list. The annotation used was "GO biological process complete". Significantly overrepresented pathways ($p<0.05$) were calculated using Bonferroni correction for multiple testing. Fold enrichment (FE) was calculated as the ratio between the expected number genes and the actual number of genes belonging to any particular biological process. The six pathways with the higher FE values for each group of genes were included in the table. If no processes were over-represented in a given gene group, the respective cell in the table was left empty.

**Supplementary Table 2.3**

| Canonical Pathway | ACTIVATION SCORE | | |
|---|---|---|---|
| | SHAM $O_3$ / SHAM AIR | DEMED $O_3$ / DEMED AIR | ADREX $O_3$ / ADREX AIR |
| Ephrin Receptor Signaling | 2.65 | 0.00 | 0.24 |
| ERK5 Signaling | 2.40 | 1.90 | 1.90 |
| Acute Phase Response Signaling | 2.31 | 0.00 | -0.78 |
| VDR/RXR Activation | 2.12 | 0.00 | 0.00 |
| 14-3-3-mediated Signaling | 2.11 | 0.00 | 0.00 |
| PCP pathway | 1.89 | 0.00 | 0.00 |
| Actin Nucleation by ARP-WASP Complex | 1.88 | 0.00 | 0.00 |
| Ceramide Signaling | 1.76 | 0.00 | -1.60 |
| p38 MAPK Signaling | 1.76 | 2.00 | 0.26 |
| CD27 Signaling in Lymphocytes | 1.70 | 0.00 | -1.13 |
| NRF2-mediated Oxidative Stress Response | 1.68 | 0.26 | 0.00 |
| Agrin Interactions at Neuromuscular Junction | 1.63 | 0.00 | 0.00 |
| nNOS Signaling in Neurons | 1.63 | 1.00 | 0.00 |
| Gαi Signaling | 1.46 | 0.00 | 0.00 |
| Apoptosis Signaling | 1.44 | 0.00 | 1.29 |
| Ephrin B Signaling | 1.41 | 0.00 | 0.00 |
| Hypoxia Signaling in the Cardiovascular System | 1.41 | 0.00 | -0.45 |
| PI3K/AKT Signaling | 1.41 | -0.24 | -1.40 |
| Cell Cycle Regulation by BTG Family Proteins | 1.26 | 2.00 | 0.00 |
| PTEN Signaling | 1.26 | 0.00 | 0.24 |
| P2Y Purigenic Receptor Signaling Pathway | -1.95 | 0.00 | 0.26 |
| Macropinocytosis Signaling | -2.00 | 0.00 | 1.26 |
| PEDF Signaling | -2.00 | 0.00 | -0.26 |
| Fc Epsilon RI Signaling | -2.06 | 0.00 | 0.00 |
| Growth Hormone Signaling | -2.12 | 0.00 | 0.90 |
| Role of NFAT in Regulation of the Immune Response | -2.12 | 0.00 | -0.73 |
| IL-9 Signaling | -2.13 | 0.00 | 0.00 |
| Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses | -2.14 | 0.00 | -1.21 |
| Role of NFAT in Cardiac Hypertrophy | -2.17 | 0.00 | 0.00 |
| VEGF Family Ligand-Receptor Interactions | -2.20 | 0.00 | 0.28 |
| Type II Diabetes Mellitus Signaling | -2.24 | 0.00 | -0.50 |
| FcγRIIB Signaling in B Lymphocytes | -2.27 | 0.00 | 0.00 |
| TREM1 Signaling | -2.27 | 0.00 | -2.89 |
| Neuropathic Pain Signaling In Dorsal Horn Neurons | -2.29 | 0.00 | 0.00 |
| Glioma Signaling | -2.38 | -0.83 | 0.50 |
| Tec Kinase Signaling | -2.39 | 0.00 | -0.89 |
| ErbB4 Signaling | -2.47 | 0.00 | 0.58 |
| eNOS Signaling | -2.48 | 0.00 | 0.00 |
| NF-κB Activation by Viruses | -2.59 | 0.00 | -1.15 |
| iCOS-iCOSL Signaling in T Helper Cells | -2.92 | 0.00 | -1.21 |

The activation scores for canonical pathways were ranked high to low. Up-regulation (>0) and down-regulation (<0) are shown for significant changes (p value < 0.05). Unchanged activation score is indicated as zero.

**Supplementary Table 2.3.** Canonical pathways that were up/down regulated by ozone in SHAM but not changed or reversed in DEMED or ADREX rats (1-day time point).

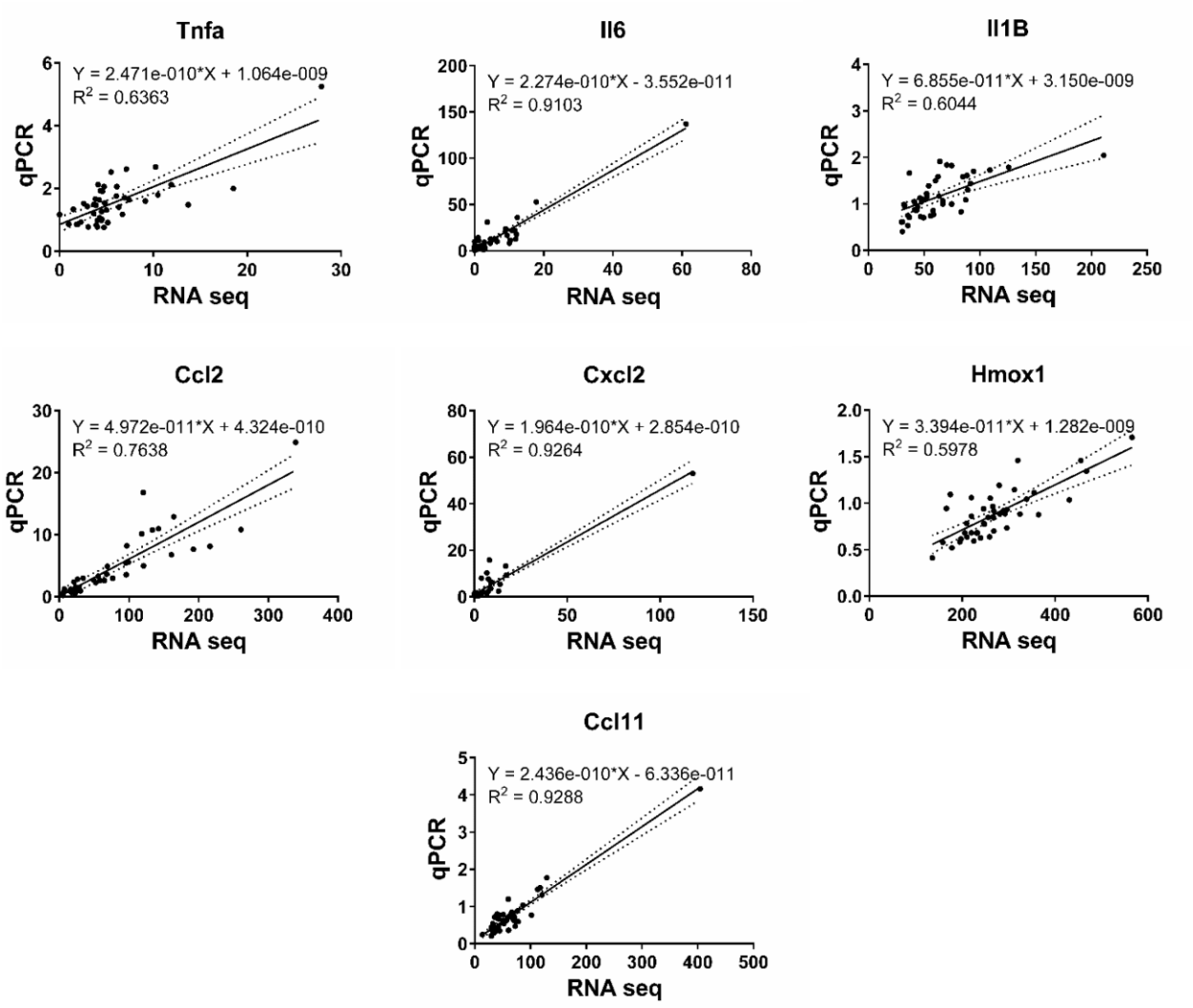
**Supplementary Table 2.4**

| Canonical Pathway | ACTIVATION SCORE | | |
|---|---|---|---|
| | SHAM $O_3$ / SHAM AIR | ADREX $O_3$ / ADREX AIR | Δ SHAM ADREX |
| Ceramide Signaling | 1.76 | -1.60 | **3.36** |
| Acute Phase Response Signaling | 2.31 | -0.78 | **3.09** |
| CD27 Signaling in Lymphocytes | 1.70 | -1.13 | **2.83** |
| PI3K/AKT Signaling | 1.41 | -1.40 | **2.81** |
| Ephrin Receptor Signaling | 2.65 | 0.24 | **2.41** |
| VDR/RXR Activation | 2.12 | 0.00 | **2.12** |
| 14-3-3-mediated Signaling | 2.11 | 0.00 | **2.11** |
| PCP pathway | 1.89 | 0.00 | **1.89** |
| Actin Nucleation by ARP-WASP Complex | 1.88 | 0.00 | **1.88** |
| Hypoxia Signaling in the Cardiovascular System | 1.41 | -0.45 | **1.86** |
| 4-1BB Signaling in T Lymphocytes | 0.63 | -1.13 | **1.77** |
| NRF2-mediated Oxidative Stress Response | 1.68 | 0.00 | **1.68** |
| IL-22 Signaling | 0.33 | -1.34 | **1.67** |
| Agrin Interactions at Neuromuscular Junction | 1.63 | 0.00 | **1.63** |
| nNOS Signaling in Neurons | 1.63 | 0.00 | **1.63** |
| HMGB1 Signaling | 1.11 | -0.45 | **1.56** |
| Toll-like Receptor Signaling | 0.94 | -0.58 | **1.52** |
| p38 MAPK Signaling | 1.76 | 0.26 | **1.50** |
| Gαi Signaling | 1.46 | 0.00 | **1.46** |
| Colorectal Cancer Metastasis Signaling | 0.00 | -1.46 | **1.46** |

Positive activation scores indicate up-regulation while negative values indicate down-regulation of any given pathway. Not significant or unchanged activation score is indicated as zero. Values in third data column are ranked by the subtraction of SHAM and ADREX to highlight the pathways which were up-regulated by ozone in SHAM rats, but not changed or even inhibited by ozone in ADREX rats.

**Supplementary Table 2.4.** Activation score for canonical pathways upregulated (>0) by ozone in SHAM rats but reduced or unchanged by ozone in ADREX rats (1-day time point).

**Supplementary Figure 2.1**




Forty-four samples regardless of treatment and exposure were correlated to test if both methods provided comparable results. $R^2$ and equation values are indicated for each graph.

**Supplementary Figure 2.1:** Correlation between qPCR (Ct) and RNAseq. (Normalized counts) for selected genes.

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