OZONE INDUCES SYSTEMIC METABOLIC DERANGEMENT THROUGH NEURONAL STRESS MECHANISMS

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ABSTRACT

Desinia B. Miller: Ozone Induces Systemic Metabolic Derangement Through Neuronal Stress Mechanisms
(Under the direction of Urmila Kodavanti)

Diabetes is reported to be the seventh leading cause of death in the United States with 90% of the cases characterized as type II diabetes. The conventional risk factors associated with diabetes are genetics, sedentary lifestyle, and high fat/high caloric diet. However, recently air pollution, such as ground level ozone, has also been postulated to contribute to diabetes, but there is limited scientific evidence. Ground level ozone is produced through the interaction of volatile organic compounds, nitric oxides and ultraviolet radiation. Ozone is known to cause pulmonary injury/inflammation but its link to peripheral metabolic effects have not been characterized. Through inhalation studies involving humans and rodent models, we examined if and how ozone exposure may change metabolism and contribute to insulin resistance, which can lead to type II diabetes. In our rodent studies, we observed that ozone exposure increases blood glucose, inhibits insulin secretion and widely affects glucose, protein and lipid metabolism. Our clinical ozone exposure study shows that humans present similar effects to rodents, especially in lipid metabolism. These types of changes over time have been linked to insulin resistance. Through surgical intervention, we also demonstrate that adrenal-derived stress hormones are essential for these ozone-induced metabolic and pulmonary effects. Further, our data indicates that these acute ozone-induced pulmonary, stress and metabolic effects persist during weekly episodic exposure subchronic exposure, but is reversible if the exposure discontinues for one week.

No insulin resistance is observed in liver or muscle after subchronic ozone exposure while a decrease in β-cell insulin secretion. Overall, these data provide insight into the mechanism of how ozone may impair systemic metabolic homeostasis through a hormonal stress response without producing peripheral insulin resistance after subchronic exposure.

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PREFACE

Explanation of my contribution to each chapter and the acknowledgement of other contributors:

Chapter 1: I was the sole author of the Introduction. The text was reviewed and revised by Urmila Kodavanti.

Chapter 2: This chapter was reprinted from Miller D.B., Karoly E.D., Jones J.C., Ward W.O., Vallanat B.D., Andrews D.L., Schladweiler M.C., Snow S.J., Bass V.L., Richards J.E., Ghio A.J., Cascio W.E., Ledbetter A.D., Kodavanti U.P., : "Inhaled Ozone (O_3) Induces Changes in Serum Metabolomic and Liver Transcriptomic Profiles in Rats", *Toxicol Appl Pharmacol*. 2015. 286, 65-79. I conceived the hypothesis and study design, assisted in or completed the sample analysis, and wrote the manuscript. Ed Karoly and Janice Jones from Metabolon INC .performed the metabolomic assessment of serum samples and statistical analysis. Beena Vallanat performed liver transcriptomic analysis, while William Ward assisted with the statistical analysis of transcriptomic data. Mette Schladweiler assisted with the necropsies and sample collection. Samantha Snow and Virginia Bass helped with metabolic endpoints. Judy Richards helped with analysis of bronchoalveolar lavage fluid (BALF) and serum lipids. Wayne Casio and Andrew Ghio reviewed and revised the manuscript. Allen Ledbetter performed and monitored exposures. Urmila Kodavanti was the senior author and oversaw the study design, data analysis and interpretation, and manuscript preparations.

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demedullation and adrenalectomy surgeries. Samantha Snow assisted with the surgeries and revision of this manuscript. Mette Schladweiler organized the necropsies and assisted with tissue and blood sample collection. Judy Richards assisted with analysis of BALF and serum lipids. Andrew Ghio reviewed and revised the manuscript. Allen Ledbetter performed and monitored all air and ozone exposures and performed whole body plethysmography on animals. Urmila Kodavanti was the senior author and oversaw the study design, data analysis and interpretation, and manuscript preparation.

Chapter 5: The findings in this chapter are unpublished. I conceived the hypothesis and study design, and assisted with all sample analysis. Samantha Snow and Andres Henriquez helped with metabolic endpoints. Mette Schladweiler organized the necropsies and assisted with tissue and blood sample collection. Judy Richards assisted with analysis of BALF and serum lipids. Allen Ledbetter performed and monitored all exposure chambers. Urmila Kodavanti was the senior author and oversaw the study design, data analysis and interpretation, and manuscript preparation.

Chapter 6: I was the sole author of the Discussion Chapter. The text was reviewed and revised by Urmila Kodavanti.

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1-D	One Day
2-D	Two Day
Aco-2	Aconitase-2
ACTH	Adrenocorticotropic Hormone
ADREX	Total Bilateral Adrenalectomy
AgRP	Agouti-Related Peptide
AKT	Protein Kinase B
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
APD	Action Potential Duration
AUC	Area Under The Curve
BCAA	Branched Chain Amino Acids
BN	Brown Norway Rat
CAA	Clean Air Act
CaMKII	Calmodulin Kinase II
CNS	Central Nervous System
CRH	Corticotrophin Releasing Hormone
CV	Cardiovascular
DEMED	Adrenal Demedullation
DAVID	Database for Annotation Visualization and Integrated Discovery
DEG	Differentially Expressed Genes
EI	Electrospray Ionization

ELF	Epithelial Lining Fluid
EPA	Environmental Protection Agency
ER	Endoplasmic Reticulum
FA	Filtered Air
FFA	Free Fatty Acids
GTT	Glucose Tolerance Test
HDL-C	High Density Lipoprotein- Cholesterol
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HPA	Hypothalamus Pituitary Adrenal
HRV	Heart Rate Variability
IL-1β	Interleukin-Beta
IL-6	Interleukin-6
IPA	Ingenuity Pathway Analysis
I.P.	Intraperitoneal
IRS	Insulin Receptor Substrate
ITT	Insulin Tolerance Test
JNK	C-Jun-NH2-terminal kinase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Locus Coeruleus
LDL-C	Low Density Lipoprotein -Cholesterol
LIMS	Laboratory Information Management System
LIT	Linear Ion Trap
LTQ	Linear Trap Quadrupole

MANOVA	Multiple Analysis of Variance
NAAQS	National Ambient Air Quality Standards
NOD	Nucleotide-Binding Oligomerization Domain
NLR	NOD-like Receptors
NO	Nitric Oxide
NPY	Neuropeptide Y
NTS	Nucleus Tractus Solitarus
O_3	Ozone
PCR	Polymerase Chain Reaction
PenH	Enhanced Pause
PFKB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1
PM	Particulate Matter
PM_{10}	Coarse Particulate Matter
$PM_{2.5}$	Fine Particulate Matter
$PM_{0.1}$	Ultrafine Particulate Matter
PNS	Parasympathetic Nervous System
Ppargc1a	Peroxisome Proliferator Activated Receptor Gamma c1a
PPM	Parts Per Million
PVN	Paraventricular Nucleus
PTT	Pyruvate Tolerance Test
RAR	Rapidly Adapting Stretch Receptors
RI	Retention Time/Index
ROS	Reactive Oxygen Species

SAR	Slowly Adapting Receptors
RSD	Relative Standard Deviation
SHAM	Sham Surgery
SNS	Sympathetic Nervous System
TCA	Tricarboxylic Acid
TLR	Toll-Like Receptor
TRPA1	Transient Receptor Ankrin 1
TRPV1	Transient Receptor Vallinoid 1
TNF-α	Tumor Necrosis Factor α
TID	Type 1 Diabetes
TIID	Type 2 Diabetes
UF	Ultrafine
UPLC	Ultra-Performance Liquid Chromatography
UPR	Unfolded Protein Response
VOC	Volatile Organic Compounds
WBC	White Blood Cell
WKY	Wistar Kyoto Rat
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Air pollution health effects: historical perspective

For over a century, atmospheric pollution has been recognized to impact human health. In 1930, a dense fog, composed of various concentrated air pollutants, filled the Meuse Valley in Belgium, which resulted in 60 deaths and severely impaired the population with adverse respiratory effects over three days (Nemery *et al.*, 2001). The respiratory signs and symptoms—dyspnea, pulmonary edema, laryngeal irritation—were diminished once the fog dissipated. Another key air pollution event occurred in a small industrial town, Donora, Pennsylvania, where over 50% of the population of 14,000 displayed respiratory function decrements as a result of thermal inversion that confined incomplete industrial combustion products near the ground (American Thorarcic Society, 1996). Similarly, the Great Smog of London occurred in 1952 where anticyclone wind conditions accumulated particulate and sulfur pollutants, from coal burning and vehicular emissions, into a fog that killed 4,000 people over three days (American Thoracic Society, 1996). The death toll of this severe air pollution episode rose to over 12,000 in subsequent weeks. These three tragic events—Meuse Valley, Belgium, Donora, Pennsylvania, and London, England—brought awareness of the potential burden of high levels of air pollution, incited epidemiological studies on adverse health effects, and prompted new emission regulations.

1.2 Air pollution regulation and human health

The World Health Organization (WHO) reported in 2012 that there were 7 million premature deaths worldwide associated with air pollution (World Health Organization, 2012). Increases in air pollution have been linked to adverse health effects as evidenced by increases in hospitalization, morbidity, and mortality for respiratory and cardiovascular diseases in humans (Schwela, 2000). In 1970, the United States Environmental Protection Agency (US EPA) mandated the Clean Air Act (CAA) to reduce air pollution emissions by implementing air standards and developing mitigation strategies and practices (EPA, 2010). Based on scientifically-determined guidelines, the US EPA established the National Ambient Air Quality Standards (NAAQS) to protect the health of the general and most susceptible populations from six criteria air pollutants: carbon monoxide, sulfur dioxide, nitrogen oxides, lead, particulate matter (PM) and ozone (EPA, 2011). Of the six common pollutants, ambient PM and ozone are the most ubiquitous and have been linked to majority of health outcomes.

1.3 PM pollution:

The primary respirable ambient PM are derived from natural and man-made sources: anthropogenic activities, incomplete combustion of fuels, vehicular emissions, unpaved roads, biomass burning, wild fires, volcanic eruptions, etc (Poschl, 2005). Ambient PM are composed of a complex mixture of solid particles, which can vary broadly in shape, size and chemical composition based on emission sources, geographical location, and meteorological conditions (i.e. season and weather). Respirable PM are classified based on their aerodynamic diameters: coarse particles (PM_{10}: 2.5 μm--10 μm), fine particles ($PM_{2.5}$: 2.5 μm-0.1μm), and ultrafine particles (UFP: <0.1μm). The US EPA currently designated

NAAQS for $PM_{2.5}$ and PM_{10} are 35 $\mu g/m^3$ and 150 $\mu g/m^3$, respectively, averaged over a 24 hour period (EPA, 2012). Common constituents of PM include inorganic and organic substances: sulfate, nitrate, ammonium, black and elemental carbon, sea salt, mineral dust, pollen, and plant components (Poschl, 2005). Gaseous pollutants are in dynamic interaction with PM surface chemicals and often form secondary particles in the atmosphere through condensation and nucleation (Poschl, 2005). Ambient particles can also undergo photochemical aging or transformation in the atmosphere by coagulation (the combination of more than two particles) (Poschl, 2005). This process in the air can increase gaseous pollutants, such as ozone.

1.4 Ozone

Ozone is a triatomic oxygen molecule and is a strong oxidizing agent. Stratospheric ozone, located in the upper atmosphere, protects the earth from harmful sun rays, while tropospheric ozone, the closest atmospheric layer to the earth's surface, can have harmful effects on living organisms, including plants and animals, due to its high oxidative potential. Ground level ozone is formed through a secondary photochemical reaction of ultraviolent radiation with ambient nitrogen oxides (NO) and volatile organic compounds (VOC). These precursor chemicals can be produced by naturally occurring processes, agricultural activities, anthropogenic fuel combustion, and vehicular and industrial emissions (Ciencewicki *et al.*, 2008). Due to the need of ultraviolent radiation, ozone concentrations can fluctuate during various seasons and throughout the day. Levels are generally high during daylight hours and in the summer with warmer temperatures. The current US EPA NAAQS for ozone is set at 0.070 parts per million (ppm) averaged over an eight hour period but is currently under review for revision of current standards (EPA, 2012).

1.5 Air Pollution and respiratory effects

The respiratory system is the primary and initial contact to inhaled pollutants, and as a result, bears many of the toxic effects caused by air pollutants. Over the years, numerous epidemiological studies have shown a positive association between air pollution exposure and increased hospitalizations, morbidity, and mortality related to respiratory conditions in humans (Schwartz, 1996; Verhoeff *et al.*, 1996; Vigotti *et al.*, 1996; Burnett *et al.*, 1997; Laumbach and Kipen, 2012). PM and ozone have also been observed to exacerbate respiratory abnormalities in people with pre-existing conditions such as asthma, pneumonia, and chronic obstructive pulmonary disease (Trasande and Thurston, 2005; Kim *et al.*, 2013a; Kim *et al.*, 2013b). Controlled acute human exposures and animal studies, especially with ozone and PM, have demonstrated decrements of lung function, airway hyperresponsiveness, lung injury, and inflammation (Schwela, 2000; Triantaphyllopoulos *et al.*, 2011; Laumbach and Kipen, 2012).

Due to the high oxidation potential of particulate and gaseous pollutants, oxidative stress is considered the primary driver of pollutant-induced pulmonary injury and inflammation. Inhalation studies have demonstrated that gaseous and particulate pollutants can increase reactive oxygen species (ROS) in the lung that can: (1) alter DNA, protein and lipids, (2) stimulate endogenous production of ROS, and (3) induce inflammatory pathways (Ciencewicki, et al., 2008) **(Figure 1.1)**. It is hypothesized that when antioxidant protective mechanisms are overwhelmed, injury to pulmonary cells, including epithelial, endothelial and immune cells ensues following pollutant exposure. Additionally, the epithelial lining fluid (ELF), which serves as a protective barrier at the air-liquid interphase, can also undergo chemical modifications and injure respiratory cells. Air pollution-associated transition metals

present on the surface of inhaled particles can participate in generation of free radicals though their interaction with macromolecules in the ELF and cellular components, leading to increased oxidative stress and initiating inflammation (Rahman, 2007).

Lipid peroxidation is considered the primary consequence of pollutant-induced damage, especially following ozone exposure. In humans and rodents, ozone has been shown to increase the reactive lipid byproduct 4-hydroxy-2-nonenal (Hamilton *et al.*, 1996; Kirichenko *et al.*, 1996) in the lung, likely involving lipid-rich airway lining fluid and epithelial cells, which has been implicated in airway injury, inflammation and remodeling (Keller *et al.*, 1997; Suc *et al.*, 1998; Tsukagoshi *et al.*, 2002)). Ozone has also been shown to modify protein structure and stability through its interaction with terminal amino side chains and formation of protein cross-links and cleavage, which can result in perturbation of protein structures (Ciencewicki, et al., 2008). Thus, ozone and PM exposures have been implicated in protein, lipid and DNA damage (Prahalad *et al.*, 2001; Cheng *et al.*, 2003).

Upon encounter of oxidatively-modified lung lining components or other particulate pollutants, activated macrophages and lung epithelial cells can express proinflammatory cytokines, which are involved in the recruitment of immune cells — neutrophils, eosinophils, macrophages — at the site of injury in the lung (Ciencewicki, et al., 2008). Air pollutants, including ozone, are thought to activate alveolar macrophages by directly interacting with cells or through secondary reactive intermediates. The inflammatory mechanisms involve activation of Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) on monocytes (Becker *et al.*, 2002; Bauer *et al.*, 2012). TLR and NLR receptors are a subset of pattern recognition proteins that are essential for the activation of defense mechanisms against foreign materials and the activation of the innate

immune response (Bauer, et al., 2012). Activation of TLR and NLR pathways are known to trigger the activation of c-Jun-NH2-terminal kinase (JNK) and degradation of IKK-β allowing translocation of nuclear factors such as the nuclear factor kappa-light-chain-enhancer of activated β cells (NFκB) that leads to the upregulation of many pro-inflammatory genes and cytokines (tumor necrosis factor tumor necrosis factor(TNF)-α, Interleukin (IL)-1β, IL-6, resistin, and more) (Huang *et al.*, 2009). These pro-inflammatory mediators can activate other local macrophages and secrete chemokines that initiate the recruitment of additional monocytes and neutrophils to the site, leading to an innate immune response. Persistence of exposure could lead to chronic inflammatory conditions that are implicated in many pulmonary diseases. Although local oxidative stress leading to inflammation is noted as the main contributor to pollutant-induced pulmonary effects, relatively less is known about potential endogenous contributors (hormones, metabolites) in pollutant-mediated respiratory effects.

The pulmonary airways are densely innervated with sensory afferent vagus fibers that carry signals through nodose and jugular ganglion, which send second-order neurons to the other areas of the brain through the brainstem. This provides a communication route between the bronchopulmonary system and the central nervous system. The neuronal circuitry regulates respiratory function and its synchronization/coordination with the cardiovascular system. There are three types of sensory neurons: C-fibers, neurons with rapidly adapting stretch receptors (RAR), and neurons with slowly adapting stretch receptors (SAR) (Carr and Undem, 2003). RAR and SAR fibres are classified as bronchopulmonary mechanosensory receptors, while chemo- and thermo-sensory C-fibers densely innervate upper and deep lung airways (Carr and Undem, 2003). These fibers extend to the brain stem

and the nucleus of solitarius tract (NTS) of the central nervous system (CNS), and stimulate vagal-mediated pulmonary refluxes and cardiopulmonary tone that control breathing and cardiac rhythms. Dysfunction of these neurons has been linked to lung function decrements including excessive coughing, bronchospasm, mucus secretion, and altered airway muscle tone (Lee and Pisarri, 2001; Canning, 2010). A number of neuroactive substances are also released at the nerve terminals in the lung and in the brain, such as substance P and neurokines, which can act in an autocrine or paracrine manner to produce local or distant effects in the brain. Transient receptor ankyrin (TRPA1) and transient receptor vanilloid (TRPV1), located in the vicinity of each other on C-fibers, detect noxious stimuli such as chemical, pH changes, osmolarity changes and heat. It is hypothesized that components of pollutants, including ozone, may interact with these receptors on pulmonary C-fibers to stimulate the central nervous system (CNS) and mediate lung refluxes; however, the specific mechanisms are not clearly understood (Taylor-Clark and Undem, 2010). Ozone has also been shown to stimulate RARs but is considered to be an indirect effector (Taylor-Clark and Undem, 2010). Thus, it is likely that pollutants may utilize C-fibers to alter pulmonary and cardiac function as well as to induce other neuronal pathways, but the mechanisms remain unclear.

1.6 Air pollution and cardiovascular effects

Prior to the 1990s, air pollution studies primarily focused on the respiratory system. High level occupational exposures such as asbestos, agricultural dusts, metal fumes, and silica were shown to cause pulmonary diseases such as asbestosis, metal fume fever, silicosis, and pneumoconiosis. It was not until the late 20th century when ambient levels of pollution were linked to adverse cardiovascular health effects. The cardiovascular system and the lung

are hemodynamically and neurohumorally-linked, and therefore, any effect on one is likely to impact the other organ. For example, changes in heart rate and heart rate variability (HRV) have been implicated in altered alveolar-arterial oxygen transfer (Sin *et al.*, 2010). Ozone exposure was demonstrated to increase heart rate and blood pressure along with inefficient pulmonary-gas exchange in healthy human subjects (Gong *et al.*, 1998). Many epidemiological, clinical and experimental studies have emerged which show that PM and ozone can induce alterations in HRV, conductance abnormalities, vasoconstriction, endothelial dysfunction and thrombosis (Delfino *et al.*, 2008; Pope, 2009; Brook *et al.*, 2010b; Farraj *et al.*, 2011), which are implicated in the rise of cardiac-related hospital admissions due to hypertension, myocardial infarctions, arrhythmias, congestive heart failure and vascular diseases such as atherosclerosis (Poloniecki *et al.*, 1997; Pope *et al.*, 2004; Pope *et al.*, 2009).

1.7 Cardiac effects

A number of cardiac effects have been linked to air pollution exposure in epidemiological and clinical studies including changes in HRV, hypertension, arrhythmia, myocardial infarction and ischemia. HRV is a clinical measure of variation in time interval between heartbeats and signifies changes in the autonomic nervous system (deBoer *et al.*, 1987; Kaushal and Taylor, 2002). Changes in HRV are indicative of perturbed depolarization and repolarization synchrony of the myocardium, involving autonomic dysregulation. A reduction in HRV suggest a poor prognosis for future adverse cardiac events, such as impairment in cardiac conductance, myocardial infarctions and sudden death (Bigger *et al.*, 1992; Dekker *et al.*, 2000). Exposure to PM has been linked to reduction in HRV in several studies (Chan *et al.*, 2004; Liao *et al.*, 2004; Chow *et al.*, 2006; Lipsett *et*

al., 2006; Adar *et al.*, 2007). However, other studies shown increases in HRV(Salvi *et al.*, 1999; Ruckerl *et al.*, 2007; Devlin *et al.*, 2012).

Air pollutants have been postulated to increase ventricular arrhythmias and atrial fibrillation (Brook *et al.*, 2010a). Specifically, a case-crossover study that followed 203 subjects given implantable cardioverter defibrillators over an average of 3.1 years reported that ozone and PM concentrations were associated with 798 ventricular arrhythmias in 84 of the subjects (Rich *et al.*, 2005). Another study demonstrated that increases in ozone concentrations were associated with increased risk for supraventricular arrhythmias in the elderly (Sarnat *et al.*, 2006). Rodent studies generally done at high concentration of ultrafine carbon black or ozone have demonstrated decreases in heart rate (Watkinson *et al.*, 2003; Ghelfi *et al.*, 2008), while other studies, conducted at relatively lower concentrations of PM, have shown increases in heart rate following exposure (Upadhyay *et al.*, 2008). Farraj et al. (2012) demonstrated that high levels of ozone caused alterations in repolarization, atrioventricular conduction block, atrial premature beats, supraventricular beats, and second degrees morbitz type I atrioventricular block in hypertensive rats (Farraj *et al.*, 2012). This study further showed that ozone exposure increased risk for ventricular tachycardia, ventricular fibrillation and cardiac arrest in rats during an aconitine challenge in a concentration-dependent manner. Overall these studies support the notion that pollutants can alter cardiac function.

In animal studies, myocardial dysfunction and cardiac ischemic injury were associated with increased inflammatory mediators, oxidative stress byproducts, and decreased antioxidant reserves following PM exposure (Kodavanti *et al.*, 2000; Liu and Meng, 2005). It has been postulated that circulating inflammatory mediators and ROS may

locally impact cardiac function by inducing myocardial contractile dysfunction through decreased systolic intracellular Ca^{2+} and activation of calmodulin kinase II (Erickson *et al.*, 2008), resulting in prolongation of action potential duration) and arrhythmogenesis (Ito *et al.*, 2008; Xie *et al.*, 2009). Subchronic episodic exposures of combustion-derived PM exposures in rats have also been link to increased inflammation, myocardial cytotoxicity, fibrosis, and changes in gene expression pattern in the heart (Kodavanti *et al.*, 2003).

1.8 Vascular effects

Several epidemiology and clinical studies have shown an association between air pollution and vascular dysfunction. An earlier study demonstrated impairment in endothelium dependent and independent vasodilation that were associated with higher concentrations of sulfate and black carbon in the air (O'Neill *et al.*, 2005). Interestingly, these effects were noted among diabetes patients. Healthy humans exposed to traffic-related PM have also displayed impairment in endothelium-dependent vasodilation (Dales *et al.*, 2007; Rundell *et al.*, 2007). Brook *et al*. (2002) demonstrated brachial artery narrowing after ozone and PM exposure in healthy volunteers (Brook *et al.*, 2002). More studies, focused primarily on diesel exhaust, have shown endothelial dysfunction and impaired vasodilation in conducting and resistant arteries in humans (Nurkiewicz *et al.*, 2004; Urch *et al.*, 2004; Mills *et al.*, 2005).

It is speculated that circulating mediators such as reactive oxidation byproducts, pro-inflammatory cytokines, and vasoactive substances may be involved in pollutant-induced vasoconstriction and endothelial dysfunction. Rats receiving antioxidant treatment were protected from ozone-induced impairment of nitric oxide (NO)-mediated coronary artery dilation (Paffett *et al.*, 2015), which suggests that increased oxidant production might be

involved in vascular effects. Similarly, reduced levels and functionality of NO are associated with increased systemic oxidative stress (Tzeng *et al.*, 2003; Li *et al.*, 2006) and inflammatory mediators (IL-6, TNF-α) within the systemic circulation after PM exposure (Ruckerl, et al., 2007). PM has also been shown to increase endothelins (Thomson *et al.*, 2004) and angiotensin II (Li *et al.*, 2005) in rats, contributing further to vasoconstrictive effects (Tzeng, et al., 2003; Sun *et al.*, 2005). Overall, pollutant-induced changes in systemic mediators have been postulated to impair peripheral physiological responses.

1.9 Proposed mechanisms of air pollutant-induced systemic and cardiovascular effects

Three mechanisms have been proposed to explain how pollutant-induced pulmonary injury/inflammation may lead to systemic inflammation and cardiovascular effects: (1) translocation of pollutant components reaching peripheral tissues and causing direct extra-pulmonary effects, (2) local lung injury causing release of vasoactive substances and spillover of pro-inflammatory cytokines from damaged lungs and affecting distant organs, and/or (3) activation of the autonomic nervous system (ANS) that perturbs function of the cardiovascular system **(Figure 1.2)**. Each of these proposed mechanisms might be involved but is likely to be influenced by the chemical composition, size, pollutant concentration, and the nature of exposure, the physical integrity of the lung and the species sensitivity.

1.9.1 Evidence for systemic translocation of air pollution components

Coarse and fine particles, due to their large size, are not systemically translocated but are phagocytosed by alveolar macrophages. The migration of these phagocytes to the lymph nodes and particle translocation have been reported in some cases. Due to their nanometer size range, UFP have the potential for systemic translocation. Although the postulate that these particles can translocate to the periphery without cells remains controversial, some

studies have demonstrated the translocation of UFPs to the circulation and various organs, but the level of translocation is generally low and is dependent upon the nature of UFPs (Nemmar *et al.*, 2001; Nemmar *et al.*, 2002). Particle-associated metals that are soluble and thus, bioavailable have been shown to translocate to the heart and extra-pulmonary organs (Wallenborn *et al.*, 2007). UFPs or soluble metals, if translocated, can induce extra-pulmonary effects by directly interacting with tissues they encounter (Wallenborn *et al.*, 2009). It is noteworthy that pollutants which do not translocate systemically can also induce extra-pulmonary effects. For example, systemic effects can be induced by reactive gaseous pollutants such as ozone, which is unlikely to translocate from the lung. Thus, it is difficult to delineate a specific mechanism for systemic effects induced by inhaled pollutant mixtures

1.9.2 The evidence for systemic inflammation induced by inhaled pollutants

During pollutant exposure, the lung was postulated to be the main source of circulating vasoactive and inflammatory mediators that can perturb cardiac and vascular function. It has been proposed that activated macrophages in the lung can, through the release of pro-inflammatory mediators, activate the bone marrow. Exposure to cigarette smoke has been shown to increase polymorphonuclear leukocytes production in the bone marrow of rabbits, which suggests that the bone marrow may be involved in pollutant-induced systemic inflammation (Terashima *et al.*, 1999). However, the precise mechanisms by which lung macrophages upon activation may signal bone marrow to release progenitor leukocytes or increase circulating cytokines is not well understood.

A number of rodent studies have shown that circulating pro-inflammatory cytokines are increased after pollution exposure and may be involved in peripheral effects of air pollutants (Nurkiewicz, et al., 2004; Finnerty *et al.*, 2007; Mutlu *et al.*, 2007; Niwa *et al.*,

2008), while many other rodent studies have demonstrated no change in circulating cytokine levels (Campen *et al.*, 2006; Kooter *et al.*, 2006; Gottipolu *et al.*, 2009). Inhalation studies showing increased circulating cytokines have generally involved long-term (subchronic) exposures in mouse models. Therefore, it remains to be determined if increased systemic inflammation will be present during acute pollutant exposure and if cytokines are the initiating factor for extra-pulmonary effects as many studies have suggested.

1.9.3 Autonomic involvement in regulation of cardiac physiological changes

Air pollutant-induced cardiac physiological changes, especially changes in HRV, are governed by autonomic regulation of sympathetic and parasympathetic tone. Ozone-induced bradycardia, along with decreased core temperature, suggests alterations in the ANS (Gordon *et al.*, 2014). Likewise, changes in HRV following ozone and diesel exposure in rats and humans can also be mediated though autonomic imbalance. The ANS, which is divided into the sympathetic and the parasympathetic axes, regulates cardiac rhythmicity and blood supply to the periphery. Dysfunction of the ANS could result in perturbation of electrical conduction and propagation of cardiac arrhythmias. Increased HRV is associated with parasympathetic nervous system (PSNS) activation, while decreased HRV indicates the predominance of the SNS activation. Reduced HRV is considered detrimental and represents an ANS imbalance and increased risk for cardiovascular morbidity and mortality. The activation of the SNS will result in increased heart rate, while the predominance of the PSNS will result in bradycardia. Many human and animal studies have examined the effects of air pollution on cardiac physiology. Arito et al. revealed that ozone-induced bradycardia was attenuated in rats, treated with a parasympathetic blocker, atropine(Arito *et al.*, 1992), while another study displayed similar reversal of ozone-induced bradycardia in vagatomized dogs

(Vaughan *et al.*, 1971), suggesting the involvement of sensory neurons. Although the involvement of autonomic regulation in air pollutant-induced cardiovascular effects has been identified, it is not clearly understood how pollutant exposure might alter autonomic regulation.

1.10 Air pollution and neuronal effects

More recently, the CNS has been reported as another target of air pollutant effects. Exposure to air pollutants have been linked to neuroinflammation, neurodegeneration, learning and psychological impairment, and decrement in cognitive function in a number of epidemiological and animals studies (Block and Calderon-Garciduenas, 2009; Costa *et al.*, 2014; Calderon-Garciduenas *et al.*, 2015; Calderon-Garciduenas and Torres-Jardon, 2015). Exposure to diesel exhaust in rats has been shown to cause microglial activation and neuroinflammation in a number of brain regions (Levesque *et al.*, 2011). Ozone exposure has been shown to cause oxidative and inflammatory changes in various areas of the brain in animals (Rivas-Arancibia *et al.*, 1998; Rivas-Arancibia *et al.*, 2010; Mokoena *et al.*, 2015). Three mechanisms have been proposed to explain how air pollutants impact the CNS. It has been thought that, depending on the physicochemical property, inhaled pollutants can induce neuronal effects (as indicated for systemic effects) through: 1) direct translocation of components, especially soluble materials and nanoparticles, through the olfactory bulb or systemic circulation, across the blood-brain barrier into the brain, 2) circulating inflammatory mediators that activate brain regions and induce local inflammation, and 3) activation of sensory neurons in the lung. Regardless of the initiating event, it has been shown that pollutant exposure is associated with oxidative stress and inflammatory responses in resident

endothelial glial cells such as astrocytes, and neurons, which may result in damage and loss of signal integration in various regions of the brain and alter behavioral outcomes.

PM and its constituents have been reported to translocate from the lung and into the brain of rodents (Oberdorster *et al.*, 2004) and humans (Calderon-Garciduenas *et al.*, 2008), which could initiate local oxidative, inflammation, and innate responses (Wang *et al.*, 2008; Block and Calderon-Garciduenas, 2009). Specifically, UFP particulate have been shown to translocate through the olfactory nerves and reach the olfactory bulb, hippocampus, and other brain areas (Wang *et al.*, 2008). These direct neuronal effects of particulates will depend upon the presence of pollutant components that are able to translocate to the brain upon inhalation.

Pollutants which do not translocate, such as ozone, have also been shown to cause a number of effects in different brain regions; however, the mechanisms still remain unclear. The activation of C-fibers on afferent sensory vagus nerves within the lung has been proposed to be involved in activation of the CNS. Acute ozone exposure has been shown to increase cFos staining, an indicator of neuronal activation, in the NTS, and in stress-responsive regions including the caudal ventrolateral medulla, parabrachial nucleus, central nucleus of the amygdala, bed nucleus of the stria terminalis and the paraventricular hypothalamic nucleus (Gackiere *et al.*, 2011). The NTS receives vagal sensory information from the cardiac and pulmonary systems, and from subdiaphragmatic organs of the gastrointestinal tract (Travagli, 2007). Ozone has been shown to activate specifically the caudal and subpostremal NTS, which overlaps with regions that receive input from the pulmonary C-fibers and RAR receptors (Gackiere, et al., 2011). Once the NTS is activated, it can relay the information to the aforementioned stress-responsive regions, specifically the

hypothalamus. These stress-responsive regions are known to stimulate the ANS to respond effectively to afferent input and lead to a plethora of responses in the body. Thus, it is likely that air pollutant exposure might induce peripheral effects through activation of central ANS mechanisms; however, the involvement of the nervous system in mediating peripheral air pollution systemic effects has not been well established.

1.11 Air pollution and metabolic effects: diabetes

In 2014, the American Diabetes Association estimated that 29.1 million people were diagnosed with diabetes (blood sugar ≥ 126 mg/dL), while 86 million were diagnosed with pre-diabetes (blood sugar levels between 100-125 mg/dL) in the United States. That same year, it was reported that 245 billion dollars were spent in medical bills associated with diabetes in America. Further, the diabetes epidemic goes beyond the United States. In 2013, the WHO estimated that 382 million people had diabetes worldwide, with 90% of the cases consisting of type II diabetes (TIID) and the remaining 10% being type I. TIID is characterized by the diminished ability of insulin to regulate glucose uptake by cells (insulin resistance), leading to increased blood glucose levels and the inability of cells to use glucose as a source of energy, resulting in a variety of other complications. Insulin resistance can begin to develop years before clinical signs of diabetes are observed (Froncesa, 2009) . It is believed that overcompensation of the pancreatic islets to produce insulin during insulin resistance will eventually result in β-cell dysfunction and often a reduction in circulating insulin, which is associated with the manifestation of TIID (Kasuga, 2006). Virtually all organ systems can be affected by diabetes as cells are not able to use glucose for energy production despite high circulating levels. Non-treatment of TIID can have an array of

consequences on the respiratory, urinary, reproductive, cardiovascular, CNS and endocrine systems.

1.12 Systemic inflammation in insulin resistance

Many studies have suggested that systemic inflammation is central to the pathogenesis of insulin resistance (Schmidt *et al.*, 1999; Barzilay *et al.*, 2001; Pradhan *et al.*, 2001; Festa *et al.*, 2002; Duncan *et al.*, 2003; Spranger *et al.*, 2003). High levels of specific pro-inflammatory markers and chemokines have been observed in diabetes patients, including TNF-α, IL-6, IL-1β, and C-reactive protein (Shoelson *et al.*, 2006). It is hypothesized that the chronic pro-inflammatory state observed in TIID patients originates in adipose tissue (Shoelson, et al., 2006). Obesity and high caloric diets are thought to initiate expansion and remodeling of adipose tissue, the activation of inflammatory mechanisms, and the systemic release of pro-inflammatory cytokines and chemokines. More specifically, recent animal studies suggest that over-nutrition and obesity can cause accumulation of free fatty acids (FFA) in adipocytes, leading to the activation of the inflammatory pathways c-Jun-NH2-terminal kinase (JNK) and IKK-β pathway. The JNK and IKK-β pathways initiate serine phosphorylation of the insulin receptor substrate (IRS)-1/2, resulting in inhibition of the insulin signaling pathway and activation of transcription factors that upregulate inflammatory mediators (Shoelson, et al., 2006). Moreover, genetic and pharmaceutical intervention of JNK and IKK-B axes have ablated obesity-induced insulin resistance in mice (Shoelson, et al., 2006). Pro-inflammatory mediators secreted from adipose tissue can also activate local macrophages that initiate the recruitment of additional monocytes to the site, leading to chronic immune activation. These activated adipose-derived monocytes are observed to transition from an M2 (suppressive) to an M1 (pro-inflammatory) phenotype

(Shoelson, et al., 2006). Increased circulating adipose-derived FFA can also negatively regulate insulin signaling in other insulin-sensitive tissues like the liver and the muscle, contributing to global progression of insulin resistance. However, it should be noted that non-obese individuals are also known to develop insulin resistance and diabetes. Thus, a variety of risk factors likely play a role in development of TIID, including calorie rich diets, sedentary lifestyle, genetic predisposition and chronic stress. The interactive contribution of these risk factors still remains unclear and thus, are under intense investigation (Finucane *et al.*, 2011).

1.13 Air pollutants and diabetes

More recently, air pollution has been postulated to be a contributing risk factor for diabetes. Epidemiological studies have shown a positive association between the incidence of TID and TIID and air pollution exposure (Liu *et al.*, 2013; Thiering and Heinrich, 2015). Pearson et al. in a cross-sectional study demonstrated that high levels of PM coincided with prevalence of diabetes in the United States (Pearson *et al.*, 2010). Specifically, increased levels of ambient PM have been associated with increases in HbA1c (a marker of chronic high levels of blood glucose) (Chuang *et al.*, 2011; Tamayo *et al.*, 2014), impairment in glucose metabolism (Teichert *et al.*, 2013), elevated homeostatic model assessment of insulin resistance (HOMA-IR), and decreased insulin sensitivity in humans (Kim and Y.C., 2012; Brook *et al.*, 2013; Thiering and Heinrich, 2015). However, the mechanisms by which air pollutants might cause metabolic effects or insulin resistance are not well understood.

Experimental studies have shown that long-term PM exposure results in liver endoplasmic reticular (ER) stress, adipose mitochondrial dysfunction, adipose inflammation, and liver insulin insensitivity (Rajagopalan and Brook, 2012a), all of which have been

implicated in TIID. PM-induced liver ER stress, also referred to as unfolded protein response (UPR), has also been implicated in the onset of insulin resistance and glucose metabolic impairment (Rajagopalan and Brook, 2012b). ER stress is induced when protein misfolding occurs due to their oxidative modifications or other conformational changes and impaired/decreased protein folding capacity in the ER (Lai *et al.*, 2007). PM exposure has been reported to elevate UPR-associated proteins, protein kinase RNA-like ER kinase, inositol-requiring enzyme-1, activating transcription factor-4, heat shock protien70, heat shock protein 90, and binding immunoglobulin protein in the liver and white adipose tissue (Rao *et al.*, 2015). The UPR can activate inflammatory and stress signaling systems, including the NF-κB and JNK pathways, which may influence cellular glucose uptake (Rajagopalan and Brook, 2012a). However, it is not known if liver ER stress is an initiating event in pollutant-induced metabolic effects or a consequence of PM-induced systemic changes. Long-term PM exposure-induced systemic increases in pro-inflammatory cytokines has been postulated to contribute to insulin resistance (Xu *et al.*, 2011). PM exposure has specifically been shown to increase inflammatory cytokines IL-6, TNF-α, IL-1β in adipose tissues (Sun *et al.*, 2009; Sun *et al.*, 2013). PM exposure was also associated with increases in systemic levels of IL-6, and reduced tyrosine phosphorylation of insulin signaling mediators: IRS-1/2, Protein Kinase B (PKB/AKT), and phosphatidylinositol-4,5-bisphosphate 3-kinase in the liver, resulting in decreased insulin-mediated glucose uptake (O'Neill *et al.*, 2007; Sun, et al., 2013). Further, long-term PM exposure has been shown to perturb mitochondrial function in adipose tissue, leading to the accumulation of different metabolites, such as diacylglycerol, that could perturb insulin signaling (Xu, et al., 2011).

The initiating events that are involved in inducing systemic inflammation or insulin resistance after air pollution exposure are still under investigation.

1.14 Neuronal involvement in air pollution-induced systemic effects

A number of studies provide clues to the initiating events that might alter immune mechanisms and induce metabolic alterations. As discussed above, air pollutants alter autonomic function and can induce brain inflammation. It has been proposed that pollutant-induced systemic effects may be mediated through the activation of key hypothalamic regions involved in the regulation of metabolic homeostasis and maintenance of energy requirements. A few studies have proposed that hypothalamic ER stress, and increased expression of pro-inflammatory cytokines such as IL-6 and transcription factors such as NF-κB in the brain may be linked to neuronal damage, hypertension, and metabolic disturbances (Purkayastha, et al., 2011). Given the evidence that air pollutants can cause cardiovascular effects through ANS involvement and also induce neuronal inflammation in various areas of the brain, we postulate that systemic inflammatory and metabolic effects are mediated through neural pathways.

Ozone is a well-studied, chemically simple prototypic pollutant, which has gained significant interest recently for its extra-pulmonary and neuronal effects. Interestingly, epidemiological studies have shown a positive association between ozone and fasting hyperglycemia, insulin resistance and exacerbation of diabetes symptoms (Stafoggia *et al.*, 2010; Zanobetti and Schwartz, 2011; Kim and Y.C., 2012), although the mechanistic link is not established. In one of our earlier studies, we showed that aged (2 year old) rats exposed to ozone episodically for a period of 4 months had increased circulating insulin levels (Gordon *et al.*, 2013). This finding prompted us to examine the link between metabolic

alterations and ozone. We subsequently reported that acute and long-term ozone exposure in Brown Norway (BN) rats were associated with hyperglycemia and impaired glucose tolerance, but these ozone-induced metabolic effects were transient (Bass *et al.*, 2013). Although inflammatory cytokines have been considered to be the main contributor to pollutant-induced systemic effects, our studies using rat models failed to demonstrate any increases in circulating cytokines with ozone or other pollutants (Gordon et al., 2013, Bass et al., 2013). No changes were noted in the inflammatory cytokines IL-6, IL-1β, C-reactive protein or TNF-α in rats following an acute or subchronic ozone exposure despite impaired glucose tolerance; however, acute phase proteins, such as acid glycoprotein and alpha-2 macroglobulin, were increased after an acute ozone exposure (Bass et al., 2013). Importantly, the metabolic effects in the Bass et al., (2013) study were evident prior to pulmonary inflammation or increases in circulating acute phase proteins following ozone exposure, suggesting that pulmonary inflammatory spillover into the systemic circulation may not be the main mechanism of hyperglycemia or glucose intolerance.

This study showed an elevation in the stress hormone, epinephrine, which suggested that the SNS may be activated during exposure. Epinephrine has been shown to exert insulin antagonistic effects by mediating increased glycogen breakdown and enhanced adipose lipolysis (Nonogaki, 2000). Sympathetic nerves can directly target the adipose tissue and stimulate lipolysis, leading to the release of non-esterified fatty acids (NEFA), which can potentially contribute to insulin resistance (Arner and Langin, 2014). Acute ozone exposure was also associated with elevated circulating corticosterone in rats, which suggests the activation of the hypothalamus pituitary adrenal (HPA) axis (Thomson *et al.*, 2013). Corticosterone, another insulin counter-regulatory hormone like epinephrine, is known to

alter glucose and lipid metabolism through decreased glucose uptake and protein synthesis, increased hepatic glucose output, stimulated adipose lipolysis and increased amino acid release (Olefsky and Kimmerling, 1976). It is hypothesized that chronically elevated circulating corticosterone or other stress hormones may result in glucotoxicity and/or lipotoxicity, which may contribute to insulin resistance and the loss of glycemic control long-term. Corticosterone has also been implicated in decreased β-cell insulin secretion (Delaunay *et al.*, 1997). Epinephrine and corticosterone are produced and secreted from the adrenal medulla and cortex, respectively, and their increases in the circulation suggests the stimulation of the hormones release from the adrenal gland through a stress response pathway. No prior air pollution studies have examined the contribution of stress hormone-mediated systemic metabolic impairment and inflammation as a mechanism that potentially can impact a number of peripheral metabolic activities and insulin sensitivity or produce other peripheral air pollution effects including systemic inflammation.

Stress hormones have a major regulatory role in mediating redistribution of immune cells and initiating an inflammatory response after stress. Specifically, stress hormones have been shown to systematically shift the pro-inflammatory state characterized by increased production of the cytokines IL-12, TNF-α, and interferon (IFN)-γ to an anti-inflammatory state, in which Il-10 and Il-4 are produced (Elenkov and Chrousos, 2002). These stress hormones can influence inflammatory responses in a tissue-specific manner based on differential distribution of adrenergic and glucocorticoid receptors. For example, in lung-derived monocytes and epithelial cells, catecholamines, such as epinephrine, enhance IL-8 production, leading to further recruitment of neutrophils to the airways (Linden, 1996; Kavelaars *et al.*, 1997). Stress hormones, especially catecholamines, to initiate mononuclear

cell activation through the induction of the NF-κB pathway (Bierhaus *et al.*, 2003), subsequently increasing expression of pro-inflammatory cytokines.

HPA axis activation leads to stimulation of the release of stress hormones from adrenal glands, which regulate peripheral metabolic homeostasis under stress. These hormones also have potent feedback inhibitory effects at the hypothalamus and pituitary levels since this homeostatic response is a transient mechanism to counter stress situations. The activation of the hypothalamus and secretion of corticotrophin releasing hormone (CRH) stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) in the circulation that directly stimulates the adrenal cortex to secrete glucocorticoids (cortisol in humans and corticosterone in rats) and other hormones, such as mineralocorticoids (Ulrich-Lai and Herman, 2009). In contrast, the adrenal medulla can be directly stimulated by the sympathetic efferent nerves, resulting in the secretion of epinephrine and norepinephrine.

Although stress-induced peripheral metabolic impairment is well characterized, air pollutants have not been linked to mediate their effects through this pathway. It has been proposed that circulating factors are responsible for air pollutant-induced peripheral effects, but no prior studies have implicated changes in circulating metabolites or neurohormones in mediating systemic metabolic or pulmonary responses. Therefore, we postulate that ozone-induced increases in epinephrine, observed in pre-studies, and corticosterone, as reported by others, may be involved in systemic metabolic impairment and pulmonary injury/inflammation and are mediated through the activation of the HPA axis and SNS **(Figure 1.3).** Since the long-term dysregulation of the HPA and autonomic nervous system has been shown to contribute to the development of insulin resistance and other cardio-metabolic effects (Olefsky and Kimmerling, 1976; Nosadini *et al.*, 1983; Bruce *et al.*, 1992;

Surwit and Schneider, 1993; Friedman *et al.*, 1996; Bjorntorp *et al.*, 1999; Laitinen *et al.*, 1999; Rask *et al.*, 2001), we postulate that subchronic episodic ozone exposure will lead to tissue specific insulin resistance in the liver and muscle.

1.15 Summary and dissertation content

For decades, studies related to air pollutants focused primarily on the lung. Now it is apparent that air pollutants that encounter the lung and produce local effects can also affect virtually all organ systems in our body. In recent years, pollutant exposure has been associated with an increased incidence of diabetes; however, causality has not been established and the mechanisms are not understood. Although systemic inflammation was implicated in a number of air pollution studies that illustrate metabolic impairment, several other air pollution studies failed to demonstrate increases in circulating pro-inflammatory cytokines. Even though a number of studies have implicated the role of the ANS in pollutant-induced cardiovascular effects, its contribution to systemic and metabolic impairment and pulmonary injury/inflammation following air pollution exposure has not been examined.

In this dissertation, we have begun to investigate whether ozone exposure produces systemic metabolic impairment and to determine if these metabolic effects are associated with the release of stress hormones in response to SNS and HPA axis activation. We hypothesized that acute ozone exposure will produce broad scale metabolic derangements through neurohormonal activation, involving stress pathways, and that longer exposures will result in insulin signaling impairment in the muscle and liver. **In Chapters 2 and 3,** we characterized the nature of systemic metabolic derangement and neurohormone responses to acute ozone exposure using a metabolomic approach, and specifically examined the

temporality of glucose intolerance, release of stress and metabolic hormones, and serum metabolite profiling in rats. In addition, we also examined the metabolomic profile of serum samples obtained from humans exposed to ozone, established the coherence between rats and humans, and identified novel metabolite biomarkers. **In Chapter 4,** I examined the contribution of stress hormones (epinephrine and corticosterone) in metabolic impairment and lung injury and inflammation. For this study, I examined ozone effects in rats that underwent total adrenal demedullation or total bilateral adrenalectomy relative to sham surgeries. We showed that adrenal-derived stress hormones are essential not only in systemic metabolic effects of ozone, but also in pulmonary injury and inflammation. In **Chapter 5**, I determined if longer (3 months, episodic) ozone exposure in rats is associated with persistent metabolic alterations and liver and muscle insulin resistance by examining the phosphorylation of critical insulin signaling mediators that promote glucose uptake and metabolism, such as the phosphorylation of AKT. We also determined the relation between insulin sensitivity and the levels of circulating cytokines, hormones, lipids and glucose. The data from our studies: 1) provide causal evidence for associations between ozone exposure and metabolic effects observed in epidemiological studies, 2) identified novel metabolite biomarkers of ozone effects in humans, 3) are the first to link a systemic stress response to air pollution exposure in rats and in humans, 4) have a potential implication in developing a new adverse outcome pathway that can be employed for many environmental stressors and exposures, 5) provide novel insights into how stress hormones might regulate pulmonary injury/inflammation induced by air pollutants, 6) challenge the current paradigm of the mechanisms by which ozone induces lung injury, vascular leakage and inflammation, and 7) examine a potential link between insulin resistance/sensitivity and air pollutant exposure.

Figure 1.1

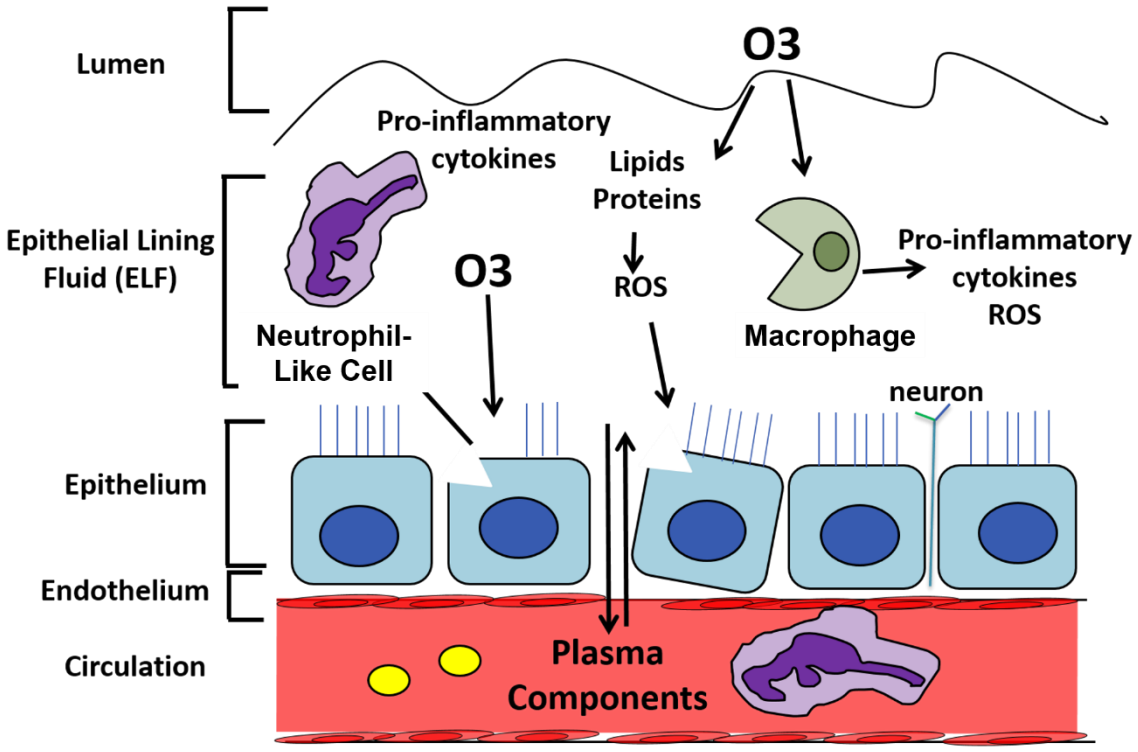

Figure 1.1: Ozone-induced lung effects. Upon inhalation, ozone (O3) can directly interact with proteins and lipids within the epithelial lining fluid (ELF) and on the surface of cells to form reactive oxygen species (ROS). These ozone-derived secondary and tertiary byproducts can injure the pulmonary endothelium and activate nerve endings, the main targets of air pollutants. Injured epithelium can release pro-inflammatory mediators, tachykinins (Substance P and neurokinins A) and enzymes that can activate local immune cells in the airway and recruit additional leukocytes to the respiratory tract. These epithelial-derived mediators or pollutant components can also lead to the activation of vagal neurons that terminate in the CNS and produced extra pulmonary effects. The alveolar leukocytes can further exacerbate the vicious cycle of ROS and inflammatory events, resulting in vascular leakage and increased lung injury/inflammation.

Figure 1.2

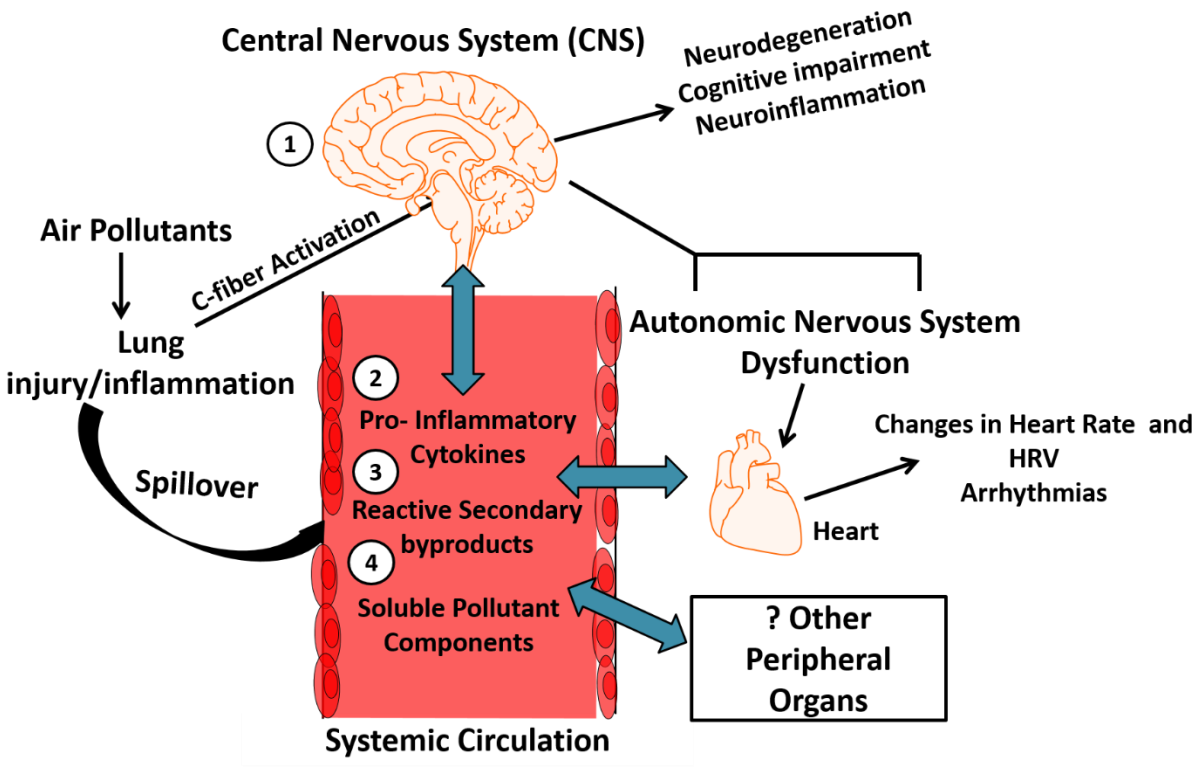

Figure 1.2 Pollutant-induced peripheral effects. It has been proposed that pollutants can elicit extra-pulmonary effects from the respiratory tract through the following mechanisms: 1) through pulmonary vagal C-fiber activation of hypothalamic regions in the brain, 2) spillover of lung pro-inflammatory cytokines or activation of the bone marrow, 3) spillover of reactive secondary oxidized by-products, and/or 4) spillover of soluble pollutant components. Air pollutants have been associated with cardiovascular and CNS adverse effects, but the specific pathway(s) by which systemic effects occur after exposure to air pollutants remain unclear.

Figure 1.3

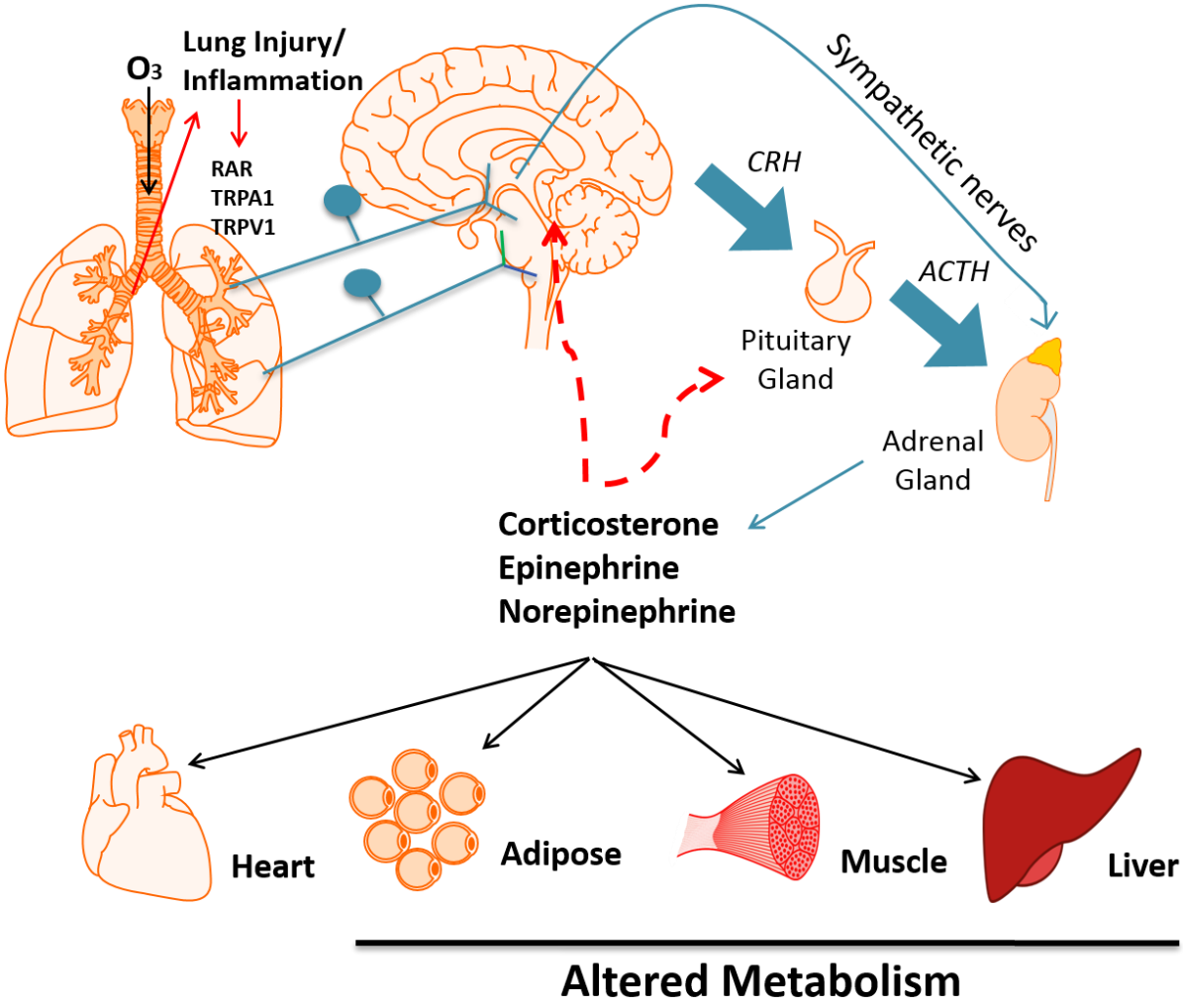
O3
Lung Injury/
Inflammation
RAR
TRPA1
TRPV1
CRH
Sympathetic nerves
ACTH
Pituitary
Gland
Adrenal
Gland
Corticosterone
Epinephrine
Norepinephrine
Heart
Adipose
Muscle
Liver
Altered Metabolism

Figure 1.3 The Proposed Mechanism of Stress Mediated Metabolic Effects of Ozone. Physiological and psychological stress are known to increase the levels of stress hormones in the circulation through activation HPA and SNS. These neuronal stress pathways are used to mobilize energy substrates to the appropriate tissues to combat stress. Specifically, the HPA leads to the activation of the adrenal cortex, which releases glucocorticoids, while sympathetic nerve terminals activate the adrenal medulla, which release catecholamines. These stress hormones are known to interact with their respective receptors on multiple organs and elicit responses: tachycardia, hyperglycemia, gluconeogenesis, glycogenolysis, adipose lipolysis, protein catabolism, etc, Chronic elevation of these stress hormones have been associated with metabolic dysfunction and insulin resistance. Ozone has been shown to activate hypothalamic neuro-stress responsive regions, potentially through stimulation of lung C-fibers (TRPA1).

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CHAPTER 2

INHALED OZONE INDUCES CHANGES IN SERUM METABOLOMIC AND LIVER TRANSCRIPTOMIC PROFILES IN RATS

2.1 Introduction

The incidence of metabolic syndrome is steadily increasing worldwide, resulting in higher prevalence of diabetes mellitus and cardiovascular morbidity and mortality (Ford et al., 2002). The common clinical components that comprise metabolic syndrome include, but are not limited to, abdominal obesity, hypertriglyceridemia, hypertension, low levels of high-density lipoprotein cholesterol (HDL-C), prothrombotic state, glucose intolerance, and insulin resistance (Ford et al., 2002; Grundy et al., 2004). Conventional etiologies, including sedentary lifestyle, diet-related obesity and genetics have been implicated as the major contributors to insulin resistance and the development of metabolic syndrome (Grundy et al., 2004). Most recently, air pollution, such as PM, has also been linked to the development of metabolic syndrome, and may serve as an effect modifier for the epidemiological associations between environmental factors and increased rate of cardiovascular diseases (Chen and Schwartz, 2008). A number of recent epidemiological and experimental studies have shown that chronic inhalation of airborne PM may increase an individual's risk for acquiring type 2 diabetes by creating a pro-inflammatory state, insulin resistance, and/or obesity (Brook et al., 2008, Rajagopalan and Brook, 2012; Liu et al., 2013)

A number of mechanistic pathways have been proposed where lung injury/inflammation initiates systemic inflammation, which is postulated to play a central role in inhaled pollutant-induced insulin resistance, but the evidence remains insufficient (Shoelson et al., 2006, O'Neill et al., 2007, Xu et al., 2011, Yan et al., 2011; Rajagopalan and Brook, 2012). Similar to PM, the ubiquitous air pollutant ozone has been associated with adverse pulmonary and cardiovascular health effects, such as lung injury/inflammation, decreased lung function and heart rate variability in animals and humans (Watkinson et al., 2001; Hollingsworth et al., 2007; Ciencewicki et al., 2008; Liu et al., 2009; Farraj et al., 2012; Wagner et al., 2014). However, the likely contribution of ozone to metabolic disorder and extra-pulmonary effects has yet to be systematically investigated.

Exposure to ozone has also been shown to induce cardiovascular functional changes through modulation of the autonomic nervous system, which regulates sympathetic and parasympathetic balance (Farraj et al., 2012; Gordon et al., 2014). More specifically, acute ozone exposure has been shown to stimulate lung vagal C-fibers through transient receptor potential member A1 (TRPA-1) receptors that lead to activation of neural stress-responsive regions in the central nervous system where lung afferents of vagus nerves terminate (Taylor-Clark and Undem, 2010 and Gackiere et al., 2011). Stress-mediated hypothalamus pituitary adrenal (HPA) axis activation is well known to modulate a variety of physiological processes including thermoregulation, immune elicitation, hormonal disposition, and systemic metabolic alterations (Ulrich-Lai and Herman, 2009). One study has recently shown that acute ozone exposure increased serum corticosterone in rats (Thomson et al., 2013), which is a marker of HPA axis activation. Recently, we have shown that ozone induces glucose intolerance and increased serum leptin and epinephrine in Brown Norway (BN) rats, in

addition to inducing hypothermia and bradycardia during exposure (Bass et al., 2013 and Gordon et al., 2014). Observed increases in serum epinephrine and corticosterone during acute pollutant exposure suggest a potential involvement of sympathetic and/or HPA-associated neurohumoral factors. However, a detailed characterization of ozone-induced metabolic impairment and involvement of neurohumoral intermediates has not been reported. This could aid in identifying potential mechanisms of ozone–induced systemic metabolic effects.

The objective of this study was to utilize serum metabolomic and liver transcriptomic techniques together with metabolic hormonal assessment to gain insight into the characteristics and potential mechanisms of metabolic alterations during ozone-induced hyperglycemia and glucose intolerance. The serum metabolomic approach used in this study is able to detect quantifiable metabolites in the serum released from biochemical processes in various tissues critical for metabolic homeostasis (Barnes et al., 2014). The detection of metabolites can provide insight regarding organs being affected by ozone exposure. Liver being the major organ for control and maintenance of metabolic processes, the assessment of liver transcriptional changes could provide mechanistic insights into its role in ozone systemic metabolic response. We hypothesized that ozone-induced hyperglycemia and glucose intolerance will be associated with broad scale systemic metabolic impairment, and that the use of serum metabolomic together with liver transcriptomic approaches will provide insights into 1) the mechanisms by which ozone perturbs metabolic processes, and 2) the potential contribution of ozone to long-term metabolic alterations.

2.2 Materials and Methods

2.2.1 Animals

Male, 10 week old, healthy Wistar Kyoto (WKY) rats (250-300g) were purchased from Charles River Laboratories Inc. (Raleigh, NC). Rats were housed (2/cage) in polycarbonate cages containing beta chip bedding in an isolated animal room in an animal facility maintained at 21 ± 1ºC, 50 ± 5% relative humidity and held to a 12hr light/dark cycle. The animal facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animals received standard (5001) Purina pellet rat chow (Brentwood, MO) and water *ad libitum* unless otherwise stated. Animal procedures were approved by the U.S. EPA NHEERL Animal Care and Use Committee (IACUC; Permit Number: 13-02-003 and 16-03-003). Animals were treated humanely and all efforts were made for alleviation of suffering.

2.2.2 Ozone generation and animal exposures

Ozone was produced from oxygen by a silent arc discharge generator (OREC, Phoenix, AZ), and its entry into the Rochester style "Hinners" chambers was controlled by mass flow controllers. The ozone concentrations in the chambers were recorded continuously by photometric ozone analyzers (API Model 400). Chamber temperature and relative humidity were measured continuously. Mean chamber air temperature and relative humidity were 23.3 °C (74 ^{0}F) and 46%, respectively. In the concentration-response study, rats were randomized by body weight into four exposure groups (n=6/group) to make sure each exposure group had the same overall average body weight. Similarly, in the time-course study, rats were randomized by body weight into six groups (n = 8/group) for two exposure conditions and each time point. In the concentration-response study, WKY rats were exposed

to either filtered air (FA) or ozone (0.25, 0.50, or 1.0 ppm), 6hr/day for two consecutive days and sacrificed 18hr after day 2. In the subsequent time-course experiment, three groups of WKY rats were used. 1) The first group was either exposed to FA or 1.0 ppm of ozone for 6hr/day for one day (1d-0hr), 2) a second group was exposed 6hr/day for two consecutive days (2d-0hr), and 3) a third group was allowed an 18hr recovery, following two consecutive days of ozone exposure (2d-18hr).

2.2.3 Glucose tolerance testing (GTT)

All rats that underwent GTT were fasted for 8-10hr in cage or during exposure prior to the assessment of blood glucose concentrations. For rats that were allowed an 18hr recovery period, food was removed for 10hr (overnight; 10pm to 8 am) before GTT. Baseline blood glucose concentrations were measured by pricking the distal surface of rats' tails using a sterile needle to obtain ~1 μl of blood. A Bayer Contour glucometer was used to determine blood glucose levels using test strips, which require 0.6 μL whole blood. After the first measurement, rats were given an intraperitoneal (I.P.) injection of glucose, 2g/kg/10 mL (20% D-glucose; 10ml/kg). Measurement with the glucometer was repeated every 30 min over the course of 2hr. In the concentration-response study, all animals underwent GTT 4 days prior to ozone exposure, immediately following ozone on day 1 and immediately following ozone on day 2. For the time-course study, rats assigned to the 18hr recovery group were used for GTT. These rats underwent GTT one day before ozone exposure, immediately following ozone exposure on day 1, immediately following ozone exposure on day 2, and also after an 18hr recovery period following two days of ozone exposure. In addition, rats assigned to the 2d-0hr time point underwent GTT immediately following one day of FA or ozone exposure. No GTT was performed on the 1d-0hr group where tissue and

serum collection were performed immediately after exposure. The 2d-0hr exposure group for tissue and serum collection did not undergo GTT on the 2nd day of ozone exposure.

2.2.4 Necropsy and sample collection

For the concentration-response study, all rats were necropsied 18hr after two consecutive days of FA or ozone exposure. For the time-course experiment, one group of rats (n=16), which did not undergo GTT, were necropsied immediately after the first day of exposure (1d-0hr). The 2nd group of rats (n=16) was necropsied immediately after the second day of ozone exposure (2d-0hr). The recovery group (n=16) was necropsied 18hr after the second day of exposure (2d-18hr). In each study, rats were fasted for 8-10hr before necropsy regardless of fasting associated with GTT. Rats were weighed and anesthetized with an overdose of sodium pentobarbital (Virbac AH, Inc., Fort Worth, TX; 50-100 mg/kg, i.p.). Blood samples were collected through an abdominal aortic puncture directly into serum separator vaccutainers without coagulant for serum preparation. Tubes were centrifuged at 3500 x g for 10 min and aliquots of serum were stored at -80°C until analysis. In the concentration-response study, bronchoalveolar lavage (BAL) was performed through tracheal tubing using Ca^{2+} and Mg^{2+} free phosphate buffer saline, 37°C at 28mL total lung capacity/kg rat weight. Aliquots of BAL fluid were used to determine total cell counts with a Z1 Coulter Counter (Coulter, Inc., Miami, FL) and cell differentials were performed on cytospin slides stained with Diff-quick as previously described (Bass et al., 2013). The cell-free BAL fluid was used to analyze albumin, as previously described (Bass et al., 2013). In the time-course experiment, in addition to collecting serum at each time point, liver tissues were collected and frozen in liquid nitrogen for RNA analysis.

2.2.5 Serum analysis

Serum samples collected from rats were analyzed for insulin and leptin using rat-specific electrochemiluminescence assays (Meso Scale Discovery, Gaithersburg, MD) via manufacturer's instructions. Total cholesterol was measured in serum samples using kits from TECO Diagnostics (Anaheim, CA), while HDL-C and low-density lipoprotein cholesterol (LDL-C) were measured with kits from Thermo Fisher Scientific, Inc. (Middletown, VA). Both types of kits were modified for use on the Konelab Arena 30 system (Thermo LabSystems, Espoo, Finland). Epinephrine circulating levels was determined by ELISA kit (Cusabio, Wuhan, China)

2.2.6 1 Metabolomic analysis

Serum global metabolomic profiling was performed by Metabolon Inc. (Durham, NC). Detailed methods are described in previous publications (Evans et al., 2009, Dehaven et al., 2010 and Reitman et al., 2011). Frozen serum samples from time-course study for 1 d–0 h and 2d–0h time points (n = 7–8/group) were used for this analysis.

2.2.6.2 Sample accessioning

Each sample received was accessioned into the Metabolon laboratory information management system (LIMS) and was assigned a unique identifier that was associated with the original source identifier only. This identifier was used to track all sample handling, tasks, results, etc. All aliquots of any sample were automatically assigned their own unique identifiers by the LIMS when a new task was created; the relationship of these samples is also tracked. All samples were maintained at -80ºC until processed.

2.2.6.3 UPLC/MS/MS

The LC/MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo-Finnigan linear trap quadrupole (LTQ) mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was dried then reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol containing 0.1% formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion. Raw data files are archived and extracted as described below.

2.2.6.4 GC/MS

The samples destined for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24hr prior to being derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramp was from 40°C to 300°C in a 16 min period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis. The information output from the raw data files was automatically extracted as discussed below.

2.2.6.5 Quality assurance (QA)/quality control (QC)

For QA/QC purposes, additional samples were included with each day's analysis. These samples included extracts of a pool of well-characterized human plasma, extracts of a pool created from a small aliquot of the experimental samples, and process blanks. QC samples were spaced evenly among the injections and all experimental samples were randomly distributed throughout the run. A selection of QC compounds was added to every sample for chromatographic alignment, including those under test. These compounds were carefully chosen so as not to interfere with the measurement of the endogenous compounds.

2.2.6.6 Metabolomic data extraction and compound identification

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. Metabolite quantification was based on area under the curve. These systems are built on a web-service platform utilizing Microsoft's .NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (m/z), and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, nominal mass match to the library +/- 0.2 amu, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library

spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals. More than 2400 commercially available purified standard compounds have been acquired and registered into LIMS for distribution to both the LC and GC platforms for determination of their analytical characteristics.

2.2.6.7 Statistical analysis of metabolomic data

Missing values (if any) are assumed to be below the level of detection. However, biochemicals that were detected in all samples from one or more groups but not in samples from other groups were assumed to be near the lower limit of detection in the groups in which they were not detected. In this case, the lowest detected level of these biochemicals was imputed for samples in which that biochemical was not detected. Following log transformation and imputation with minimum observed values for each compound, Welch's two-sample t-test was used to identify biochemicals that differed significantly between experimental groups. Pathways were assigned for each metabolite, allowing examination of overrepresented pathways. Significant ($p<0.05$) pathway enrichment output (cumulative hypergeometric distribution) was assessed for each of the selected contrasts (1d-0hr; 2d-0hr and 2d-18hr) using MetaboSync, version 1.0 (Metabolon, Inc. RTP, NC) to determine the metabolic processes impacted by ozone.

2.2.7.1 Gene Array

Liver tissue samples from FA or 1 ppm ozone-exposed rats (n=5-6/group) for all three time points from the time-course study were used for this analysis. Total liver RNA was isolated from ~20 mg tissue with a commercially available RNeasy mini kit (Qiagen, Valencia, CA) using silica gel membrane purification. Liver RNA was resuspended in 30µl

of RNAse-free water. RNAse inhibitor was added and RNA yield was determined spectrophotometrically on a NanoDrop 1000 (Thermo Scientific, Wilmington, DE). RNA integrity was assessed by the RNA 6000 LabChip® kit using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). We examined global gene expression changes using the Affymetrix platform (RG-230 PM Array strip). Biotin-labeled cRNA was produced from total RNA using an Affymetrix IVT-express labeling kit (cat# 901229). Total cRNA was then quantified using a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and evaluated for quality on a 2100 Bioanalyzer. Fragmented cRNA were also evaluated for quality using 2100 Bioanalyzer. Following overnight hybridization at 45ºC to Affymetrix RG-230 PM array strip in AccuBlock Digital Dry Baths (Labnet International Inc.), the arrays were washed and stained using an Affymetrix GeneAtlas fluidics station as recommended by the manufacturer. Arrays were scanned on an Affymetrix Model GeneAtlas scanner. After scanning, raw data (Affymetrix.cel files) were obtained using Affymetrix Command Console Operating Software. This software also provided summary reports by which array QA metrics were evaluated including average background, average signal, and 3'/5' expression ratios for spike-in control GAPDH.

2.2.7.2 Normalization and determination of differentially expressed genes

The Affymetrix GeneAtlas array data for each sample was normalized by Affymetrix Expression Console software using the plier algorithm with perfect match only probes. The resulting expression table was downloaded from Affymetrix Expression Console software into a text file. Statistical contrasts were calculated at each time point for ozone versus the FA control. Each contrast was computed on a text file containing all assayed genes as rows and only the contrast samples as columns by a Bayes t-test using R. Subsequently, a multiple

test correction using the Benjamin-Hochberg method with an alpha of 0.05 was applied to the Bayes t-test output in Excel. To support subsequent analysis, the differentially expressed genes (DEGs) from the three time-point contrasts were consolidated into an ozone DEG expression table.

2.2.7.3 Functional gene list preparation

The DEG list for each time point was also submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 for determination of significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using a modified Fisher Exact test with a p-value cutoff of 0.05. Functional gene lists were generated by NetAffx queries at the Affymetrix website (www.affymetrix.com). The query terms were "apoptosis", "diabetes", "gluconeogenesis", "glycolysis", "mitochondria", "steroid metabolism", "tricarboxylic acid cycle", "unfolded protein response" and "cytokines". The eight lists were exported separately from NetAffx as text files. For each gene list, Affymetrix probeset IDs were used to select the corresponding gene from the ozone DEG expression table to build an expression table that only contains the genes for a given function that were also ozone DEGs. Hierarchical clustering was computed for each functional gene list using Cluster 3.0 (de Hoon et al., 2004) and the clusters were displayed using Java Treeview (Saldanha, 2004). Three functional probeset lists based on queries of "steroid receptor", "insulin receptor" or "fatty acid" were obtained from NetAffx. Each of these lists was compared to the list of DEGs for the 1d–0hr time point to identify DEGs in each of the three functional categories. Each functional list was processed separately by Ingenuity Pathway Analysis (IPA) to produce a direct relationship graph. The graph constructed from the Ingenuity knowledgebase depicts some of

the biological relationships among the probe sets on the list. The microarray data are publically available through Gene Expression Omnibus (accession # GSE59329).

2.2.7.4 Real time PCR confirmation of gene array findings

We selected 4 genes increased in expression from the gene array (insulin receptor substrate-2, IRS-2; 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1, PFKFB1; aconitase-2, Aco-2; and peroxisome proliferator activated receptor gamma c1a, Ppargc1a) and a control transcript (β-actin) to determine the validity of gene array findings. RT-PCR for RNA from FA or ozone-exposed rat livers at 1d-0hr time point was conducted on an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) as described previously (Bass et al., 2013). Primers were purchased from ABI as inventoried TaqMan Gene Expression Assays, each containing a 6-carboxy-fluorescein (FAM dye) label at the 5' end. Data were analyzed using ABI sequence detection software (SDS version 2.2). For each PCR plate, cycle threshold (cT) was set to an order of magnitude above background. For each individual sample, target gene cT was normalized to a control gene cT (β-actin) to account for variability in the starting RNA amount. Expression of exposure group was quantified as fold difference over FA control.

2.2.8.1 General statistical analysis

Graphpad prism 4.03 software was used for statistical analysis of GTT and biomarker data. Dose response study GTT was analyzed using a two-way analysis of variance (ANOVA) where each time blood glucose measurement was independently assessed. The two independent variables were day and dose. One-way ANOVA was used for data analysis of neutrophils and albumin in the BAL fluid. The time-course study GTT was analyzed by two-way repeated measures MANOVA (multivariate ANOVA). The two independent

variables were day and dose. The time course study biomarker measurements were analyzed using a two-way ANOVA followed by Duncan's multiple range test. Pair-wise comparisons were performed as subtests of the overall ANOVA. The nominal Type I error rate (α) was set at 0.05. No adjustments were made for multiple comparisons.

2.3 Results

2.3.1 Ozone induces pulmonary injury and inflammation in a concentration-dependent manner

Although ozone is well studied for its potential to induce lung injury in many experimental settings, we wanted to determine the concentration-dependent cellular responses and inflammation in our experimental model to better correlate these changes with systemic metabolic alterations. Therefore, we first confirmed whether ozone-induced pulmonary cellular responses in WKY rats by examining the BAL fluid for increases in the lung vascular leakage of albumin and neutrophilic inflammation at 18hr post two consecutive days of ozone exposure. Inflammation and cellular responses have been shown to peak on the second day of ozone exposure (van Bree et al., 2001). The concentration response analysis showed that 1.0 ppm ozone increased the number of neutrophils in the BAL fluid (Fig. 2.1A). Albumin, a lung protein leakage marker that exists at lower levels in the normal lung, was also elevated at 1.0 ppm ozone (Fig. 2.1B). These changes were not evident in rats exposed to 0.5 or 0.25 ppm ozone.

2.3.2 Ozone exposure induces concentration-and time-dependent hyperglycemia and glucose intolerance

GTT in the concentration-response study was conducted to determine whether ozone-induced cellular and inflammatory lung responses were associated with prior changes in

blood glucose regulation. In comparison to the FA group, ozone-exposed rats at 0.5 and 1.0 ppm on day 1 displayed marked fasting hyperglycemia (0 min time point; Fig. 2.1C). By contrast, the 2d-0hr ozone exposure group illustrated diminished fasting hyperglycemia (Fig. 2.1D). Both the 0.5 and 1.0 ppm experimental groups exhibited glucose intolerance when examined immediately after ozone exposure on day 1 (Fig. 2.1C) and day 2 (Fig. 2.1D). Ozone at 0.25 ppm caused neither hyperglycemia nor glucose intolerance (Fig. 2.1C and D). GTT was also performed in the time-course study where exposure was to FA or 1.0 ppm ozone. As noted in the concentration-response study (Fig. 2.1C), ozone at 1.0 ppm induced hyperglycemia and glucose intolerance when examined immediately after a 6hr exposure (1d-0hr; Fig. 2.2A). Like ozone exposure on day 1, hyperglycemia and glucose intolerance persisted on day 2 of exposure (Fig. 2.2B). Ozone-induced hyperglycemia and glucose intolerance were largely reversed after 18hr in the recovery group (Fig. 2.2C).

2.3.3 Ozone exposure alters the serum metabolic hormones and lipids

To determine the potential cause of hyperglycemia and glucose intolerance, we assessed the serum levels of metabolic hormones and cytokines through electroimmunochemiluminescence and colorimetric ELISA techniques. Variability was noted in baseline (FA control) levels of insulin between three time points, where FA-exposed rats after 2d-0hr had high levels of insulin relative to the other two groups. This increase in insulin could relate to the sustained stimulation of its release in response to the injection of a high dose of glucose immediately following the day 1 of FA or ozone exposure for performing GTT. When compared to time-matched FA group, serum insulin levels were lower in ozone-exposed rats at 2d-0hr time point (Fig. 2.3A). Serum leptin, a satiety hormone, increased after day 1 (1d-0hr) of ozone exposure, but not on day 2 (2d-0hr) or

following an 18hr of recovery (2d-18hr) (Fig. 2.3B). Circulating epinephrine levels were increased after 1d-0hr and 2d-0hr but was reversible in rats allowed an 18 hour recovery after two consecutive days of ozone exposure (Fig. 2.3C). There was a significant diurnal variation in cholesterol levels of FA-exposed rats such that rats necropsied in the afternoon times for 1d-0hr and 2d-0hr had higher levels than rats necropsied in the morning at 2d-18hr time point. Ozone exposure resulted in elevated serum total cholesterol and HDL-C at 2d-18hr time point (Fig. 2.3D and F). The levels of LDL-C were increased on day 2 (2d-0hr and 2d-18hr) after ozone exposure (Fig. 2.3E).

2.3.4 Metabolomic analysis

We conducted metabolomic analysis of serum samples from FA or 1.0 ppm ozone-exposed rats at 1d-0hr and 2d-0hr to gain understanding of the nature of metabolic changes and potential involvement of multiple organs in homeostatic control of metabolic processes. Comparison of global biochemical profiles for rat serum revealed several key metabolic differences between ozone-exposed rats vs. FA control. The metabolomic analysis identified strong effects of ozone exposure on metabolites that reflect changes in central energy metabolism. Of 313 named biochemicals identified in the serum, 81 metabolites were significantly increased in ozone-exposed rats and 48 decreased at 1d-0hr, while 71 metabolites were increased and 80 were decreased at 2d-0hr. Pathway analysis identified a number of pathways that were significantly altered after ozone exposure. Those included lysine catabolism, branch chain amino acids (BCAA) metabolism, protein degradation, urea cycle, sphingolipids metabolism, fatty acid synthesis, primary and secondary bile acid metabolism and glutathione metabolism at 1d-0hr. Many of these pathways also remained changed at 2d-0hr, in addition to alteration in β-oxidation pathway. A detailed analysis of

metabolic processes affected based on individual metabolites impacted by ozone is given below.

2.3.5 Metabolomic analysis: Ozone impairs glucose homeostasis through perturbation of glycolytic pathways

The metabolomic analysis confirmed hyperglycemia at 1d-0hr following ozone exposure (Table 2.1), as evident during GTT. In addition, the metabolite 1,5-anhydroglucitol, a biomarker inversely related to long-term glycemic control, was markedly decreased in ozone-exposed animals at both time points (Table 2.1). Fructose levels were also increased at 1d-0hr but not at 2d-0hr. The glycolysis end-product pyruvate was increased on day 1 of ozone exposure while the anaerobic glycolytic metabolite, lactate, was significantly decreased at day 2 in ozone-exposed animals (Table 2.1). Several tricarboxylic acid (TCA) cycle intermediates (citrate, α-ketoglutarate, fumarate and malate) were less abundant in the serum samples of ozone-exposed rats especially on day 2 (Table 2.1).

2.3.6 Metabolomic analysis: Ozone exposure increases serum amino acids

A number of BCAA and their metabolites were increased in the serum following ozone exposure (Table 2.1). The data showed several signatures of altered protein and amino acid catabolism. Urea, generated to eliminate nitrogenous waste made from amino acid catabolism, was more abundant with ozone exposure. One muscle-specific protein catabolite (3-methylhistidine) and one possible muscle protein catabolite (N-acetyl-1-methylhistidine) were more abundant after 2 days of ozone exposure, suggesting their release from muscle (Table 2.1). Increases were observed in the BCAA themselves (leucine, valine and isoleucine) as well as several metabolites generated when BCAA are catabolized to enter the TCA cycle. Some of these metabolites (alpha-hydroxyisocaproate, alpha-hydroxyisovalerate and 2-hydroxy-3-methylvalerate) are typically present at low abundance unless dehydrogenase reactions are defective, or with mitochondrial dysfunction. Combined with the observed decrease in TCA cycle intermediates, these metabolite changes may reflect mitochondrial dysfunction.

2.3.7 Metabolomic analysis: Ozone exposure increases serum free fatty acids (FAA) and cholesterol while decreasing bile acids

All detectable short- and long-chain FFA were noticeably more abundant in ozone-exposed rats at both time points. These increases included essential, non-essential, saturated, polyunsaturated and hydroxy fatty acids ranging in length from C12 to C22, as well as, palmitoyl and stearoyl sphingomyelin (Table 2.2). Additionally, ozone exposure reduced mitochondrial ω-oxidation metabolites (beta-hydroxybutyric acid, propionylcarnitine and butyrylcarnitine) (Table 2.2).

Bile acids are synthesized in the liver from cholesterol, further metabolized by the gut microbiome, and released into the intestine to facilitate dietary fat absorption. In this study, several cholesterol and bile acid metabolites were changed by ozone exposure. Serum cholesterol was elevated, while virtually all serum bile acid metabolites were decreased in ozone-exposed rats at both time points (Table 2.3). The observed increase in diet-derived phytosterols (fucosterol, beta-sitosterol and campesterol) suggests increased dietary absorption of cholesterols or decreased metabolism/excretion of cholesterol. While rats were not provided food during ozone exposure, the changes in circulating cholesterol and bile acids might be due to decreased metabolism and/or release in the circulation. The observed decreases in several bile acids and an intermediate in bile acid synthesis from cholesterol (7-hoca) suggest decreased bile acid production may contribute to elevated serum cholesterol (Table 3).

2.3.8 Liver transcriptomic profiling reveals its role in homeostatic control of ozone-induced systemic metabolic alterations

Because the liver is the primary organ that regulates glucose, amino acid, cholesterol and fatty acid metabolism, we conducted a global gene expression profiling of liver to determine its role in systemic metabolic impairment induced by ozone exposure. Overall, the effect of ozone on the liver transcriptome was greatest at 1d-0hr with reduction in number of genes affected at 2d-0hr and 2d-18hr. At 1d-0hr, 2335 genes were found to be significantly different in ozone-exposed rats compared to FA controls, while on 2d-0hr there were only 72 genes and 2d-18hr there were 247 genes differentially expressed. KEGG pathway analysis of liver DEGs at day 1 indicated that steroid biosynthesis, TCA cycle, and glyoxylate and decarboxylate metabolism pathways were significantly altered by ozone. When genes

associated with various metabolic processes, apoptosis, mitochondria, unfolded protein response and diabetes were separated from master DEGs list, probe sets assigned to each of this process represented 11-34% of the total probes on the array, suggesting widespread liver gene expression impact of ozone exposure implicating metabolic processes (Appendix A. Table 2.1). The heatmaps of cytokine network showed inhibition of many genes with induction of some genes (Appendix A. Fig. 2.1A-C). Many genes belonging to apoptosis pathway were impacted by ozone exposure (Appendix A. Fig. 2.2A-F). Genes involved in steroid and fatty acid metabolism and insulin signaling were of interest and showed that ozone impacted key regulatory genes involved in these processes (Appendix A. Fig. 3 and Fig. 4A-C).

Ozone increased hepatic expression of numerous glycolytic genes including insulin receptor kinase-2, lactate dehydrogense A, aldolase A, phosphoglycerate mutase-1, enolase 1-alpha, pyruvate kinase, 6-phosphofructo-2-kinase-fuctose-2,6 bisphosphatase, and glycerol-3-phosphate dehydrogenase-2 at 1d-0hr and 2d-0hr (Fig. 2.4), suggesting a central stimulation to increase glycolysis. However, the expression of glucokinase, an important enzyme for conversion of circulating glucose to glucose-6-phosphate, was decreased in ozone-exposed rats. Interestingly, ozone exposure also increased the expression of some of the gluconeogenic genes, such as the phosphoenolpyruvate carboxykinase 1-soluble, glucose-6-phosphatase, and phosphofructo-2-kinase-fuctose-2,6 bisphosphatase, which is a multi-functional protein that plays a role in gluconeogenesis, as well as, glycolysis. In addition, ozone increased expression of genes involved in amino acid metabolism that feed into gluconeogenesis. Those included glutamate-ammonia ligase, tyrosine aminotransferase, serine dehydratase, and glycine- N-methyltransferase (Fig. 2.4). Ozone also elevated

expression of genes encoding the TCA cycle enzymes such as fumarate hydratase, acontinase-2, and isocitrate dehydrogenase 3 NAD-alpha and gamma suggestive of enhanced energy expenditure in the liver.

Because many genes involved in glycolysis and TCA cycle were induced, we presumed that liver mitochondrial function might also be affected by ozone exposure. Many of the observed glycolysis, gluconeogenesis, and TCA genes modified by acute ozone exposure were seen in heat maps generated for mitochondria gene cluster (Fig. 2.5A and B). These also included genes involved in steroid metabolism. Mitochondrial heat maps (Fig. 2.5A and B) showed that ozone decreased the expression of genes involved in cholesterol and steroid metabolism. These genes included farnesyl diphosphate synthase, ATP citrate lyases, and MLX interacting protein. Meanwhile, ozone increased the expression of genes involved in glycolytic and fatty acid catabolic processes, such as acyl CoA thioesterase 1, acyl CoA thioesterase 2, acyl CoA thioesterase 7, 3-hydroxybutarte dehydrogense type 1, and pyruvate dehydrogenase phosphatase catalytic subunit 2 (Fig. 2.5B). Interestingly, ozone increased expression of genes encoding enzymes involved in mitochondria biogenesis and homeostasis in the liver, such as the peroxisome-proliferator-activated receptor gamma, involved in liver homeostatic control. Ozone-exposed rats also showed increases in mitochondria apoptotic genes such as the Bcl-2 interacting protein containing the BH3 subunit and other genes involved in apoptotic pathways (Fig. 2.5B; Appendix A. Fig. 2).

While metabolomics data indicated marked changes in FFA and steroid metabolism, DEGs belonging to these processes were separated from a master DEGs list of liver genes. Since these metabolic pathways operate in mitochondria, some of these genes were readily seen in the heat maps for mitochondrial genes. A heat map of steroid metabolism genes

further confirmed that ozone exposure decreased expression of genes that encode enzymes necessary for cholesterol synthesis in the liver, including but not limited to, lanosterol synthase, farnesyl diphosphate synthase, mevalonate diphospho-decarboxylase, isopentenyl-diphosphate delta isomerase, sterol regulatory element binding factor 1/2, and more (Fig. 2.6A). In contrast, ozone increased the expression of genes that encode for the proteins arginase, argininosuccinate lyase, and argininosuccinate synthetase, which are essential for liver ammonia detoxifying processes and the progression of the urea cycle (Fig. 2.6B). Ozone-exposed rats also demonstrated higher expression of genes that encode for nuclear receptors, such as, estrogen, thyroid, and RAR-related orphan receptor A (Fig. 2.6B).

Since increases in circulating BCAA occurred likely due to increased muscle protein catabolism, we wanted to determine whether genes involved in liver amino acid metabolism responded to this systemic response. Some of the genes involved in the use of amino acids for gluconeogenesis providing precursors for TCA cycle were induced by ozone, as indicated earlier. These gene included dihydrolipamide S–succinyltransferase, serine dehydratase, aminoadipate-semialdehyde synthase, butyrobetaine, 2 –oxoglutarate dioxygenase 1 and more (Fig. 2.7).

We confirmed the validity of the liver gene array findings using RT-PCR for four transcripts at 1d-0hr time point (Fig. 2.8). All four transcripts that are important in metabolic regulation, including IRS-2, PFKFB1, Aco-2, and Ppargc1a, were found to be increased when expression was analyzed using RT-PCR, confirming that the array technique provided satisfactory results (Fig. 2.8).

2.4 Discussion

Recent epidemiological and experimental studies have shown that ozone and ambient PM, in addition to causing respiratory effects in humans and animals, may also contribute to a number of systemic health outcomes such as neuronal, reproductive, cardiovascular and metabolic effects, including insulin resistance (Campbell, 2004; Thomson et al., 2013; Liu et al., 2013; Pedersen et al., 2014). The mechanisms of these systemic effects are poorly understood. We have recently shown that acute ozone exposure in BN rats induces glucose intolerance, leptinemia and catecholamine release generally observed during a stress response (Bass et al., 2013). Ozone exposure has been shown to induce lung injury and inflammation (van Bree et al., 2001), activate stress responsive regions in the brain (Gackiere et al., 2011), and induce hypothermia and bradycardia (Gordon et al., 2014). In this study, our goal was to characterize the homeostatic metabolic response of the WKY rat to ozone exposure using serum metabolomic and liver transcriptomic approaches to better understand the potential mechanisms responsible for systemic metabolic alterations.

In WKY rats, ozone-induced fasting hyperglycemia, glucose intolerance, and serum leptin increases that were much more pronounced than responses we recently reported in BN rats (Bass et al., 2013). These metabolic and hormonal changes were accompanied by rapid and reversible increases in serum protein degradation byproducts, FAA and cholesterol, depictive of a systemic metabolic response, and decreases in TCA cycle intermediates, which might relate to hypothermia observed in rats after an ozone exposure (Gordon et al., 2014). Liver gene expression changes were coherent with changes in serum metabolites and showed inhibition of genes involved in steroid biosynthesis but stimulation of genes involved in gluconeogenesis, glycolysis, ω-oxidation and energy expenditure. Ozone-induced release of

short- and long-chain FFA into the circulation, likely involving adipose lipolysis, was coincided with decreased expression of hepatic lipid biosynthesis genes. The concomitant increase in circulating cholesterol may be a result of reduced catabolism in the liver as a consequence of inhibition of bile acid synthesis evidenced by the decrease in circulating bile acid metabolites. Further, our data show increased flux of BCAA into the circulation, which corroborated with increased expression of hepatic enzymes that utilize precursor amino acids for gluconeogenesis. Overall, we show that these acute systemic effects of ozone exposure involve a broad scale derangement of glucose, lipid, and protein metabolism reflective of a sympathetic and HPA-mediated stress response. If and how this reversible derangement might relate to chronic development of metabolic disorders will require studies involving long-term exposures.

As observed in our study, ozone-induced lung injury and inflammation peaks one day after an acute exposure (van Bree et al., 2001). We have shown that ozone initially induces acute hypothermia during exposure but this is followed by hyperthermia and lung inflammation the following day. The temporal pattern of metabolic changes seen in the present study suggests that neuronal response might be stimulated prior to inflammation. A number of animal studies have shown that ozone can induce neuroinflammation and activate catecholeminergic neurons likely through lung vagus afferents (Gackiere et al., 2011; Martinez-Lazcano et al., 2013). Activation of these central nervous system locations can stimulate sympathetic and HPA-mediated release of adrenal stress hormones in the systemic circulation, which can perturb metabolic functions involving glucose, lipids, and amino acids (Seematter et al., 2004). We have also observed an increase of epinephrine after an acute ozone exposure in BN rats (Bass et al., 2013). In addition, ozone has been shown to increase

corticosteroidal activities in multiple tissues likely through activation of HPA axis (Thomson et al., 2013). The systemic metabolic changes such as hyperglycemia, glucose-intolerance, lipidemia, and amino acid influx induced by ozone are similar to those induced during a neuronally-mediated stress response following an organ injury (Molina, 2005; Wang et al., 2007). Although stress-mediated metabolic disorder has been very well established, no prior air pollution studies have examined a stress response and subsequent systemic metabolic impairment.

Temporal differences were noted in ozone-mediated hyperglycemia, leptinemia and glucose intolerance. Leptinemia was observed only on day 1, which coincided with fasting hyperglycemia, increased circulating pyruvate and 2-hydroxybutarate on day 1 and decreased lactate on day 2, while glucose intolerance persisted over both days. This may relate to a number of potential interactive mechanisms governing glucose intolerance and could explain the reversible nature of metabolic effects upon termination of ozone exposure. It is not clear what stimulates leptin release immediately after ozone exposure, which coincides with release of FFA on day 1 but not on day 2. The stimulation of sympathetic nerves is known to result in inhibition of leptin secretion (Sandoval and Davis, 2003), but the activation of HPA has been associated with its increase (Roubos et al., 2012). Leptin is made primarily in adipocytes and has major impact on metabolic and immune processes (Mainardi et al., 2013). Since adipose lipolysis is likely stimulated after ozone exposure, it is possible that the release of FFA into the circulation stimulates concomitant leptin release. However, leptin increase was not sustained on day 2 while FFA continued to be increased. Acute release of leptin from adipose tissue can directly signal the hypothalamus to mediate peripheral fat oxidation and suppress food intake (Roubos et al., 2012). It has been shown that increased leptin can inhibit

the neurons co-expressing neuropeptide Y (NPY) and Agouti-related peptide (AgRP) in the arcuate nucleus in the medio-basal hypothalamus (Morton, 2007). Thus, leptin can contribute to the reduction of acute fasting hyperglycemia observed on day 2 of ozone exposure.

Enhanced expressions of some of the genes involved in glycogenolysis, gluconeogenesis, and glycolysis after ozone exposure are suggestive of the changes induced following a stress response (Raz et al., 1991; Dufour et al., 2009). The ozone-induced increases in corticosterone (Thomson et al., 2013) may augment hepatic glucose production by stimulating glycogenolysis and gluconeogenesis. However, the increase in blood glucose is accompanied by increases in 2-hydroxybutarate on day 1 and decreases in 1,5 – anhydroglucitol in serum at both time points, suggesting compromised glycemic control (Buse et al., 2003). Concomitantly, the glycolytic metabolite pyruvate accumulated only on day 1 with a slight reduction on day 2, suggesting that glycolysis does not progress into the TCA cycle at least in the early phase of ozone exposure in the tissues. In contrast, lactate decrease in the ozone-exposed group on day 2 may indicate subsequent reduction of anaerobic glycolysis in the peripheral tissues that may coincide with the rebound effect on body temperature in rats following termination of exposure as previously noted (Gordon et al., 2014). Ozone exposed rats showed reduced serum levels of TCA cycle and β-oxidation intermediates despite stimulation of genes in the liver. These changes suggest that the ozone-induced metabolic response likely involves reduced energy expenditure by peripheral tissues, such as the muscle, while maintaining homeostatic activities in the liver.

Ozone exposure decreased serum insulin levels despite glucose intolerance, particularly on day 2, suggesting that ß-cells in the pancreas may be impacted. It has been shown that the autonomic nervous system can have inhibitory effects on insulin production

and secretion into systemic circulation by islet ß-cells (Bloom et al., 1978; Buijs et al., 2001; Kiba, 2004). Additionally, short-term leptin increases have also been observed to suppress ß-cells' insulin production through sympathetic activation in rodents (Park et al., 2010). It is possible that this is an adaptive mechanism involving short-term insulin resistance in the peripheral tissues in the midst of ozone-induced stress to ensure adequate glucose supply to the brain and other organs actively involved in maintenance of homeostasis, such as the liver.

The release of FFA into the circulation from adipose lipolysis is one of the hallmark features of stress-induced metabolic alterations. Ozone-induced increases in circulating FFA could be due to sympathetic action on white adipocytes (Nonogaki, 2000). It is has been shown that catecholamines during a stress response stimulate lipolysis and the mobilization of FFA in adipose tissue through activation of the ß-adrenergic receptors (Carey, 1998). ß-adrenergic receptor activation results in stimulation of adenylyl cyclase, followed by increases in intracellular cAMP, leading to the degradation of triglycerides and shuttling of FFA into the systemic circulation (Carey, 1998). In addition, activation of the HPA axis can also cause increased release of FFA from adipose tissues (Carey, 1998). Increased circulating FFA are known to induce insulin resistance by increasing serine phosphorylation of IRS-1 and halting insulin-mediated glucose uptake (Schulman and Zhou, 2009), which may explain ozone-induced glucose intolerance.

Ozone-induced increase in circulating cholesterol further supports the contribution of sympathetic or HPA axis activation (Kunihara and Oshima, 1983). Despite, rats being fasted during ozone exposure, the observed increases in circulating cholesterol and phytosterols suggest decreased catabolism of cholesterol. Bile acid production is one important mechanism for eliminating cholesterol through excretion. The observed decreases in several

bile acids and an intermediate of bile acid synthesis from cholesterol suggest that decreased bile acid production may contribute to elevated serum cholesterol. These data are supported by evidence that the transcription of essential genes involved in cholesterol and steroid synthesis was suppressed in the liver.

Notably, ozone-induced increases in several circulating BCAA including muscle-specific protein catabolites, combined with the observed decrease in TCA cycle intermediates, may reflect increased protein catabolism and mitochondrial dysfunction. Given the fact that BCAA are known to regulate insulin production (Lu et al., 2013), their increases in the circulation after ozone exposure might influence insulin action in peripheral tissues. Stress has been shown to augment the catabolic state and therefore increases the utilization of BCAA for energy production in organs such as liver (Biolo et al., 1997; Porter et al., 2013). It has been shown that muscle protein catabolism is stimulated under stress to provide necessary precursor amino acids for the liver to synthesize large amounts of acute phase proteins (Biolo et al., 1997). In addition, the increases in the expression of gluconeogenic enzymes involving BCAA suggests their use in energy production by the liver.

Changes in liver gene expression were not restricted to metabolic processes, rather genes involved in apoptosis and mitochondrial function were remarkably impacted by ozone. Ozone exposure also changed the expression of several genes involved in inflammatory processes that tended to be inhibited rather than induced. Air pollution has been recently linked to non-alcoholic liver disease, also known as liver steatohepatitis (Zheng et al., 2013; Lin et al., 2014). One experimental study involving long-term exposure to concentrated ambient PM has shown liver inflammation, fibrosis and changes in markers of insulin

signaling (Zheng et al., 2013). Marked acute effects of ozone on liver gene expression reflecting apoptosis, alteration of mitochondrial function and metabolic processes in the present study support the hypothesis that air pollutants could, over a long period of time, induce liver disease. The mechanisms by which ozone may lead to acute changes in liver gene expression and contribute to long-term disease will need to be examined further.

Although a number of studies have shown an increased release of inflammatory mediators from the lung into the circulation following exposure to air pollutants, specifically PM (Finnerty, et al., 2007; Mutlu et al., 2007; Delfino et al., 2008), ozone did not significantly increase serum IL-6 levels in this study. We and others have previously shown that acute ozone alone did not influence other serum inflammatory biomarkers such as TNF-α (Urch et al., 2010; Bass et al., 2013). Additionally, metabolic impairment was noted at 0.5 ppm without changes in indicators of lung injury or inflammation, suggesting that the immediate metabolic response likely resulted from neurohumoral activation and not from cytokines released systematically.

Some of the metabolic effects of ozone observed immediately following day 1 were reduced upon the second day of exposure, and most were reversed upon an 18 hour recovery period. This is critical in considering the relevance of these observations to humans who are exposed episodically over their lifetime. The reduction of ozone effects upon subsequent exposure might relate to the adaptation that has been widely reported in pulmonary injury and inflammation (Brink et al., 2008; Hamade and Tankersley, 2009); however, the mechanisms are poorly understood. We observed that the adaptation response to ozone might also involve reduced metabolic impairments upon subsequent exposure. It is likely that the degree of adaptation responses vary between animal species and strain (Hamade and

Tankersley, 2009). In our prior study, we observed that episodic subchronic ozone exposure might reduce some of the acute metabolic effects in BN rats; however, glucose intolerance was still apparent after a 13 week episodic exposure (Bass et al., 2013). Thus, the metabolic consequences of low-level episodic ozone exposure are likely dependent upon the nature of that episodic exposure, the underlying genetic susceptibility and the biomarker of interest. Chronic stress has been linked to a rise in circulating lipids, insulin resistance and increased incidence of diabetes (Kelly and Ismail, 2015). Thus, it remains to be established if long-term ozone exposure might be linked to insulin resistance and diabetes predisposition through chronic stress and persistent metabolic alterations. The EPA.gov site states that the average ozone concentration that humans are exposed to across the United States is at or below the National Ambient Air Quality Standard of 0.075 ppm, with higher levels (0.2 to 0.3 ppm) seen frequently in regions with hot climates (Calderón-Gacidueñas et al., 2000). The 1.0 ppm ozone concentration used in this study for most analysis is considered high compared to human relevant doses. However, it has been shown that rats require three to four times the concentration of ozone to acquire an equivalent dose to humans (Hatch et al., 1994). Thus, the concentrations of ozone used in our study, although higher than ambient levels, are appropriate for rodent exposures.

In conclusion, we show that the inhaled pulmonary irritant ozone is able to induce systemic metabolic changes, as seen by hyperglycemia, glucose intolerance, leptinemia, increased serum FFA, cholesterol, and BCAA together with varied changes in liver transcriptome expression involving the processes of apoptosis, mitochondrial dysfunction and the alterations of glucose, protein and lipid metabolism. The known increases in epinephrine (Bass et al., 2013) and corticosterone (Thomson et al. 2013), together with the

nature of systemic metabolic changes involving multiple central and peripheral tissues, provides supportive evidence for the involvement of a stress response in ozone-induced systemic metabolic impairment. Further studies will be required to determine the likely long-term consequences of these metabolic alterations on insulin resistance. Although the immediate metabolic responses are not associated with the systemic increases in inflammatory cytokines, the contribution of chronic systemic inflammation might be important in the development of metabolic disorders.

Table 2.1. Metabolomic analysis: ozone-induced changes in serum metabolites for glucose and amino acid metabolism.

		Fold Change			
Biological Processes	**Metabolite**	**Ozone FA 1d**	**p-Value**	**Ozone FA 2d**	**p-Value**
Glycolysis, Gluconeogenesis, Pyruvate Metabolism	Glucose	1.47	0.0000	1.07	0.2801
	Fructose	1.64	0.0168	1.16	0.5933
	1,5 androhydroglucitol	0.77	0.0073	0.75	0.004
	Pyruvate	1.87	0.0011	0.86	0.2283
	Lactate	1.10	0.3613	0.72	0.0029
Kreb Cycle	citrate	1.07	0.5455	0.83	0.0778
	alpha-ketoglutarate	0.70	0.0067	0.67	0.0048
	succinate	0.88	0.4810	0.91	0.3771
	fumarate	1.08	0.9269	0.66	0.0177
	malate	0.78	0.1529	0.73	0.0414
Tryptophan Metabolism	Kinurenate	3.13	0.0004	2.11	0.0137
	Kinurenine	1.63	0.0001	1.23	0.0468
Amino Acid Metabolism	3-Methylhistidine	1.03	0.7168	1.22	0.0117
	N-acetyl-1-methylhistidine	0.85	0.1509	1.60	0.0008
	3-methyl-2-oxobutyrate	1.62	0.0001	1.46	0.0009
	3-methyl-2-oxovalerate	1.84	0.0000	1.60	0.0001
	isoleucine	1.47	0.0000	1.47	0.0000
	leucine	1.57	0.0000	1.39	0.0001
	valine	1.69	0.0000	1.49	0.0000
	4-methyl-2-oxopentanoate	1.89	0.0000	1.59	0.0000
	beta-hydroxyisovalerate	1.85	0.0005	1.78	0.0006
	beta-hydroxyisovalerate	2.03	0.0000	1.53	0.0001
	N-acetylleucine	5.72	0.0000	1.36	0.1878
	N-acetylvaline	2.16	0.0000	1.00	1.0000
	N-acetylisoleucine	5.79	0.0000	1.81	0.0180
	3-hydroxyisobutyrate	1.91	0.0000	1.40	0.0038
	4-methyl-2-oxopentanoate	1.89	0.0000	1.59	0.0000
	alpha-hydroxyisovalerate	2.06	0.0000	1.47	0.0071
	isobutyrylcarnitine	1.20	0.1633	0.61	0.0011
	2-hydroxy-3-methylvalerate	1.87	0.0000	1.34	0.0041
	2-methylbutyrylcarnitine (C5)	1.31	0.0891	1.06	0.6531
	isovalerylcarnitine	1.63	0.0029	1.26	0.0973
	Urea	1.24	0.0143	1.18	0.0398

Table depicting ozone-induced changes in circulating metabolites reflecting changes in glucose metabolism following 1d or 2d of FA or ozone exposure. Values indicate relative fold differences for each biochemical between ozone and FA samples at each time point (n=6-7/group). Fold change >1 indicates increase, while fold change <1 indicates decrease. When p-value is <.05, the change is considered significant.

Table 2.2 Metabolomic analysis: ozone exposure increases circulating FFA.

Biological Processes	Metabolite	Fold Change			
		Ozone FA 1d	p-Value	Ozone FA 2d	p-Value
Essential fatty acid	linoleate (18:2n6)	1.57	0.0034	2.14	0.0000
	linolenate [alpha or gamma; (18:3n3 or 6)]	1.63	0.0107	2.17	0.0001
	dihomo-linolenate (20:3n3 or n6)	1.67	0.0014	1.13	0.296
	eicosapentaenoate (EPA; 20:5n3)	2.12	0.0000	1.29	0.0426
	docosapentaenoate (n3 DPA; 22:5n3)	2.02	0.0000	1.3	0.0268
	docosapentaenoate (n6 DPA; 22:5n6)	2.22	0.0000	1.7	0.0001
	docosahexaenoate (DHA; 22:6n3)	1.93	0.0000	1.59	0.0001
Long chain fatty acid	myristate (14:0)	1.31	0.0087	1.49	0.0001
	myristoleate (14:1n5)	1.33	0.0358	1.84	0.0001
	pentadecanoate (15:0)	1.37	0.0048	1.39	0.0018
	palmitate (16:0)	1.59	0.0009	1.8	0.0000
	palmitoleate (16:1n7)	1.51	0.0417	2.47	0.0001
	margarate (17:0)	1.51	0.0016	1.68	0.0001
	10-heptadecenoate (17:1n7)	1.31	0.0119	1.47	0.0003
	stearate (18:0)	1.33	0.0034	1.36	0.001
	oleate (18:1n9)	1.61	0.0069	2.38	0.0000
	cis-vaccenate (18:1n7)	1.65	0.0091	1.46	0.027
	nonadecanoate (19:0)	1.27	0.1076	1.38	0.0156
	10-nonadecenoate (19:1n9)	1.4	0.1337	1.63	0.0077
	eicosenoate (20:1n9 or 11)	1.29	0.0804	1.39	0.0227
	dihomo-linoleate (20:2n6)	1.6	0.0021	1.45	0.0057
	mead acid (20:3n9)	1.77	0.0003	1.51	0.0032
	arachidonate (20:4n6)	1.22	0.0819	1.23	0.0474
	docosadienoate (22:2n6)	1.42	0.0077	1.07	0.5246
	adrenate (22:4n6)	1.88	0.0001	1.26	0.0814
Fatty acid, monohydroxy	3-hydroxypropanoate	0.99	0.9762	0.73	0.0091
	3-hydroxyoctanoate	0.93	0.7825	0.99	0.8814
	2-hydroxystearate	1.3	0.0029	1.26	0.0066
	2-hydroxypalmitate	1.34	0.0007	1.31	0.0007
Fatty acid and BCAA metabolism	propionylcarnitine	0.82	0.1495	0.64	0.008
	butyrylcarnitine	0.72	0.0168	0.54	0.0001
	butyrylglycine	0.87	0.168	0.94	0.4452

Table depicting ozone-induced changes in circulating FFA following 1d or 2d of FA or ozone exposure. Values indicate relative fold differences for each biochemical between ozone and FA samples at each time point (n=6-7/group). Fold change >1 indicates increase, while fold change <1 indicates decrease. When p-value is <.05, the change is considered significant.

Table 2.3 Metabolomic analysis: ozone exposure alters circulating metabolites indicative of impairment in cholesterol and bile acid metabolism.

Biological Processes	Metabolite	Fold Change			
		Ozone FA 1d	p-Value	Ozone FA 2d	p-Value
Bile acid metabolism	cholate	0.22	0.0100	0.24	0.0019
	glycocholate	0.21	0.0061	0.18	0.001
	chenodeoxycholate	0.27	0.0401	0.16	0.0062
	hyodeoxycholate	0.32	0.0030	0.62	0.1782
	taurodeoxycholate	0.5	0.0279	1.81	0.1032
	glycodeoxycholate	0.07	0.0042	0.16	0.0671
	glycochenodeoxycholate	0.15	0.0219	0.08	0.0041
	6-beta-hydroxylithocholate	0.17	0.0005	0.45	0.1509
	beta-muricholate	0.33	0.0251	0.30	0.0011
	hyocholate	0.38	0.0974	0.33	0.011
	alpha-muricholate	0.19	0.0130	0.12	0.0006
	tauroursodeoxycholate	0.46	0.0058	1.09	0.8176
	tauro-alpha-muricholate	0.54	0.0222	0.89	0.6976
	taurohyodeoxycholic acid	0.25	0.0011	1.14	0.5012
Monoacyl-glycerol	1-linoleoylglycerol (1-monolinolein)	0.64	0.0009	0.54	0.0000
	2-linoleoylglycerol (2-monolinolein)	0.64	0.0004	0.52	0.0000
	1-arachidonylglycerol	0.64	0.0065	0.79	0.0689
	2-arachidonoyl glycerol	0.67	0.0172	0.75	0.0496
Sphingolipid	palmitoyl sphingomyelin	1.57	0.0004	1.75	0.0000
	stearoyl sphingomyelin	2.73	0.0018	2.66	0.0002
Sterol/Steroid	cholesterol	1.33	0.0003	1.27	0.0008
	beta-sitosterol	1.44	0.0003	1.10	0.2589
	campesterol	1.31	0.0047	1.07	0.5349
	fucosterol	1.43	0.0101	1.25	0.0895

Table depicting ozone-induced changes in circulating cholesterol and bile acid metabolites following 1d or 2d of FA or ozone exposure. Values indicate relative fold differences for each biochemical between ozone and FA samples at each time point (n=6-7/group). Fold change >1 indicates increase, while fold change <1 indicates decrease. When p-value is <.05, the change is considered significant.

Figure 2.1.

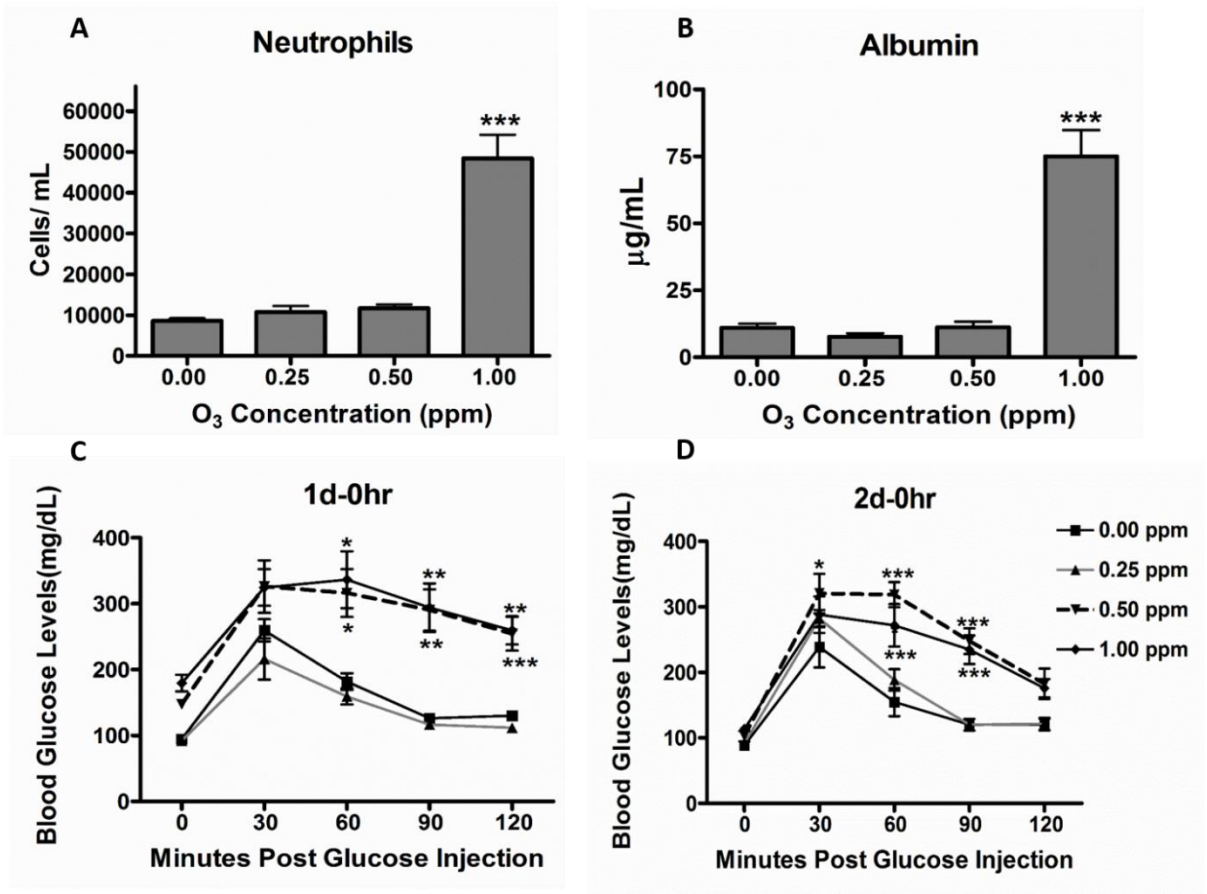
A
Neutrophils
Cells/ mL
60000
50000
40000
30000
20000
10000
0
***
0.00
0.25
0.50
1.00
O3 Concentration (ppm)
B
Albumin
µg/mL
100
75
50
25
0
***
0.00
0.25
0.50
1.00
O3 Concentration (ppm)
C
1d-0hr
Blood Glucose Levels(mg/dL)
400
300
200
100
0
0
30
60
90
120
Minutes Post Glucose Injection
D
2d-0hr
Blood Glucose Levels(mg/dL)
0.00 ppm
0.25 ppm
0.50 ppm
1.00 ppm
Minutes Post Glucose Injection

Figure 2.1 Ozone-induced cellular and inflammatory responses in the lung are associated with hyperglycemia and glucose intolerance in WKY rats.

Rats were exposed to FA or ozone (0.25, 0.5 or 1.0 ppm O_3) for 6h/day for two consecutive days. Immediately after day 1 (1d-0hr) and day 2 (2d-0hr) of exposure GTT was performed. Rats were necropsied 18hr after day 2 exposure (2d-18hr) and BAL fluid was analyzed for lung toxicity markers. (A) Neutrophils, as a marker of inflammation. (B) Albumin, as an indicator of vascular protein leakage. (C) Blood glucose at baseline and during GTT in rats exposed to FA or ozone (1d-0hr). (D) Blood glucose at baseline and during GTT in rats exposed to FA or ozone (2d-0hr). The values in the bar graphs are displayed as mean ± SE of n=6/exposure group. The GTT curve shows mean value ± SE of n=6/ group with repeated measures over two hours. The 0 min time point shows fasting glucose levels in each group. * Indicates significant difference from respective FA-exposed groups at a given time (*=p<.05, **=p<.01, ***=p<.001).

Figure 2.2.

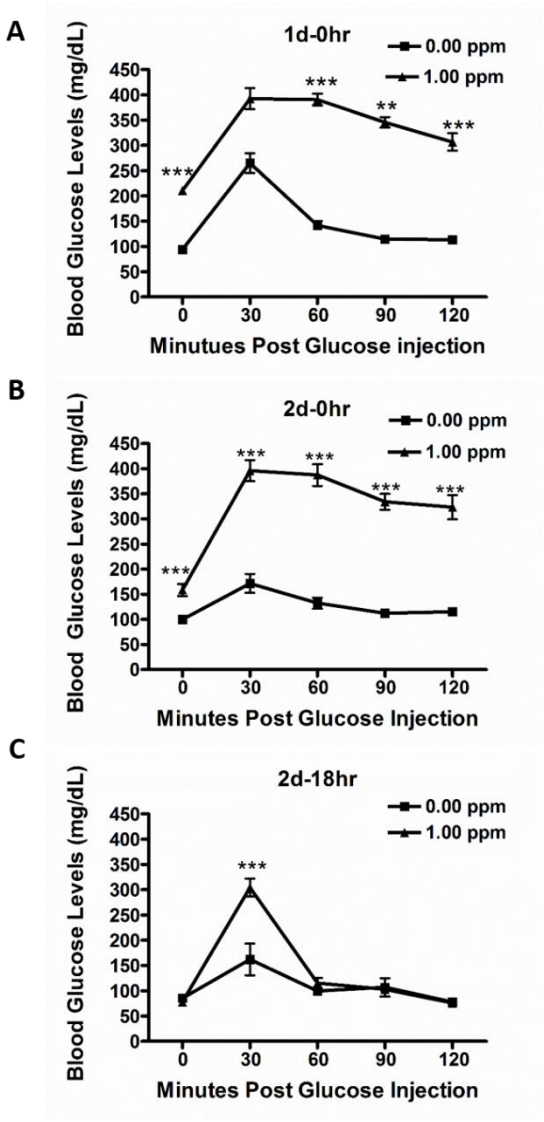

Figure 2.2 Acute ozone exposure induces reversible hyperglycemia and glucose intolerance.

GTT was performed in rats exposed to FA or 1.0 ppm of ozone immediately after first day of 6h ozone (1d-0hr) (A); immediately after second day of 6h ozone (2d-0hr) (B); and at 18h after second day of 6h ozone exposure (2d-18hr) (C; recovery group). Each value represents mean ± SE of n=8/group. *Indicates significant difference from FA exposed rats for a given time point (**=p<.01, ***=p<.001).

Figure 2.3

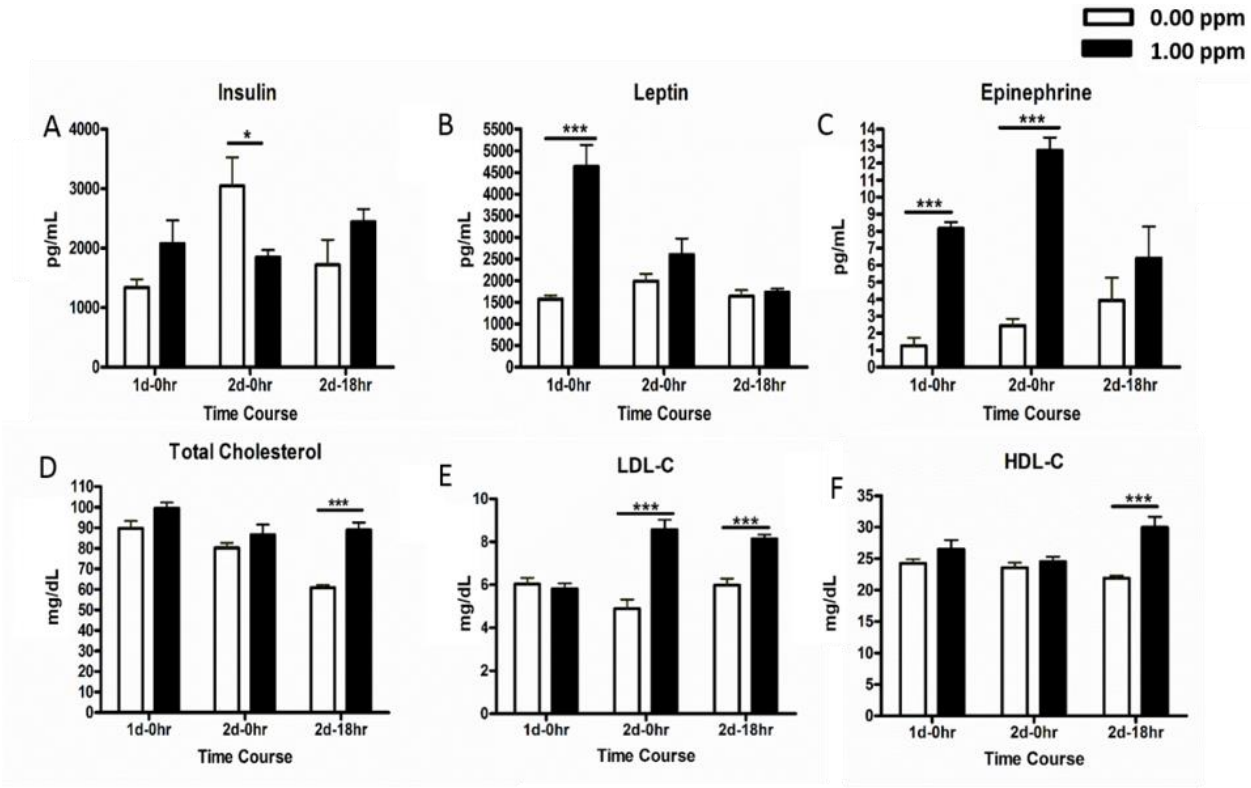

Figure 2.3 Acute ozone exposure alters circulating mediators, metabolic hormones and lipids in rats.

Metabolic hormones and cholesterols were measured in the serum of FA and ozone (1.00 ppm) exposed rats at various time points (1d-0hr, 2d-0hr, 2d-18hr). Each value indicated in the bar graphs represents mean ± SE (n=6-8/group). *Indicates significant difference from time-matched FA exposed rats (*=p<.05, **=p<.01, ***=p<.001).

Figure 2.4

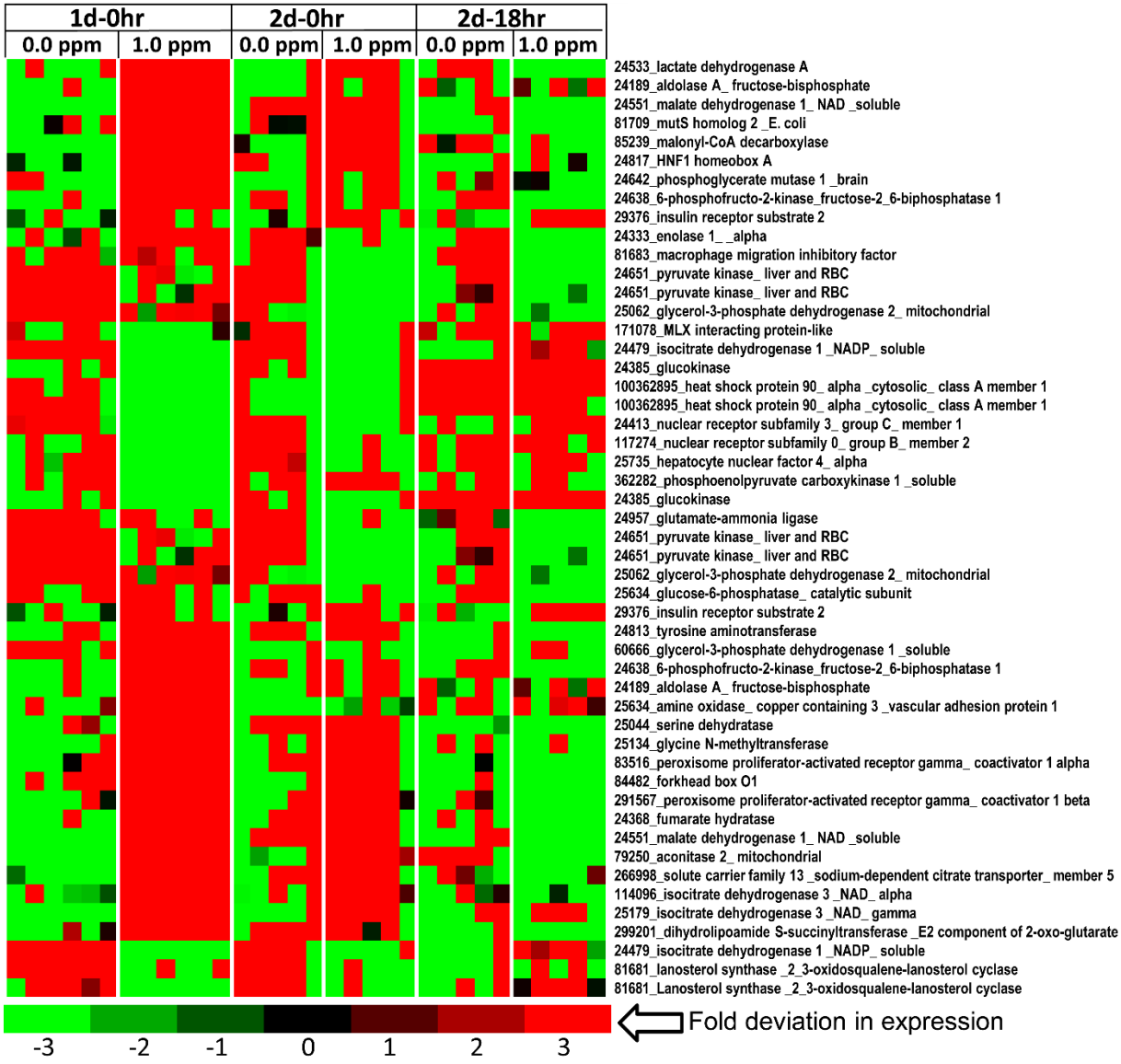
1d-0hr
2d-0hr
2d-18hr
0.0 ppm
1.0 ppm
0.0 ppm
1.0 ppm
0.0 ppm
1.0 ppm
24533_lactate dehydrogenase A
24189_aldolase A_ fructose-bisphosphate
24551_malate dehydrogenase 1_ NAD _soluble
81709_mutS homolog 2 _E. coli
85239_malonyl-CoA decarboxylase
24817_HNF1 homeobox A
24642_phosphoglycerate mutase 1 _brain
24638_6-phosphofructo-2-kinase_fructose-2_6-biphosphatase 1
29376_insulin receptor substrate 2
24333_enolase 1_ _alpha
81683_macrophage migration inhibitory factor
24651_pyruvate kinase_ liver and RBC
24651_pyruvate kinase_ liver and RBC
25062_glycerol-3-phosphate dehydrogenase 2_ mitochondrial
171078_MLX interacting protein-like
24479_isocitrate dehydrogenase 1 _NADP_ soluble
24385_glucokinase
100362895_heat shock protein 90_ alpha _cytosolic_ class A member 1
100362895_heat shock protein 90_ alpha _cytosolic_ class A member 1
24413_nuclear receptor subfamily 3_ group C_ member 1
117274_nuclear receptor subfamily 0_ group B_ member 2
25735_hepatocyte nuclear factor 4_ alpha
362282_phosphoenolpyruvate carboxykinase 1 _soluble
24385_glucokinase
24957_glutamate-ammonia ligase
24651_pyruvate kinase_ liver and RBC
24651_pyruvate kinase_ liver and RBC
25062_glycerol-3-phosphate dehydrogenase 2_ mitochondrial
25634_glucose-6-phosphatase_ catalytic subunit
29376_insulin receptor substrate 2
24813_tyrosine aminotransferase
60666_glycerol-3-phosphate dehydrogenase 1 _soluble
24638_6-phosphofructo-2-kinase_fructose-2_6-biphosphatase 1
24189_aldolase A_ fructose-bisphosphate
25634_amine oxidase_ copper containing 3 _vascular adhesion protein 1
25044_serine dehydratase
25134_glycine N-methyltransferase
83516_peroxisome proliferator-activated receptor gamma_ coactivator 1 alpha
84482_forkhead box O1
291567_peroxisome proliferator-activated receptor gamma_ coactivator 1 beta
24368_fumarate hydratase
24551_malate dehydrogenase 1_ NAD _soluble
79250_aconitase 2_ mitochondrial
266998_solute carrier family 13 _sodium-dependent citrate transporter_ member 5
114096_isocitrate dehydrogenase 3 _NAD_ alpha
25179_isocitrate dehydrogenase 3 _NAD_ gamma
299201_dihydrolipoamide S-succinyltransferase _E2 component of 2-oxo-glutarate
24479_isocitrate dehydrogenase 1 _NADP_ soluble
81681_lanosterol synthase _2_3-oxidosqualene-lanosterol cyclase
81681_Lanosterol synthase _2_3-oxidosqualene-lanosterol cyclase
Fold deviation in expression
-3
-2
-1
0
1
2
3

Figure 2.4 Altered transcription levels of genes involved in glucose metabolism in the livers of ozone-exposed rats.

Functional gene lists were generated by NetAffx queries at the Affymetrix website (www.affymetrix.com) and identified from DEGs list based on the query terms, "glycolysis", "tricarboxylic acid" cycle and "gluconeogenesis". These genes were selected to prepare expression value tables for each sample. Genes were then median centered with average linkage, hierarchically clustered using Cluster 3.0 and displayed through Java Treeview. Red indicates genes that have high expression values across all groups, green indicates genes that have low expression values across all groups, and black indicates median expression. Note that this heat map is truncated to show important clusters affected by ozone exposure (n=5-6/group).

Figure 2.5

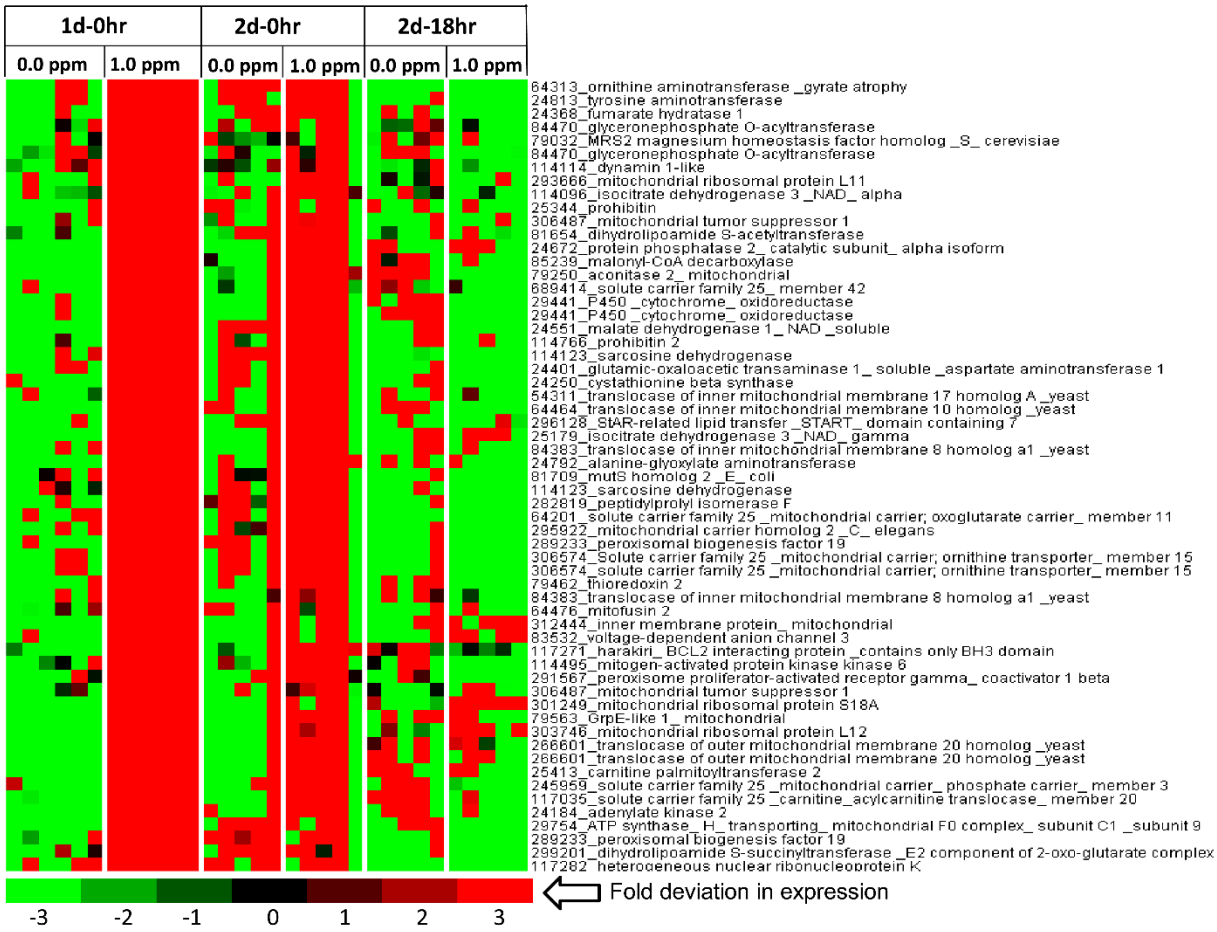
1d-0hr
2d-0hr
2d-18hr
0.0 ppm
1.0 ppm
0.0 ppm
1.0 ppm
0.0 ppm
1.0 ppm
64313_ornithine aminotransferase _gyrate atrophy
24813_tyrosine aminotransferase
24368_fumarate hydratase 1
84470_glyceronephosphate O-acyltransferase
79032_MRS2 magnesium homeostasis factor homolog _S_ cerevisiae
84470_glyceronephosphate O-acyltransferase
114114_dynamin 1-like
293666_mitochondrial ribosomal protein L11
114096_isocitrate dehydrogenase 3 _NAD_ alpha
25344_prohibitin
306487_mitochondrial tumor suppressor 1
81654_dihydrolipoamide S-acetyltransferase
24672_protein phosphatase 2_ catalytic subunit_ alpha isoform
85239_malonyl-CoA decarboxylase
79250_aconitase 2_ mitochondrial
689414_solute carrier family 25_ member 42
29441_P450 _cytochrome_ oxidoreductase
29441_P450 _cytochrome_ oxidoreductase
24551_malate dehydrogenase 1_ NAD _soluble
114766_prohibitin 2
114123_sarcosine dehydrogenase
24401_glutamic-oxaloacetic transaminase 1_ soluble _aspartate aminotransferase 1
24250_cystathionine beta synthase
64311_translocase of inner mitochondrial membrane 17 homolog A _yeast
64464_translocase of inner mitochondrial membrane 10 homolog _yeast
296128_StAR-related lipid transfer _START_ domain containing 7
25179_isocitrate dehydrogenase 3 _NAD_ gamma
84383_translocase of inner mitochondrial membrane 8 homolog a1 _yeast
24792_alanine-glyoxylate aminotransferase
81709_mutS homolog 2 _E_ coli
114123_sarcosine dehydrogenase
282819_peptidylprolyl isomerase F
64201_solute carrier family 25 _mitochondrial carrier; oxoglutarate carrier_ member 11
295922_mitochondrial carrier homolog 2 _C_ elegans
289233_peroxisomal biogenesis factor 19
306574_Solute carrier family 25 _mitochondrial carrier; ornithine transporter_ member 15
306574_solute carrier family 25 _mitochondrial carrier; ornithine transporter_ member 15
79462_thioredoxin 2
84383_translocase of inner mitochondrial membrane 8 homolog a1 _yeast
64476_mitofusin 2
312444_inner membrane protein_ mitochondrial
83532_voltage-dependent anion channel 3
117271_harakiri_ BCL2 interacting protein _contains only BH3 domain
114495_mitogen-activated protein kinase kinase 6
291567_peroxisome proliferator-activated receptor gamma_ coactivator 1 beta
306487_mitochondrial tumor suppressor 1
301249_mitochondrial ribosomal protein S18A
79563_GrpE-like 1_ mitochondrial
303746_mitochondrial ribosomal protein L12
266601_translocase of outer mitochondrial membrane 20 homolog _yeast
266601_translocase of outer mitochondrial membrane 20 homolog _yeast
25413_carnitine palmitoyltransferase 2
245959_solute carrier family 25 _mitochondrial carrier_ phosphate carrier_ member 3
117035_solute carrier family 25 _carnitine_acylcarnitine translocase_ member 20
24184_adenylate kinase 2
29754_ATP synthase_ H_ transporting_ mitochondrial F0 complex_ subunit C1 _subunit 9
289233_peroxisomal biogenesis factor 19
299201_dihydrolipoamide S-succinyltransferase _E2 component of 2-oxo-glutarate complex
117282_heterogeneous nuclear ribonucleoprotein K
Fold deviation in expression
-3
-2
-1
0
1
2
3

Figure 2.5 Acute ozone exposure modulates the expression of genes involved in liver mitochondrial function.

Functional gene lists were generated by NetAffx queries at the Affymetrix website (www.affymetrix.com) and identified from DEGs list based on the query term, "mitochondrial genes". Genes were then median centered with average linkage, hierarchically clustered using Cluster 3.0 and displayed through Java Treeview. (A) A cluster of mitochondria genes showing lower expression after ozone exposure relative to FA. (B) A cluster of genes showing higher expression in rats exposed to ozone relative to FA-exposed rats. Red indicates genes that have high expression values across all groups, green indicates genes that have low expression values across all groups, and black indicates median expression (n=5-6/group).

Figure 2.6.1

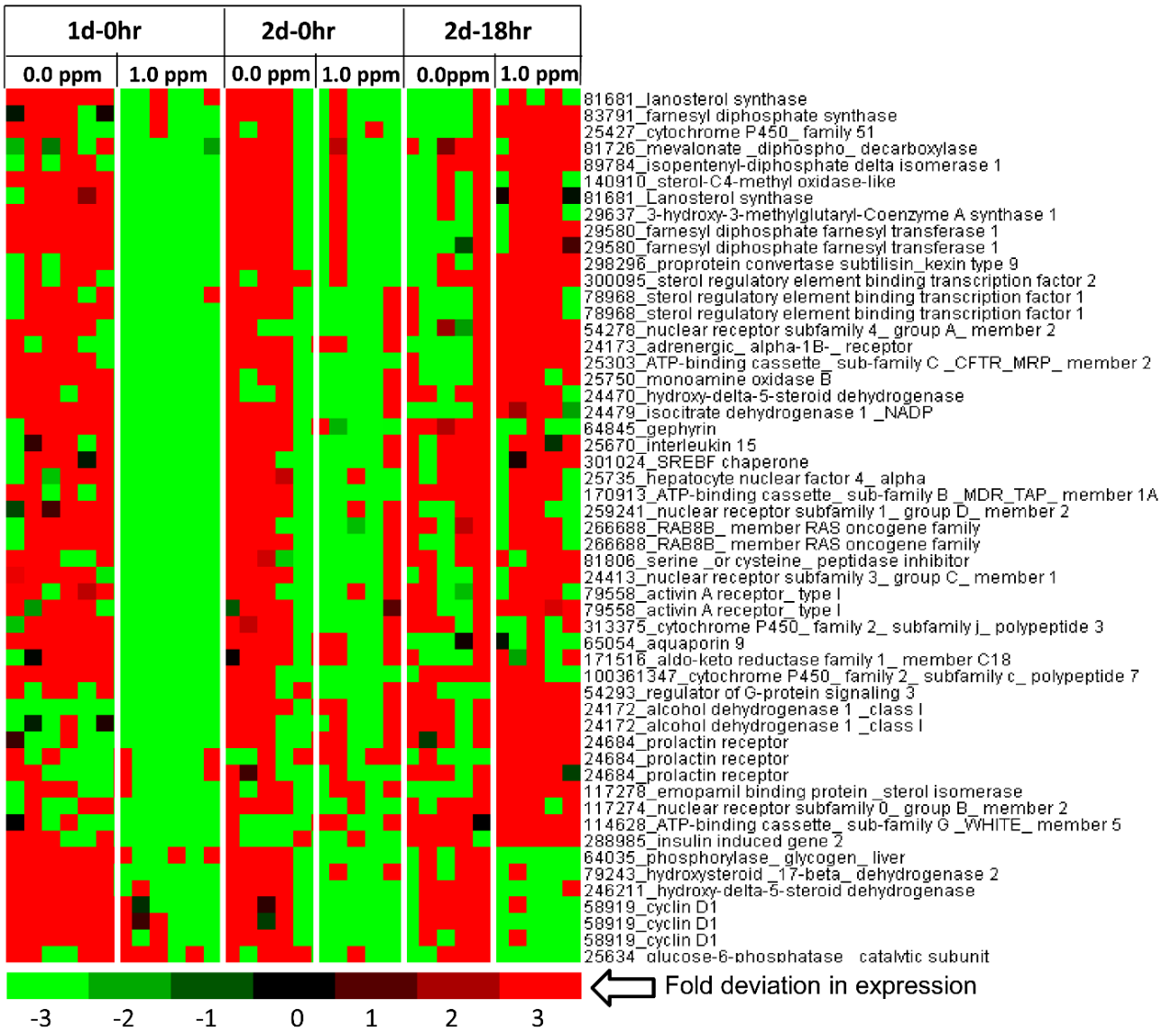
1d-0hr
2d-0hr
2d-18hr
0.0 ppm
1.0 ppm
0.0 ppm
1.0 ppm
0.0ppm
1.0 ppm
81681_lanosterol synthase
83791_farnesyl diphosphate synthase
25427_cytochrome P450_ family 51
81726_mevalonate _diphospho_ decarboxylase
89784_isopentenyl-diphosphate delta isomerase 1
140910_sterol-C4-methyl oxidase-like
81681_Lanosterol synthase
29637_3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1
29580_farnesyl diphosphate farnesyl transferase 1
29580_farnesyl diphosphate farnesyl transferase 1
298296_proprotein convertase subtilisin_kexin type 9
300095_sterol regulatory element binding transcription factor 2
78968_sterol regulatory element binding transcription factor 1
78968_sterol regulatory element binding transcription factor 1
54278_nuclear receptor subfamily 4_ group A_ member 2
24173_adrenergic_ alpha-1B-_ receptor
25303_ATP-binding cassette_ sub-family C _CFTR_MRP_ member 2
25750_monoamine oxidase B
24470_hydroxy-delta-5-steroid dehydrogenase
24479_isocitrate dehydrogenase 1 _NADP
64845_gephyrin
25670_interleukin 15
301024_SREBF chaperone
25735_hepatocyte nuclear factor 4_ alpha
170913_ATP-binding cassette_ sub-family B _MDR_TAP_ member 1A
259241_nuclear receptor subfamily 1_ group D_ member 2
266688_RAB8B_ member RAS oncogene family
266688_RAB8B_ member RAS oncogene family
81806_serine _or cysteine_ peptidase inhibitor
24413_nuclear receptor subfamily 3_ group C_ member 1
79558_activin A receptor_ type I
79558_activin A receptor_ type I
313375_cytochrome P450_ family 2_ subfamily j_ polypeptide 3
65054_aquaporin 9
171516_aldo-keto reductase family 1_ member C18
100361347_cytochrome P450_ family 2_ subfamily c_ polypeptide 7
54293_regulator of G-protein signaling 3
24172_alcohol dehydrogenase 1 _class I
24172_alcohol dehydrogenase 1 _class I
24684_prolactin receptor
24684_prolactin receptor
24684_prolactin receptor
117278_emopamil binding protein _sterol isomerase
117274_nuclear receptor subfamily 0_ group B_ member 2
114628_ATP-binding cassette_ sub-family G _WHITE_ member 5
288985_insulin induced gene 2
64035_phosphorylase_ glycogen_ liver
79243_hydroxysteroid _17-beta_ dehydrogenase 2
246211_hydroxy-delta-5-steroid dehydrogenase
58919_cyclin D1
58919_cyclin D1
58919_cyclin D1
25634_glucose-6-phosphatase_ catalytic subunit
Fold deviation in expression
-3
-2
-1
0
1
2
3

Figure 2.6.2.

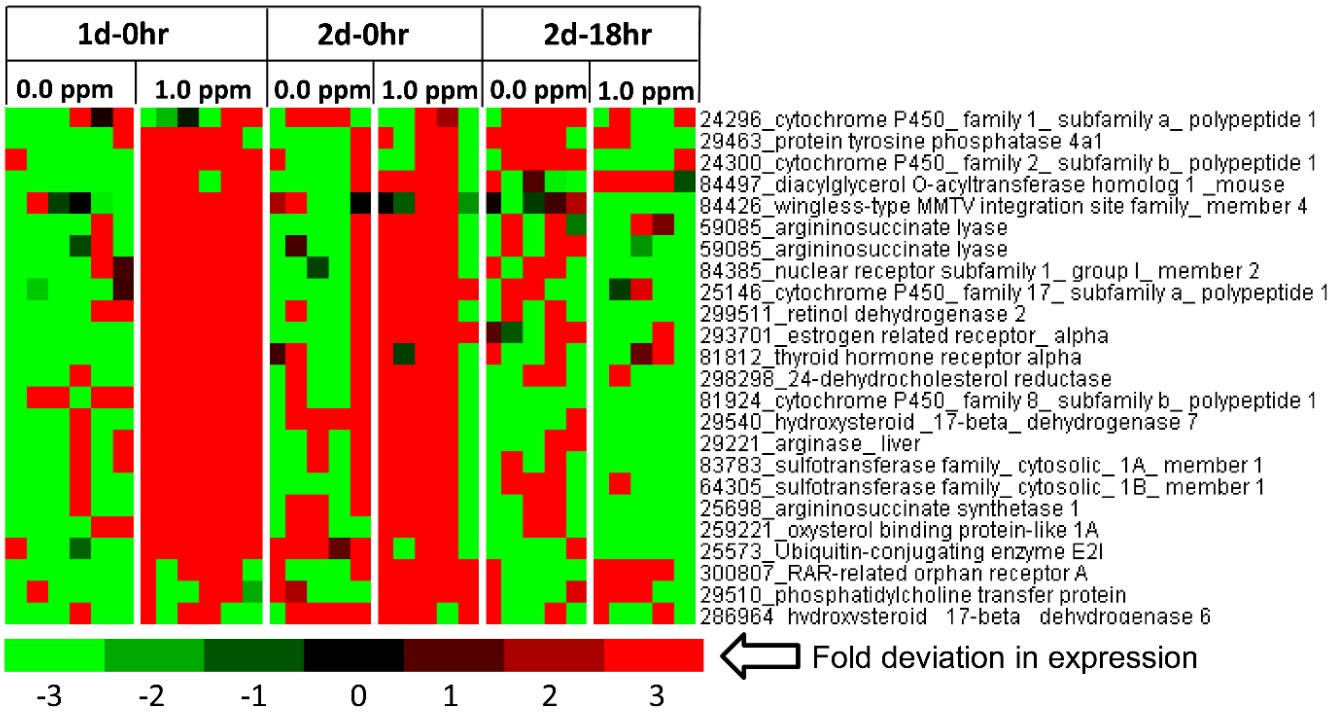

Figure 2.6 Modification of steroid metabolism genes in the livers of rats after ozone exposure.

Functional gene lists were generated by NetAffx queries at the Affymetrix website (www.affymetrix.com) and identified from DEGs list based on the query term, "steroid metabolism genes". Genes were then median centered with average linkage, hierarchically clustered using Cluster 3.0 and displayed through Java Treeview. (A) A cluster of steroid metabolism genes showing lower expression after ozone exposure relative to FA. (B) A cluster of steroid metabolism genes showing higher expression in rats exposed to ozone relative to FA. Red indicates genes that have high expression values across all groups, green indicates genes that have low expression values across all groups, and black indicates median expression (n=5-6/group).

Figure 2.7

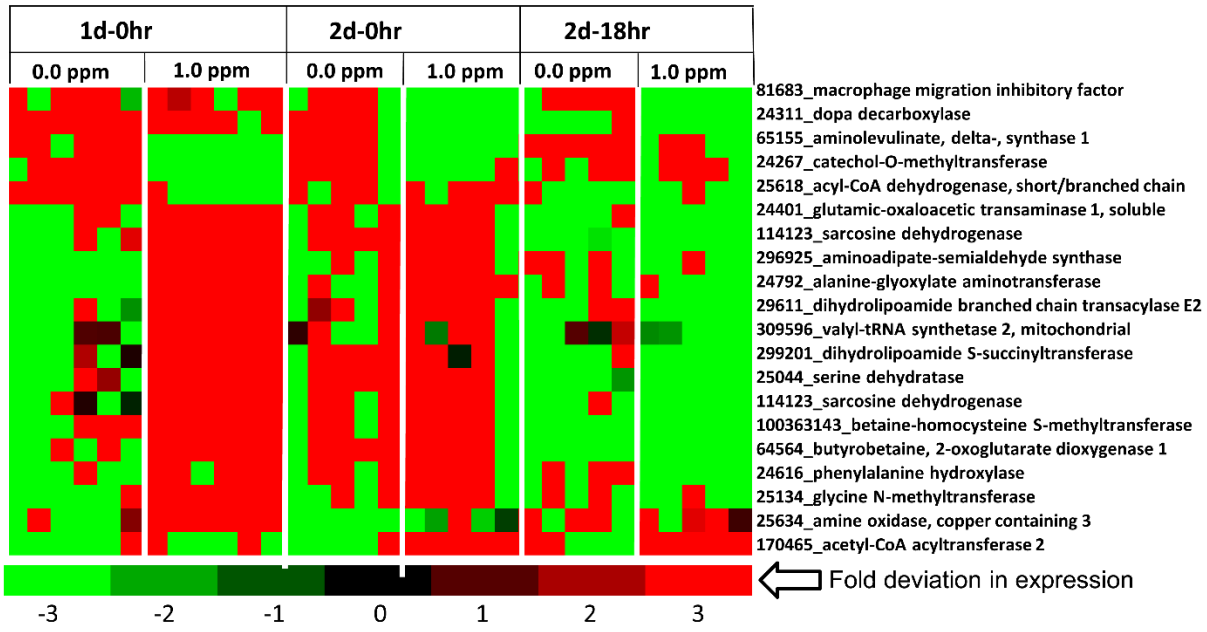

Figure 2.7 Ozone exposure alters expression of genes involved in amino acid metabolism in the liver.

Functional gene lists were generated by NetAffx queries at the Affymetrix website (www.affymetrix.com) and identified from DEGs list based on the query term, "amino acid metabolism". Genes were then median centered with average linkage, hierarchically clustered using Cluster 3.0 and displayed through Java Treeview. Heat map of DEGs with significant ozone effect. Red indicates genes that have high expression values across all groups, green indicates genes that have low expression values across all groups, and black indicates median expression (n=5-6/group).

Figure 2.8

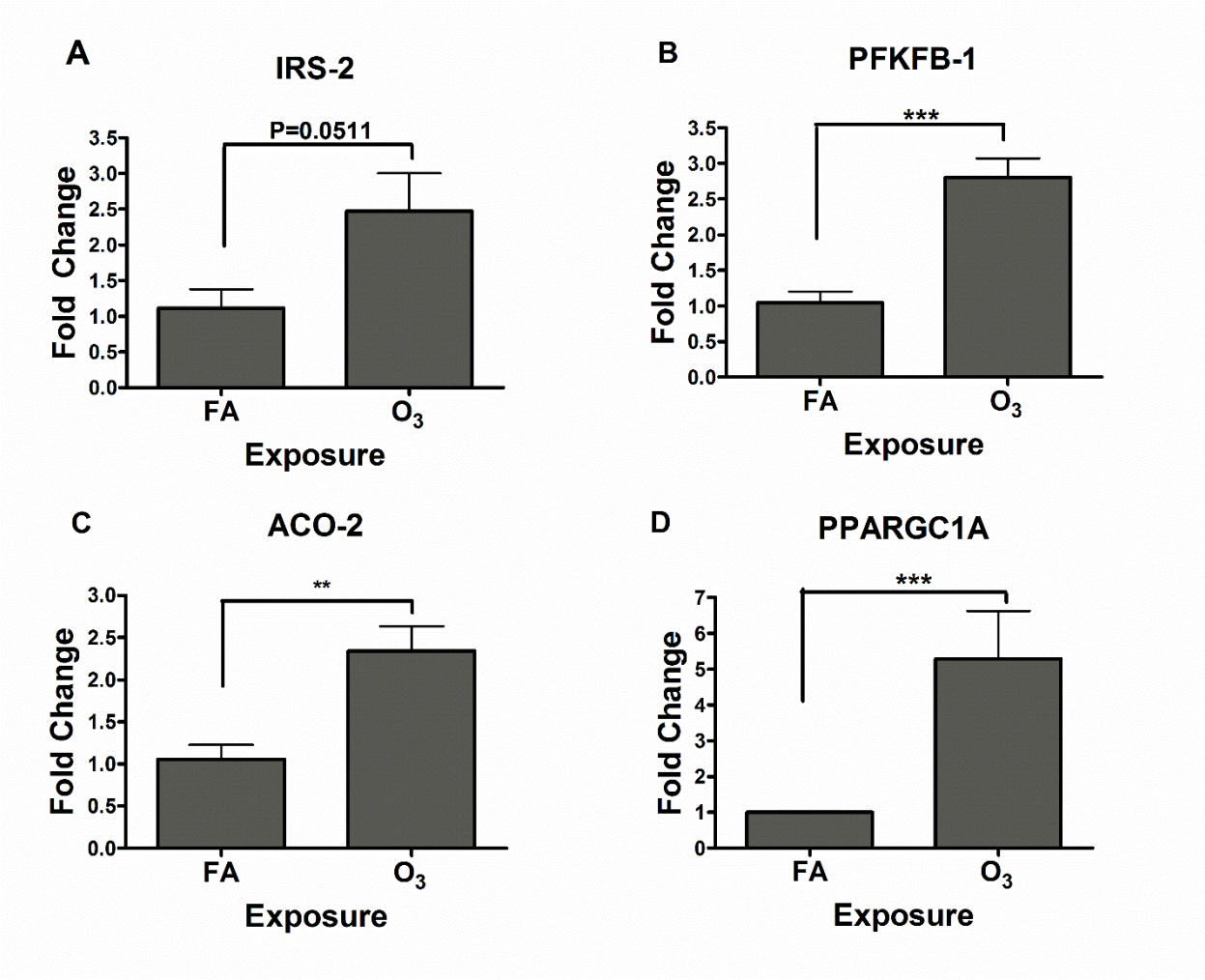

Figure 2.8 Ozone-induced increases in expression of selected genes confirmed using RT-PCR.

Total liver RNA from FA and ozone (O_3)-exposed rats (1d-0hr) was used for RT-PCR (n=6/group). The expression values were first normalized for individual rats using □-actin as a control transcript and then relative fold change from ozone was calculated using FA values. *Indicates significant difference from FA-exposed rats for a given time point (**=p<.01, ***=p<.001).

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CHAPTER 3

OZONE EXPOSURE INCREASES CIRCULATING STRESS HORMONES AND LIPID METABLITES IN HUMANS

3.1 Introduction

Several epidemiological studies nationally and internationally have predicted a link between air pollution and prevalence of diabetes (Pearson et al., 2010; Zanobetti et al., 2014; Eze et al., 2015; Nicole, 2015; Thiering and Heinrich, 2015). It is apparent that the conventional risk factors such as sedentary lifestyle, obesogenic high caloric diets, and/or genetics alone do not fully explain the causal relationship. The contribution of stress and environmental factors has been postulated. Near road air pollution exposure has also been linked to diabetes (Brook et al., 2008; Brook et al., 2013; Weinmayr et al., 2015) and a recent study has associated roadway proximity with hyperglycemia in women (Ward et al., 2015). Although both particulate matter (PM) and gaseous pollutants have been associated with diabetes, in one study this association was presumed to be stronger with gaseous pollutants than PM (Janghorbani et al., 2014).

Subchronic ambient PM exposure studies using rodent models have shown increased adipose and brain inflammation, and liver insulin resistance (Rajagopalan and Brook, 2012;Liu et al., 2014). It has been postulated that pulmonary injury/inflammation, following

inhalation of air pollutants, leads to increased release of cytokines and biologically active mediators causing systemic inflammation and metabolic effects.

However, most air pollution studies examining circulating cytokines fail to demonstrate their increases in the blood. More recently, epidemiological and experimental studies have associated inhaled pollutants with a variety of neural outcomes (Allen et al., 2014; Costa et al., 2014; Calderon-Garciduenas and Torres-Jardon, 2015; Wilker et al., 2015). Specifically, exposure to ozone activates the nucleus tractus solitarius (NTS) and stress responsive regions of the hypothalamus through stimulation of pulmonary vagal C-fibers (Gackiere et al., 2011). Acute ozone exposure can increase levels of circulating stress hormones such as epinephrine and corticosterone in rats (Bass et al., 2013; Thomson et al., 2013; Miller et al., 2015a). Ozone exposure also induces cardiac autonomic effects in humans (Arjomandi et al., 2015), hypothermia and bradycardia in rats (Gordon et al., 2014), and leptinemia, hyperglycemia, glucose intolerance, and global changes in circulating metabolites involved in peripheral glucose, lipid, and amino acid metabolism in rats (Miller et al., 2015b). These changes are reflective of classical stress response-mediated homeostatic alterations involving activation of sympathetic neurons and hypothalamus pituitary adrenal (HPA) axis (Miller et al., 2015a).

We have previously performed global metabolomic assessment of serum to characterize the metabolic response to ozone and gain mechanistic insights in rats (Miller et al., 2015a). The goal of this exploratory study was to perform a global metabolomic assessment of archived human serum samples from a prior clinical study involving ozone

exposure, and determine ozone-induced metabolic changes in humans, establish rodent to human coherence, and identify novel non-protein biomarkers of ozone exposure. We hypothesized that global metabolomic assessment of serum samples collected after air or ozone exposure in humans will reveal metabolic derangements through a neurohormonally-mediated stress response, comparable to rats. Since most human ozone exposures are performed during intermittent exercise and day-light cycle, we predicted that there would be some discrepancies in the metabolite profile of the serum between non-exercising rats (nocturnal) exposed during their non-active circadian cycle and exercising humans. Some of the results of this study have been previously reported in the form of an abstract (Miller et al., 2015b).

3.2 Materials and Methods

3.2.1 Original study design: study population and ozone exposure

Serum samples for this exploratory study were obtained from Clinical Trial NCT01492517. In this trial, subjects were recruited under an EPA contract with Westat Corporation (Rockville, MD). Cardiac and pulmonary function, and systemic effects of ozone on coagulation and inflammatory markers have been previously reported from this study (Devlin et al., 2012). A total of 24 volunteers were involved in the study. The protocol and consent forms were approved by the University of North Carolina, School of Medicine Committee on the Protection of the Rights of Human Subjects and the US Environmental Protection Agency's Institutional Review Board. The exposure was conducted in a randomized crossover design where two clinical visits of each individual were separated by at least two weeks. During each visit, the subjects were exposed to either 0.3 ppm ozone or clean air for 2 hrs in the morning time in a blinded manner. During the exposure, subjects

alternated between 15 minutes of rest and 15 minutes of exercise on a cycle ergometer (Devlin et al., 2012). Exposures were conducted at the US Environmental Protection Agency, Human Studies Facility on the campus of the University of North Carolina, Chapel Hill. Ozone was generated by a silent electric discharge method (model 502; Meckenheim, Bonn, Germany). The exposure chambers were maintained at 40% relative humidity for all exposures. For more details of the exposure system, see the Appendix B and Devlin et al., 2012 publication.

3.2.2 Serum samples and metabolomic analysis

Serum samples collected prior to the start of exposure, immediately post exposure (within 1 hr) and during the next day morning follow up visit were assessed for glucose, triglycerides and cholesterols (LabCorp Inc, Durham, NC) when the clinical study was performed. In the present study, archived serum samples collected immediately post (within 1 hr) after air or ozone exposure were used for exploratory metabolomic assessment. These samples were sent to Metabolon, Inc. (Durham, NC) for global metabolomic analysis (see Appendix B, methods for details). Briefly, each sample was accessioned into the Metabolon Laboratory Information Management System (LIMS) system and assigned a unique identifier. Samples were prepared using the automated MicroLab STAR® system from Hamilton Company (Appendix B, methods). The LC-MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution (Evans et al., 2014). The GC-MS portion of the platform utilized a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron

impact ionization (EI). The informatics system consisted of four major components: the LIMS, the data extraction and peak-identification software, data processing tools, and data interpretation and visualization tools (see methods, and Figures E1 and E2 in the Appendix B). The hardware and software foundations for these informatics components were the LAN backbone and a database server running Oracle 10.2.0.1 Enterprise Edition. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Peaks were quantified using area-under-the-curve. Statistical analysis included log transformation and imputation followed by one way analysis of variance with repeated measures. An estimate of the false discovery rate (q-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. Significant ($p<0.05$) pathway enrichment output (cumulative hypergeometric distribution) was assessed for each of the selected contrasts using MetaboLync, version 1.1.2 (Shannon et al., 2003) to determine the metabolic processes impacted by ozone.

Serum samples collected immediately post air or ozone exposure were also analyzed for insulin, leptin, interleukin-6 (IL-6), and tumor necrosis factor (TNF)-□, using human-specific antibody-based electrochemiluminescence assays (Meso Scale Discovery, Gaithersburg, MD) according to manufacturer's instructions.

3.3 Results

3.3.1 Demographics of human subjects

The clinical study from which serum samples were obtained included 24 healthy, young adult participants; 20 males and 4 females (Table 3.1). The average age of the subjects was 25.6 with a range of 22–30 years. Weights averaged 78.5 kg with a range from 50.7 to 97.3 kg. Eleven subjects had a body mass index between 18.5 and 24.9, and 13 subjects were between 25 and 29.9 (see online data supplement #2 for details on individual subjects). Most of the subjects never smoked. Only three subjects were previous smokers, all before the year 2006. All subjects participated in weekly exercise: 2 mild level (90 to 180 min/week), 9 moderate level (180 to 360 min per week) and 13 high level (over 360 min per week). Participants did not have a recent history of prescription medication use, cardiopulmonary disease, or allergies as determined by a detailed medical history and physical examination. Their specific characteristics are summarized in Table 3.1 and detailed in the Appendix B and online supplement #2.

Heart rate, blood pressure, and serum glucose, triglycerides, and cholesterols measured prior to, immediately post air or ozone exposure, and after one day follow up visit for each subject are provided as line graphs in Appendix B (Figures E3-E4) and individual subject's data in the online supplement #2. There were no marked pre or post air or ozone exposure differences noted in heart rate and blood pressure; however, between subject variability was noted in the levels of triglycerides and cholesterols. Between subject glucose levels varied slightly at pre exposure time but not after post exposure or next day follow up visit.

3.3.2 Ozone exposure increases circulating cortisol but does not change cytokines or HOMA-IR

Cortisol and corticosterone were significantly increased in the serum samples collected after ozone exposure when compared to air (Figure 3.1). In addition, a 1.62-fold increase in corticosterone metabolite 11-dehydrocorticosterone, but not cortisone (see data in the online supplement #2) suggests HPA axis involvement in acute ozone-induced extra pulmonary effects. Serum levels of TNF-α and IL-6 as reported earlier (25) showed no significant difference between air and ozone immediately post exposure (data not shown). Serum leptin and insulin (see Figure E5 in the Appendix B and data in the online supplement #2) were not significantly different between air and ozone samples immediately post exposure. High individual sample variability was noted for 3-4 individuals in the levels of insulin and leptin. Homeostatic model assessment for insulin resistance (HOMA-IR) provided no significant differences between air and ozone exposure (see Figure E5 in the Appendix B for line graph and online supplement #2 for data).

3.3.3 Correlations of subject characteristics with individual metabolites and ozone-induced changes

We attempted to correlate ozone-induced changes (fold-increase) in selected metabolites, including steroid hormones with individual subject's BMI, but no significant relationships appeared to occur (see Figure E6 in the Appendix B.). Although the number of subjects included in the study were small, especially when stratified for their prior exercise habit (as mild, moderate or heavy) or sex, we have provided the correlation plots and results in Figures E7-E8 and results section of the Appendix B. Likewise, circulating levels of metabolites (glucose, triglycerides and cholesterols) post air or post ozone exposure were

analyzed for correlations with BMI, prior exercise habit and sex, and these plots are provided in Figures E9-E11 in the Appendix B. No significant differences were noted between air and ozone exposure for pre exposure normalized glucose, triglycerides and lipids levels (see online data supplement #2 for all individual subject's clinical data).

3.3.4 Ozone exposure changes the profile of circulating metabolites

There were 663 compounds of known identity that were detected in human serum samples (see online data supplement #2 for all original data, normalized data, heat maps and scatter plots). Of those, statistically significant differences were found for 121 biochemicals ($p<0.05$) between air and ozone exposure as determined by ANOVA. Specifically, 85 were increased and 36 were decreased after ozone exposure compared to air. There were also a number of biochemicals for which the difference between the air and ozone exposures approached significance ($0.05<p<0.10$;23 increased and 29 decreased). A pathway enrichment analysis using MetaboLync (27) indicated seven biochemical pathways (sphingolipid metabolism, endocannabinoid synthesis, fatty acid metabolism, β-oxidation, dicarboxylic acid metabolism, steroid hormone biosynthesis and phospholipid metabolism) to be significantly altered by ozone exposure (Figure 3.2). All of these pathways are associated with lipid metabolism.

3.3.5 Ozone exposure increases circulating free fatty acids and lysolipids in humans

One of the most consistent and strongest effects associated with ozone exposure in this study was the pronounced increases in serum free fatty acids (Table 3.2A). There were significant ($p<0.05$) increases in medium- and long-chain free fatty acids in the serum samples collected after ozone exposure relative to air, along with a significant increase in serum glycerol, a marker of lipolysis (Table 3.2A). Additionally, higher levels of a variety of

lysolipids were found in the serum samples collected after ozone exposure relative to air (Table 3.2B), which may be indicative of membrane phospholipid hydrolysis contributing to fatty acid generation. Finally, increasing trends were also noted in sphinganine (P=0.0511) and sphingosine 1-phosphate (P<0.00005) in combination with elevated long-chain fatty acids in the serum samples obtained after ozone exposure relative to the clean air.

3.3.6 Ozone exposure during intermittent exercise increases fatty acid oxidation in human.

There were significant changes in biochemicals associated with fatty acid β-oxidation (Table 3.3). These changes included significantly higher levels of ocanoylcarnitine, decanoylcarnitine and cis-4-decanoylcarnitine together with a trend of an increase in acetylcarnitine (p=0.079; a surrogate for the fatty acid oxidation end product acetyl-CoA) in the serum samples collected after ozone relative to air exposure. Ozone exposure was also associated with increases in serum laurylcarnitine, myristoylcarnitine, palmitoylcarnitine, oleoylcarnitine, linoleoylcarnitine and myristoleoylcarnitine. Long-chain fatty acids (>12 carbons) are conjugated with carnitine to facilitate transport into the mitochondrial matrix where they may undergo fatty acid β-oxidation for energy. These changes were also accompanied by lower levels of carnitine in the serum samples collected after ozone exposure, which may be a marker of increased utilization for acylcarnitine conjugation. Moreover, the dicarboxylates, azelate and 2-hydroxyglutarate were significantly ($p<0.05$) increased in the serum after ozone exposure when compared to air (Table 3.3), and may suggest that the β-oxidation process was saturated, thereby shifting to fatty acid ω-oxidation. Taken together, these findings support increased fatty acid β-oxidation for energy generation following the 2 hr ozone exposure during intermittent exercise. Further indication of increased fatty acid β-oxidation was revealed by the elevation in the ketone body, 3-

hydroxybutyrate (BHBA; Table 3.3), a marker of hepatic fatty acid β-oxidation which can accumulate in cases where the capacity of the TCA cycle is overwhelmed by the availability of substrate.

3.3.7 Ozone exposure increased polyunsaturated fatty acids in the serum.

In addition to the observed increases in free fatty acids and fatty acid oxidation byproducts, n3 and n6 polyunsaturated fatty acids were also significantly ($p<0.05$) increased in the serum from ozone-exposed subjects relative to air and may contribute to production of inflammatory mediators (Figure 3). These included linolenate alpha or gamma (18:3n3 or n6; Figure 3.3A), eicosapentaenoate (EPA 20:5n3; Figure 3.3B), docosapentaenoate (n3 DPA 22:5n3; Figure 3C), docosahexaeonate (DHA 22:6n3; Figure 3.3D), linoleate (18:2n6; Figure 3.3E), dihomo-linolenate (20:3n3 or n6 (DHGLA); Figure 3.3F), and arachidonate (AA) (20:4:6; Figure 3.3G).

3.4 Discussion

Epidemiological studies have associated air pollutants with increased prevalence of type-2 diabetes and changes in markers of insulin resistance (Brook et al., 2008; Pearson et al., 2010; Kim and Hong, 2012; Brook et al., 2013; Fleisch et al., 2014; Zanobetti et al., 2014; Eze et al., 2015; Nicole, 2015; Thiering and Heinrich, 2015; Weinmayr et al., 2015). However, controlled human studies examining systemic metabolic effects and potential mechanisms have not been performed. A number of environmental stressors are postulated to be interactively involved in the development of insulin resistance over a long period of time and linked to lipidemia and high blood glucose. We have recently reported that acute ozone exposure in rodents produces systemic homeostatic changes in circulating metabolites reflective of activation of neuronal stress response pathways (Bass et al., 2013; Miller et al., 2015a). The goal of the current study was to determine if metabolomic assessment of serum samples obtained from a prior clinical study where humans acutely exposed to ozone show similar systemic metabolic alterations as rodents. Serum metabolomic analysis revealed increases in circulating cortisol and corticosterone, but not cortisone, in samples collected after ozone exposure, reflective of an activation of a neurohormonally-mediated stress response in humans. Pathway analysis of metabolites significantly ($p<0.05$) changed in serum after ozone exposure implicated altered lipid metabolic processes. As observed in rodents, increased levels of a number of circulating free fatty acids and glycerols in the serum from ozone-exposed individuals suggested stimulation of adipose lipolysis of triglyceride stores and their liberation into the circulation. Increased lysolipids, likely released from hydrolysis of cellular and membrane phospholipids, and serum polyunsaturated fatty acids in ozone-exposed humans may be linked to proinflammatory mechanisms. Unlike rodents,

samples obtained from humans who exercised intermittently during ozone exposure showed elevated circulating metabolites of β-oxidation, such as ketone bodies, dicarboxylates, and metabolites of ω–oxidation. No ozone effects were noted in HOMA-IR. Overall, this study demonstrates that ozone exposure in humans is associated with increased release of stress hormones causing lipolysis as in rodents, likely through activation of HPA axis. Chronic elevations of these metabolites in the circulation might contribute to insulin resistance and systemic inflammation (Kelly and Ismail, 2015; Magomedova et al., 2015).

In non-exercising rats, acute ozone exposure induces glucose intolerance and increases circulating leptin and epinephrine (Bass et al., 2013; Miller et al., 2015a). Both leptin and epinephrine changes are associated with a reversible decrease in body temperature (Gordon et al., 2014), suggesting involvement of the sympathetic axis and changes in hypothalamic thermoregulation. Increased ACTH and cortisol levels after ozone exposure have also been noted in other rodent studies (Soulage et al., 2004; Thomson et al., 2013). In this study, humans exposed to ozone also presented elevated circulating cortisol and corticosterone, suggesting the activation of the HPA axis, similar to rats. However, unlike rats (Bass et al., 2013; Miller et al., 2015a), no differences were noted in leptin levels in serum samples obtained after ozone exposure in humans (see Figure E5 in the Appendix B). This might be due to the differences in the study design and exposure protocol. The present human study did not involve ozone exposure during resting and exposure occurred only for 2 hrs. Acute ozone exposure has been shown to activate neuro-stress responsive regions and the NTS where pulmonary vagal nerves terminate in the brain (Soulage et al., 2004; Gackiere et al., 2011). Thus, pulmonary C-fibers, likely through regional neurotransmission in the brain, can stimulate sympathetic and/or the HPA axis, leading to the release of stress

hormones from sympathetic nerve endings and also from the adrenal gland (Patterson et al., 2014; Nicolaides et al., 2015). As a result, these stress hormones can target metabolic organs in a tissue specific manner and alter glucose and lipid metabolism through activation of cellular glucocorticoids and adrenergic receptors.

Acute ozone exposure in humans increased circulating long- and medium-chain fatty acids as observed in rats (Miller et al., 2015a). This is likely induced through stress hormone-mediated adipose lipolysis. Endogenous and ingested free fatty acids are re-esterified in adipose tissue as triacylglycerides for storage (Papakova and Cahova, 2015). During a stress response, circulating epinephrine and cortisol through β-adrenergic and glucocorticoid receptors, respectively, can increase hormone-sensitive lipase-mediated lipolysis of adipose triglycerides into free fatty acids and glycerol (Papakova and Cahova, 2015). Ozone-induced elevation of free fatty acids in the serum could lead to uptake of these lipid metabolites by the peripheral tissues, including liver and muscle, for oxidation. In a previous study, we noted that genes involved in mitochondrial metabolism and biogenesis were markedly altered in the livers of ozone-exposed rats (Miller et al., 2015a). Since the metabolic changes occurring immediately following an ozone exposure in humans are likely reversible after termination of exposure, as noted in rats after a single exposure (Miller et al., 2015a), there is likely to be minimal long-term impact on healthy individuals. However, in those with underlying metabolic impairment or those with defects in homeostatic mechanisms, exposure to ozone might be more impactful and may contribute to metabolic imbalance.

Lysolipids, also known as lysophospholipids, were elevated in serum samples obtained after ozone exposure, indicating likely increased phospholipid turnover and plasma membrane remodeling by phospholipases. Although specific tissue sources cannot be

ascertained through serum metabolomic assessment, these lipids through their action on G-protein-coupled lysophospholipid membrane receptors (LPL-R) have been shown to impact a variety of cellular responses, such as cytoskeletal integrity/stability, mitogenesis, inflammation, energy production and lipid metabolism (Gijon and Leslie, 1999). Ozone exposure also increased circulating polyunsaturated fatty acids (Figure 3). In the lung, ozone exposure has been shown to increase polyunsaturated fatty acids, which are formed by a combination of activation of phospholipases and inhibition of free fatty acid esterification pathways (Wright et al., 1994; Madden et al., 1998). The resulting increases in arachidonic acid metabolites can be involved in homeostatic and acute inflammatory responses. For example, arachidonic acid released by phospholipase A2 is metabolized through the lipoxygenase or cyclooxygenase pathways to form eicosanoids, including leukotrienes, prostaglandins, and thromboxanes (Serhan et al., 1996). Ozone exposure was associated with significant increases in plasma thromboxane B2, 6-keto prostaglandin F1 alpha and prostaglandin E1 in guinea pigs (Miller et al., 1987). It is unclear whether the identified mediators shown in Figure 3 of ozone induced phospholipase activation (Kafoury et al., 1998) can exert extra-pulmonary effects. Enzymes involved in lipid metabolism were shown to be altered in a primate model after subchronic exposure to 0.3 ppm ozone (Rao et al., 1985). Interestingly, it has also been demonstrated that cortisol inhibits phospholipase activity and prostaglandin production (Thompson, 2003). Since these lipids were increased in the serum samples obtained after ozone exposure done with intermittent exercise in humans, but not in rats which were exposed during inactivity, the potential mechanism and implication of these findings will require further studies.

Acute ozone exposure might alter mitochondrial β-oxidation involved in catabolism of free fatty acids. Decreases in carnitine and increases in acylcarnitines together with elevated 3-hydroxybutyrate (BHBA), a marker of hepatic β-oxidation, in serum after ozone exposure relative to air suggests changes in fatty acid oxidation that may be attributed to mitochondrial involvement (Aon, Bhatt and Cortassa, 2014). Additionally, increases in dicarboxylic acids (DCAs) in serum after ozone exposure may relate to increased ω-oxidation, a NADPH- and cytochrome P450-dependent process where the omega carbon of a fatty acid is oxidized to an alcohol and then to a carboxylic acid, thereby generating DCA. DCAs are produced by peroxisomes when the supply of fatty acids to the liver exceeds the capacity for β-oxidation and re-esterification. Because subjects during air and ozone exposure underwent an exercise regimen, and exercise can influence the metabolic status, it is likely that the ozone response was influenced by exercise. Exercise-induced oxidative stress (Holland et al., 2015) was postulated to contribute to ozone-induced cytogenetic damage to human lymphocytes after ozone exposure (Powers, Nelson and Hudson, 2011). Interestingly, these changes were not observed in rats exposed to ozone (Costa et al., 2014)) during inactivity. It is noteworthy that the ozone-induced hypothermia in non-exercising rodents exposed during their inactive (daytime) cycle (Gordon et al., 2014) may lead to diminution of peripheral metabolic processes (muscle), while this response may not be apparent in humans exposed during their active cycle (daytime).

There are several limitations of our study. We only performed metabolomic analysis post air or ozone exposure during two clinical visits thus, this study is not a complete crossover design except for clinical measures. Ozone effects were not determined in humans during inactivity/resting and thus, the contribution of exercise and interaction with ozone

can't be assessed directly. Since we are attempting to compare the ozone effects in exercising humans to non-exercising rats, only some speculation of the role of exercise can be made. Moreover, human exposures occurred over 2 hrs, whereas rats were exposed for 6 hrs. The discrepancies in effective lung ozone dose could be partially explained by the evidence that exercising humans will inhale a larger ozone dose relative to non-exercising rats (Hatch et al., 2013). We have demonstrated that acute ozone-induced metabolic changes are reversible the following day despite a continued inflammatory response in rodents (Miller et al., 2015), but we were neither able to analyze the reversibility of these changes in humans nor study the long-term ozone effects. In a previous study involving subchronic episodic exposure of Brown Norway rats, we noted that glucose intolerance still occurred after 12 weekly exposures (Bass et al., 2013). Since, humans are exposed episodically to ozone throughout their lifetime, it is important to determine if persistent episodic metabolic derangement contributes to insulin resistance.

This study is the first to characterize the global metabolic derangement in humans after ozone exposure using a metabolomic assessment. We show that ozone exposure in humans is associated with marked increases in a variety of fatty acids in the circulation together with increases in cortisol and corticosterone, suggesting activation of the neurohormonal stress response pathway and subsequent changes in peripheral metabolic homeostasis. This study establishes the coherence between humans and rodents in ozone-induced stress hormone increase and metabolic effects. Through this approach, novel biomarkers such as cortisol, free fatty acids, monoacylglycerols, and lysolipids for acute pollutant-induced health effects are identified. Further studies will be needed to examine if these responses will persist during chronic exposure and contribute to insulin resistance.

Table 3.1 Demographics of study participants*.

Demographics	Ratio
Gender	
Male	20/24
Female	4/24
Race	
White	22/24
Hispanic	2/24
Smoking	
Never Smoker	21/24
Former Smoker†	3/24
Medication ‡	3/24
Exercise§	
Mild	2/24
Moderate	9/24
Heavy	13/24
	Mean
Age (yr)	25.6±3.8
Height (cm)	177.9±9.3
Weight (kg)	78.5±13.9
Body Mass Index	24.7±3.0
18.5-24.9	11/24
25-29.9	13/24
Systolic	121.0± 8.4
Diastolic	73.9± 6.8
Heart Rate (bpm)	73.4±14.4

*For details on individual subject demographics, see the Appendix B.

†Smoking before the year 2006.

‡Currently on medication (Multivitamins, and/or Lexapro).

§Exercise level

Mild	90 to 180 minutes per week
Moderate	180 to 360 minutes per week
Heavy	over 360 minutes per week

Table 3.2a. Acute ozone exposure increases circulating free fatty acids and monoacylglycerols in humans.

		Fold Change		
Free Fatty Acids	**Metabolite**	**Ozone * / Air**	**p-Value †**	q-Value
Medium-Chain Fatty Acid	caproate (6:0)	1.17	0.0041	0.048
	laurate (12:0)	1.17	0.0649	0.218
	5-dodecenoate (12:1n7)	1.57	0.0058	0.053
Long-Chain Fatty Acid	myristate (14:0)	1.42	0.0102	0.072
	myristoleate (14:1n5)	1.80	0.0005	0.025
	palmitoleate (16:1n7)	1.72	0.0019	0.042
	margarate (17:0)	1.30	0.0097	0.070
	10-heptadecenoate (17:1n7)	1.48	0.0041	0.048
	oleate (18:1n9)	1.38	0.0014	0.034
	cis-vaccenate (18:1n7)	1.20	0.0759	0.238
	10-nonadecenoate (19:1n9)	1.54	0.0042	0.048
	eicosenoate (20:1n9 or 11)	1.44	0.0082	0.065
Fatty Acid, Monohydroxy	3-hydroxyoctanoate	1.48	0.0195	0.115
	3-hydroxydecanoate	1.78	0.0031	0.045
	3-hydroxylaurate	1.77	0.0021	0.043
Glycerolipid Metabolism	glycerol	1.34	0.0072	0.059
Monoacylglycerol	1-stearoylglycerol (1-monostearin)	2.32	0.0046	0.048
	2-stearoylglycerol (2-monostearin)	1.89	0.0028	0.044
	1-oleoylglycerol (1-monoolein)	1.56	0.0000	0.004
	2-oleoylglycerol (2-monoolein)	1.85	0.0047	0.048

*Values indicate relative fold differences for each biochemical between ozone and filtered air samples (n=24/group). A fold change >1 indicates an increase, while fold change <1 indicates a decrease.

† When the p-value is <0.05, the change is considered significant.

Table 3.2b Ozone exposure elevates circulating lysolipid and sphingolipid metabolites in humans.

		Fold Change		
	Metabolite	Ozone * / Air	p-Value†	q-Value
Lysolipids	1-pentadecanoylglycerophosphocholine (15:0)	1.10	0.0901	0.256
	1-palmitoylglycerophosphocholine (16:0)	1.08	0.0035	0.047
	2-palmitoylglycerophosphocholine (16:0)	1.06	0.0787	0.239
	1-palmitoleoylglycerophosphocholine (16:1)	1.11	0.0313	0.155
	1-margaroylglycerophosphocholine (17:0)	1.24	0.0004	0.023
	1-stearoylglycerophosphocholine (18:0)	1.16	0.0027	0.044
	2-stearoylglycerophosphocholine (18:0)	1.19	0.0108	0.075
	1-oleoylglycerophosphocholine (18:1)	1.18	0.0014	0.034
	2-oleoylglycerophosphocholine (18:1)	1.11	0.0665	0.219
	1-linoleoylglycerophosphocholine (18:2n6)	1.08	0.0614	0.218
	2-linoleoylglycerophosphocholine (18:2n6)	1.14	0.0324	0.158
	1-linolenoylglycerophosphocholine (18:3n3)	0.82	0.0856	0.250
	2-linolenoylglycerophosphocholine(18:3n3)	0.85	0.0406	0.176
	1-nonadecanoylglycerophosphocholine(19:0)	1.29	0.0046	0.048
	1-dihomo-linoleoylglycerophosphocholine (20:2n6)	1.22	0.0007	0.028
	1-arachidoylglycerophosphocholine (20:0)	1.44	0.0112	0.076
	1-eicosenoylglycerophosphocholine (20:1n9)	1.33	0.0029	0.044
	2-eicosenoylglycerophosphocholine(20:1n9)	1.48	0.0012	0.033
	1-eicosatrienoylglycerophosphocholine (20:3)	1.22	0.0023	0.044
	2-eicosatrienoylglycerophosphocholine (20:3)	1.20	0.0009	0.032
	1-arachidonoylglycerophosphocholine (20:4n6)	1.15	0.0006	0.028
	2-arachidonoylglycerophosphocholine (20:4n6)	1.16	0.0002	0.014
	1-eicosapentaenoylglycerophosphocholine (20:5n3)	1.17	0.0378	0.170
Sphingolipid Metabolism	sphingosine 1-phosphate	1.37	0.0000	0.042
	sphinganine	1.20	0.0511	0.198

* Values indicate relative fold differences for each biochemical between ozone and filtered air samples (n=24/group). A fold change >1 indicates an increase, while fold change <1 indicates a decrease.

† When the p-value is <0.05, the change is considered significant.

Table 3.3 Ozone exposure during intermittent exercise increases circulating β-oxidation metabolites in humans.

		Fold Change		
Pathway	**Metabolite**	**Ozone * / Air**	**p-Value †**	**q-Value**
Fatty Acid Metabolism (Acyl Carnitine)	acetylcarnitine	1.09	0.0787	0.239
	hexanoylcarnitine	1.22	0.0827	0.246
	octanoylcarnitine	1.32	0.0224	0.127
	decanoylcarnitine	1.42	0.0132	0.085
	cis-4-decenoylcarnitine	1.27	0.0235	0.132
	laurylcarnitine	1.79	0.0094	0.069
	myristoylcarnitine	1.57	0.0360	0.169
	palmitoylcarnitine	1.25	0.0319	0.157
	oleoylcarnitine	1.38	0.0022	0.043
	linoleoylcarnitine	1.31	0.0130	0.085
	myristoleoylcarnitine	1.81	0.0051	0.048
Fatty Acid, Dicarboxylate	2-hydroxyglutarate	1.26	0.0320	0.187
	azelate	1.55	0.0002	0.004
Carnitine Metabolism	carnitine	0.92	0.0102	0.072
Ketone Bodies	3-hydroxybutyrate (BHBA)	1.82	0.0451	0.187

* Values indicate relative fold differences for each biochemical between ozone and filtered air samples (n=24/group). A fold change >1 indicates an increase, while fold change <1 indicates a decrease.
† When the p-value is <0.05, the change is considered significant.

Figure 3.1

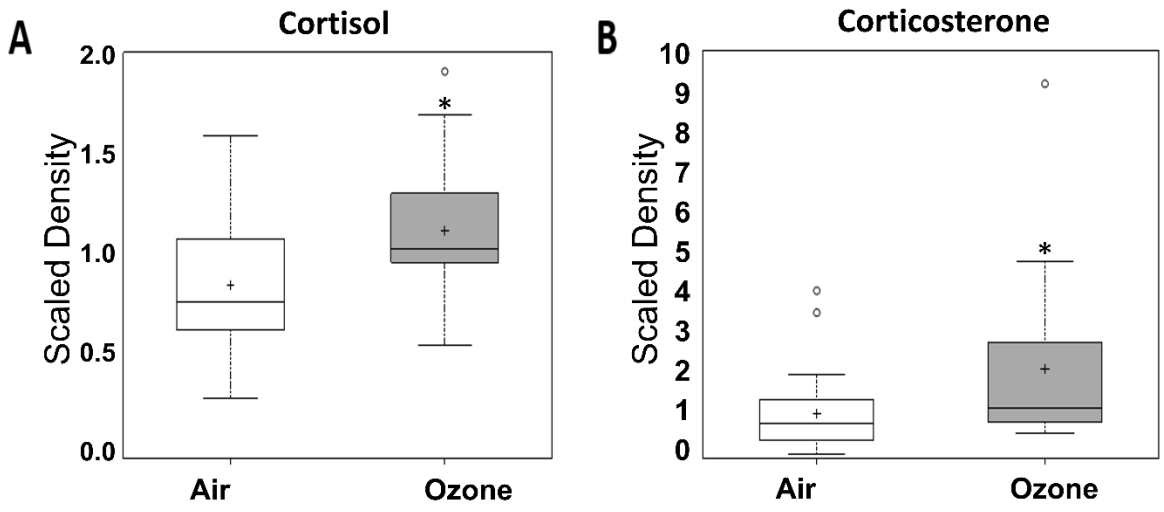

Figure 3.1 Acute two hour ozone exposure increases serum levels of stress hormones, cortisol and corticosterone, in humans. Boxplots convey the spread of the data with the interquartile range represented by the shaded box and the range of the data shown by the whiskers (n=24/group). Outlier values are defined as those that exceed, in either direction, 1.5 times the interquartile range (shown as "o" in each plot). The solid bar across the box represents the median value while the "+" represents the mean. * indicates significantly different from air group ($p<0.05$). For each biochemical, data are median scaled with the median value across all samples set to 1.0. The y-axis thus reflects scaled intensity for each metabolite.

Figure 3.2

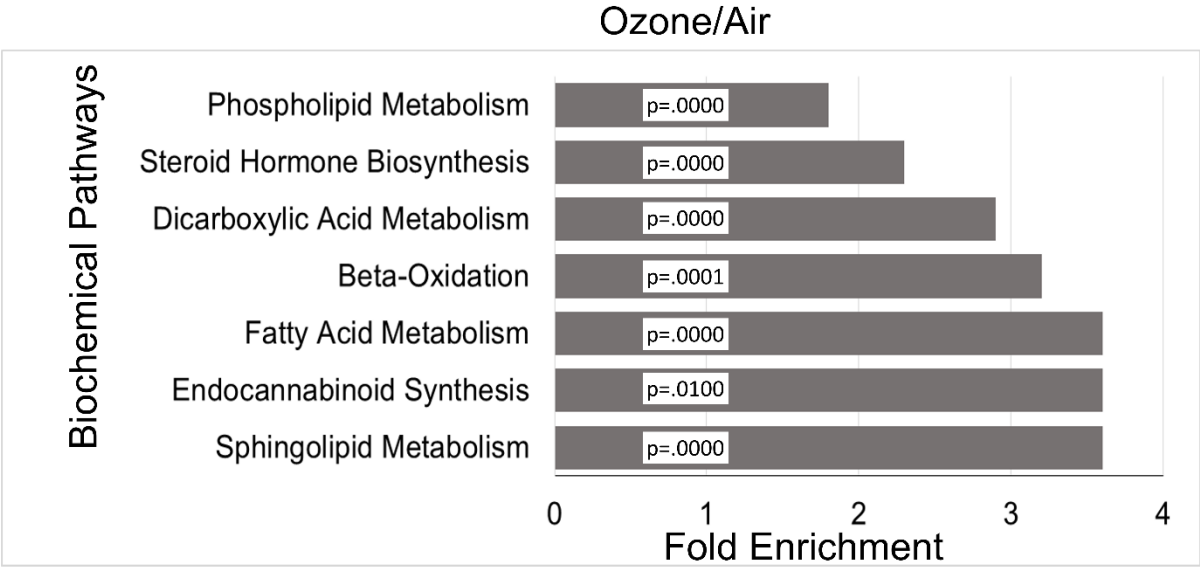

Figure 3.2 Ozone altered lipid metabolism pathways in humans. Pathway enrichment analysis (using MetaboSync) identified seven specific biochemical pathways - sphingolipid metabolism, endocannabinoid synthesis, fatty acid metabolism, β-oxidation, dicarboxylic acid metabolism, steroid hormone biosynthesis and phospholipid metabolism - as having a significant ($p<0.05$) fold enrichment value for ozone exposure. This analysis was based on a number of parameters including the total number of detected metabolites in the study and selected pathway, as well as the abundance of total metabolites associated with each pathway.

Figure 3.3

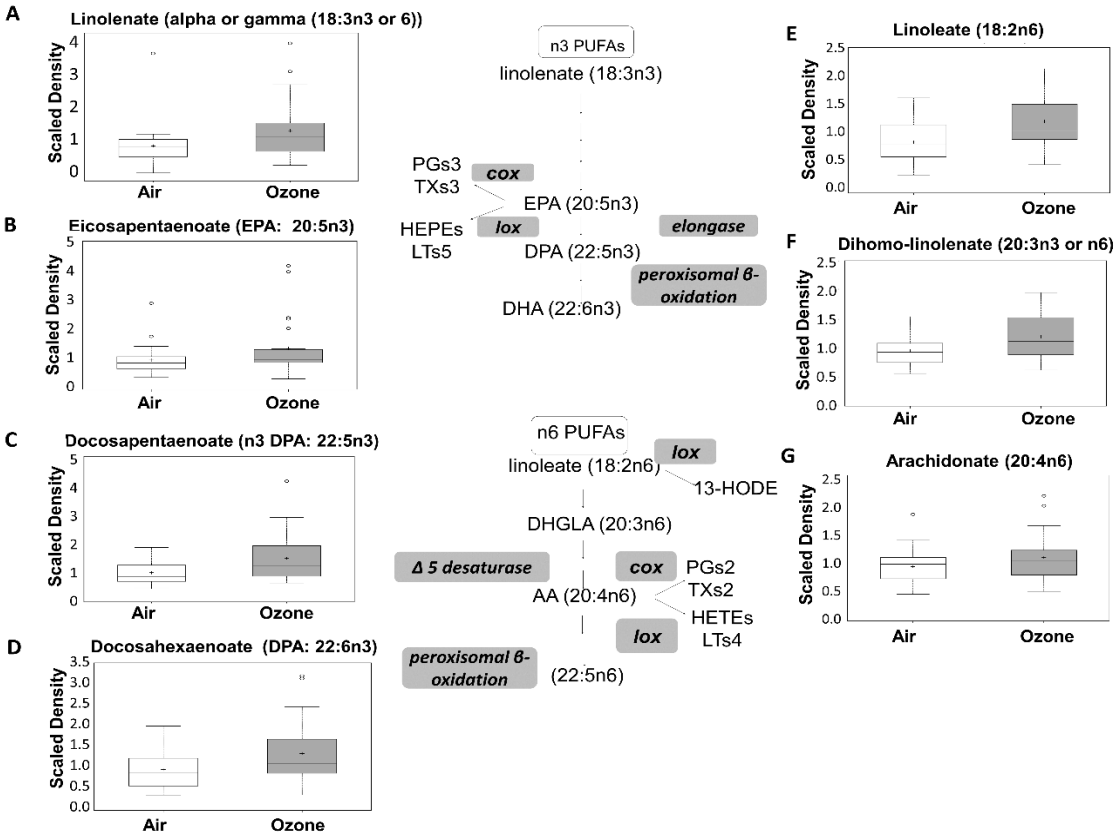
A
Linolenate (alpha or gamma (18:3n3 or 6))
Scaled Density
Air
Ozone
B
Eicosapentaenoate (EPA: 20:5n3)
C
Docosapentaenoate (n3 DPA: 22:5n3)
D
Docosahexaenoate (DPA: 22:6n3)
n3 PUFAs
linolenate (18:3n3)
PGs3
TXs3
cox
EPA (20:5n3)
HEPEs
LTs5
lox
elongase
DPA (22:5n3)
peroxisomal β-oxidation
DHA (22:6n3)
n6 PUFAs
linoleate (18:2n6)
lox
13-HODE
DHGLA (20:3n6)
Δ 5 desaturase
cox
PGs2
TXs2
AA (20:4n6)
HETEs
LTs4
lox
peroxisomal β-oxidation
(22:5n6)
E
Linoleate (18:2n6)
F
Dihomo-linolenate (20:3n3 or n6)
G
Arachidonate (20:4n6)

Figure 3.3 Acute ozone exposure increases circulating polyunsaturated lipids in humans. Polyunsaturated lipids, (A) Linolenate (alpha or gamma; 18:3n3 or n6), (B) Eicosapentaenoate (EPA, 20:5n3), (C) Docosapentaenoate (n3 DPA, 22:5n3), (D) Docosahexaeonate (DHA 22:6n3), (E) Linoleate(18:2n6), (F) Dihomo-linolenate (DHGLA, 20:3n3 or n6) and (G) Arachidonate (AA, 20:4:6) were increased significantly in the serum of ozone-exposed subjects. The metabolic pathways in which these metabolites were generated is summarized in the middle panel. Boxplots convey the spread of the data with the interquartile range represented by the shaded box and the range of the data shown by the whiskers (n=24/group). Outlier values are defined as those that exceed, in either direction, 1.5 times the interquartile range (shown as "o" in each plot). The solid bar across the box represents the median value while "+" represents the mean. For each biochemical, data are median scaled with the median value across all samples set to 1.0. The y-axis thus reflects scaled intensity for each metabolite. * indicates significantly different from air group ($p<0.05$).

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CHAPTER 4

ACUTE OZONE-INDUCED PULMONARY AND SYSTEMIC METABOLIC EFFECTS ARE DIMINISHED IN ADRENALECTOMIZED RATS

4.1 Introduction

Epidemiological studies have demonstrated a positive association between air pollution and increases in type II diabetes, hyperglycemia, glucose intolerance and increased homeostasis model assessment (HOMA) index (Brook et al., 2008; 2013; Rao et al., 2015; Thiering and Heinrich, 2015; Hu et al., 2015). The mechanisms by which air pollutants impact metabolic processes and change clinical indicators of insulin resistance are still largely unknown. Also not known is how air pollutants can induce changes in pathways involving lipid and amino acid metabolism (Miller et al., 2015), which can contribute to insulin resistance and steatohepatitis. It has been hypothesized that systemic inflammation, endoplasmic reticulum stress, and/or autonomic activation induced by air pollutant exposure are involved in insulin resistance (Rajagopalan and Brook, 2012).

Ambient ozone has been studied for decades for its pulmonary and cardiovascular effects

Recently, the interest in ozone health effects has been rejuvenated for two reasons: 1) its levels are anticipated to increase as a result of climate change (Fann et al., 2015), and 2) its has been increasingly recognized that even though ozone is less likely to translocate beyond the lung, it causes a myriad of systemic effects (Miller et al., 2015). While ambient particulate matter (PM) exposures have been linked to the onset and/or exacerbation of peripheral insulin resistance (Rajagopalan and Brook, 2012), ozone has also been associated with the incidence of diabetes (Ren et al., 2010; Janghorbani et al., 2014). In addition, new studies show that ozone exposure induces a variety of neurological effects (Akhter et al., 2015; Chounlamountry et al., 2015 Calderón-Garcidueñas et al., 2015). Ozone exposure stimulates afferent vagal sensory nerves that terminate in stress-responsive hypothalamic regions in the central nervous system (Gackiere et al., 2011). Acute ozone exposure has been shown to alter neuronal catecholamine biosynthesis and increase circulating stress hormones (Soulage et al., 2004; Thompson et al. 2013; Bass et al., 2013; Miller et al., 2015). Our recent studies have demonstrated that rats exposed acutely to ozone develop lung injury/inflammation, hyperglycemia, glucose intolerance, and lipidemia, which are associated with increases in circulating epinephrine and cortisol (Bass et al. 2013; Miller et al. 2015). We have also shown that acute ozone produces hypothermia and bradycardia (Gordon et al., 2014). Collectively, these data suggest that ozone peripheral metabolic effects are likely mediated by central sympathetic and hypothalamus-pituitary-adrenal (HPA) stress axis activation.

The neuronal stress response, activated as a result of bodily injury or threat, initiates physiological mechanisms that provide integrated communication between all organ systems (Smith and Vale, 2006). The initial event includes the release of hypothalamic

paraventricular nucleus (PVN)-derived corticotrophin releasing hormone and the activation of catecholaminergic neurons within the locus coeruleus (LC), which results in the activation of the HPA axis and sympathetic axis, respectively (Navarro-Oliveira et al., 2000; Ulrich-Lai and Herman, 2009). The priority of the neuronal stress response is to channel energy substrates to appropriate tissues to restore equilibrium, which is mainly achieved by elevated circulating stress hormones (i.e. induction of gluconeogenesis, glycogenesis, muscle proteolysis, and adipose lipolysis) (Smith and Vale, 2006). Acute ozone-induced peripheral metabolic changes we observed in humans and rats are similar to homeostatic changes in response to stress (Bass et al., 2013; Miller et al., 2015, 2016). However, it is not known if these stress hormones can contribute to pulmonary injury and inflammation induced by exposure to air pollutants.

In response to HPA activation, the adrenal cortex secretes glucocorticoids (cortisol in humans and corticosterone in rats). In contrast, the adrenal medulla can be directly stimulated by the sympathetic efferent nerves, resulting in the secretion of epinephrine and/or norepinephrine (Goldstein, 2010). Adrenalectomy has been used to examine the role of these hormones in 1) neuronal regulation of the HPA axis (Kaminski and Watts, 2012; Weidenfeld and Feldman, 2000; Helmreich et al., 1996), 2) regeneration of adrenals over time (Rebuffat et al., 2007), and 3) endogenous corticosteroid biogenesis (Freel et al., 2007; Bykowski et al., 2007). Surgical removal of adrenal medulla can allow one to examine the role of epinephrine while total bilateral adrenalectomy can be used for examining the roles of epinephrine and cortical steroidal hormones.

In the present study, we test the postulate that acute ozone-induced pulmonary and systemic metabolic effects are mediated through adrenal-derived epinephrine and

corticosterone. Further, that bilateral adrenal demedullation (DEMED) will selectively dampen some effects, while bilateral total adrenalectomy (ADREX) will have more profound effect on ozone-induced systemic metabolic impairment, and pulmonary injury and inflammation. We examined pulmonary and systemic metabolic effects of ozone exposure together with an assessment of adrenal-derived hormones in rats that underwent sham surgery, DEMED or ADREX.

4.2 Materials and Methods

4.2.1 Animals

Healthy male Wistar Kyoto (WKY) rats (250-300g) were purchased from Charles River Laboratory (Raleigh, NC) at 6-8 weeks of age. Rats were housed (n=2/cage or as indicated below) in polycarbonate cages containing beta chip bedding in an isolated room in an animal facility maintained at 21 ± 1ºC, 50 ± 5% relative humidity and held to a 12h light/dark cycle. The animal facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animals received standard (5001) Purina pellet rat chow (Brentwood, MO) and water ad libitum unless otherwise stated. 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 (IACUC; Permit Number: 16-03-003). Animals were treated humanely and all efforts were made for alleviation of suffering.

4.2.2 Animal surgery and recovery protocol

At 12-13 weeks of age, rats underwent surgical procedures. Anesthesia was achieved using an intraperitoneal injection of ketamine/xylazine (25 mg/2 mg in 1mL saline/kg), followed by a subcutaneous injection of buprenorphine (0.02 mg/kg) for analgesia. Eye

ointment was applied to rats to prevent drying during surgery. Isoflurane was also used if any signs of movement were noticed during the procedure. Following anesthesia, rats were placed in sternal recumbency for Charles River Surgeons to perform SHAM, DEMED or ADREX) using surgical protocols established at Charles River Inc. Both (right and left) glands were removed for ADREX, while the DEMED involved bilateral removal of medulla only. The SHAM group underwent similar procedures as DEMED or ADREX rats except that adrenals were not removed. The muscle wall incisions were closed with absorbable suture. Skin incisions were then closed with stainless steel surgical wound clips. Immediately following surgery, animals were placed on heated pads and observed until recovery from anesthesia. Once recovered from anesthesia, animals were injected subcutaneously with Meloxicam analgesic (0.2mg/mL/kg). Additional two doses of Buprenorphine were injected (0.02mg/mL/kg) subcutaneously every 8-12 hours. All drugs used in surgery were purchased from Henry Schein Animal Health Inc. (Dublin, OH). Following surgery, the animals were single housed using Enviro Dry enrichment/nesting material and provided powdered food. The rats that underwent ADREX were provided with saline (0.9% NaCl) as drinking water to maintain water/salt balance. All animals were allowed 96 hours to recover prior to their exposure to air or ozone. We believe that 4 days was an appropriate time as the weight gain was stablized (Table 4.1), there were no clinical signs of distress noted, food and water consumption were normal and the recovery appeared near complete. Furthermore, a number of studies involving adrenalectomy previously used a 4 day recovery time (Osterlund et al., 2013.; Sakakibara et al., 2014). We wanted to avoid the influence of secondary changes that might occur with a longer recovery time frame in the absence of aldosterone-mineralocorticoids, which regulate salt-fluid balance, blood volume and cardiovascular

function. The timeline of the surgery, ozone exposure, in-life testing and necropsy are shown in figure 4.1.

4.2.3 Ozone generation and animal exposures

Ozone was produced from oxygen by a silent arc discharge generator (OREC, Phoenix, AZ), and its entry into the Rochester style "Hinners" chambers was controlled by mass flow controllers (Coastal Instruments Inc., Burgaw, NC). The ozone concentrations in the chambers were recorded continuously by photometric ozone analyzers (API Model 400, Teledyne Instruments; San Diego, CA). Mean chamber air temperature and relative humidity were 23.3°C (74^0F) and 46%, respectively. Each surgery group (SHAM, DEMED, ADREX; n=20-24/surgery group) was randomized and subdivided into air or 1.0 ppm ozone exposure, 4h/day for either 1 day or 2 days (n=4 for SHAM-air, n=6 for all other exposure groups). This ozone concentration is much higher than what one would expect environmentally. However, ozone concentrations as high as 0.4 ppm can be expected in tropical hot climates with high anthropogenic pollution (WHO, 1974). It has been shown that in rats once inhaled, ozone deposition in the lung is 3-4 times less than what humans will experience (Hatch et al, 1994). We have shown that humans experience metabolic effects of ozone at 0.3 ppm (Miller et al., 2016).

For the 1 day group, rats were necropsied immediately following a 1 day, 4h exposure (1-D group), whereas for the 2 day group, rats were necropsied immediately following the second day of a 4h ozone exposure (2-D group). Only the 2-D group underwent glucose tolerance testing (GTT) immediately following the first day of exposure. As observed in our prior study, the ozone effects are evident immediately following the first and also second day exposure. Since GTT takes ~ 3 hours and a large bolus of glucose is injected in the animals,

which may require several hours for blood glucose levels to return to baseline, we decided to do GTT immediately following first day exposure in rats assigned for 2-D exposure. Another group of rats, which did not undergo GTT, was necropsied immediately following first day of exposure (1-D) to correlate with changes in GTT. Since lung injury and inflammation generally takes several hours to peak after ozone exposure, and the ozone effects are maximum on the second day, we believed that 2-D exposure was necessary to determine the extent of lung damage/injury, while at the same time determining systemic responses immediately following the second day.

4.2.4 Glucose tolerance test (GTT)

Immediately following the first day of ozone exposure, rats assigned to the 2-D exposure group underwent GTT (rats were fasted for ~6h prior to testing). Baseline blood glucose concentrations were measured by pricking the distal surface of rats' tails using a sterile needle. A Bayer Contour glucometer (Leverkusen, Germany) was used to determine blood glucose levels using test strips. After the first measurement, rats were given an intraperitoneal injection of glucose (20% D-glucose; 10ml/kg in saline; Sigma-Aldrich, St Louis, MO). Measurement with the glucometer was repeated every 30 min over the course of 2h.

4.2.5 Whole body plethysmography

Pulmonary ventilatory parameters were examined prior to the first day of exposure in the morning (6:00am), immediately following first ozone exposure (11:30am) and in the morning prior to second day of exposure (6:00am) in the 2-D group. A four chamber whole-body plethysmography system using Buxco BioSystem XA software (Buxco Electronics, Wilmington, NC) was used to measure minute volume, tidal volume, breathing frequency,

and the enhanced pause (Penh), which is presumed to provide an index of airflow limitation and often used as a surrogate for bronchoconstriction (Hamelmann et al. 1997). Each rat was placed in a plethysmograph chamber and allowed 1 min prior 5 min to adapt prior to assessing the respiratory parameters. Respiratory parameters were computed as described earlier (Kodavanti et al., 2005).

4.2.6 Necropsy and sample collection

The 1-D and 2-D groups were necropsied immediately following the first and second 4h ozone exposure, respectively. Rats were weighed and anesthetized with an overdose of Nembutal (Virbac AH, Inc., Fort Worth, TX; 50-100 mg/kg, i.p.). Blood samples were collected through an abdominal aortic puncture. Complete blood counts were performed using a Beckman-Coulter AcT blood analyzer (Beckman-Coulter Inc., Fullerton, CA, USA). All blood samples were centrifuged and aliquots of serum and plasma stored at -80°C until analysis. Bronchoalveolar lavage (BAL) was performed through tracheal tubing using 37°C phosphate buffer saline at a volume of 28mL/kg body weight. Aliquots of BAL fluid were used to determine total cell counts with a Z1 Coulter Counter (Coulter, Inc., Miami, FL) and cell differentials were performed on cytospin slides stained with Diff-quick (Fischer Scientific, Pittsburgh, PA) as previously described (Bass et al., 2013). The cell-free BAL fluid (BALF) was used to analyze protein, albumin, and lactate dehydrogenase (LDH) activity as previously described (Bass et al., 2013).

4.2.7 Plasma and serum snalysis

Adrenaline and noradrenaline plasma levels were measured using kits from Rocky Mountain Diagnostics (Colorado Springs, CO) per the manufacturer's protocol. Serum corticosterone concentrations were analyzed employing an immunoassay kit (Arbor Assay,

Ann Arbor, MI) per the manufacturer's protocol. Total cholesterol and triglycerides were measured in serum samples using kits from TECO Diagnostics (Anaheim, CA). Non-esterified free fatty acids were measured in serum by a coupled enzymatic reaction and the resultant hydrogen peroxide detection using a colorimetric probe as per the manufacturer's protocol (Cell Biolabs, Inc, San Diego, CA). The kit protocols for measuring lipids were modified for use on the Konelab Arena 30 system (Thermo LabSystems, Espoo, Finland). Branched chain amino acids (BCAA) were measured in serum using ELISA kits and the protocol based on chemiluminescence detection (Abcam, Cambridge, MA).

4.2.8 Statistics

GTT data were analyzed using a two-way repeated measure multivariate analysis of variance (MANOVA) where serial blood glucose measurements were incorporated in the analysis (Graphpad prism 4.03 software). Area under the curve (AUC) for GTT was determined by the trapezoidal method and analyzed for statistical significance by ANOVA followed by a Duncan's multiple range test. Other variables were analyzed using a two-way ANOVA model (independent variables being exposure and surgical manipulation). The two assumptions associated with ANOVA models are equality of variances and normality of errors (residuals). We used Levene's test to assess the equality of variances and Shapiro Wilk's test to examine the normality of errors. The ANOVA assumptions for serum adrenaline, serum corticosterone, serum cholesterol, serum FFA, BALF protein, BALF albumin, BALF LDH, BALF neutrophils, BALF eosinophils and PenH did not satisfy desired equal variance and/or normality. These data were then transformed to satisfy two assumptions (log or square root as appropriate). Pairwise comparisons were performed as

subsets of the overall ANOVA. The level of significance was set at 0.05. No adjustments were made for multiple comparisons.

4.3 Results

4.3.1 Body weights

Body weights were closely examined after surgery, throughout recovery and during ozone exposure. The SHAM and DEMED groups did not lose body weight as a result of surgery as determined 4 days after surgery or during the course of air exposure over 2 days (Table 4.1). In general, ADREX group showed a slight but significant decrease in body weight gain after surgery compared to other surgery groups. Ozone exposure on the second day tended to reduce body weight in all rats; however, this effect was not significant relative to time-matched air groups.

4.3.2 ADREX, DEMED and ozone impacted circulating adrenal-derived hormones

To determine ozone-induced changes in adrenal-derived hormones and the effectiveness of surgical intervention, we evaluated levels of circulating adrenaline, noradrenaline and corticosterone. Concentrations of adrenaline, derived from the medulla, were markedly reduced in all DEMED and ADREX rats, regardless of exposure (Fig. 4.2A). Ozone significantly increased adrenaline plasma levels in SHAM rats on day 1 and day 2 compared to air exposure (Fig. 4.2A). Noradrenaline plasma levels were not altered by exposure or surgical interventions (Fig. 4.2B). DEMED caused a slight reduction in the levels of circulating corticosterone, which was significant only in 2-D air-exposed rats. Ozone exposure in SHAM rats tended to increase levels of serum corticosterone on day 1, but the difference was statistically insignificant when compared to time-matched air group

(Fig. 4.2C). Corticosterone levels dropped to nearly zero in all ADREX rats. Ozone did not affect corticosterone levels in the DEMED and ADREX groups (Fig. 4.3C).

4.3.3 Acute ozone-induced glucose intolerance is inhibited by ADREX

GTT was conducted in SHAM, DEMED, and ADREX groups immediately following the first day of ozone exposure in the 2-D group to determine the relative contribution of adrenal-derived hormones to acute ozone-induced glucose metabolic effects. Glucose tolerance was not affected by ADREX or DEMED in air-exposed rats (Fig. 4.3). The SHAM group exposed to ozone demonstrated significant fasting hyperglycemia and glucose intolerance compared to its matching air group (Fig 4.3A) as observed in our previous study (Miller et al., 2015). The DEMED rats showed marked suppression of ozone–induced hyperglycemia and glucose intolerance throughout the testing period (Fig. 4.3A). There was nearly a complete reversal of ozone-induced fasting hyperglycemia and glucose intolerance in ADREX rats (Fig. 4.3A). AUC calculations further revealed that ozone-induced glucose metabolic effects were almost completely eliminated in ADREX rats (Fig. 4.3B).

4.3.4 Acute ozone-induced increases in circulating lipids and protein metabolites are reduced in rats with prior ADREX

The activation of the stress response pathway and subsequent release of adrenal stress hormones are central to changes in peripheral metabolism. Our prior study demonstrated that exposure to ozone resulted in increased circulating FFA and BCAA in rats (Miller et al., 2015). Although prior metabolomic assessment showed a small increase in serum cholesterol after a 6h ozone exposure in our previous study (Miller et al., 2015), in this study 4h ozone exposure for 1-D or 2-D did not affect serum cholesterol levels in any group regardless of prior surgical procedure (Fig. 4.4A). ADREX rats exposed to air had increased triglycerides

at 1-D compared to SHAM rats (Fig. 4.4B). Serum triglycerides were significantly increased after day 1 of ozone exposure in SHAM rats (Fig. 4.4B). Ozone-induced triglyceride increase was smaller in DEMED rats while ADREX was associated with nearly a complete negation of ozone effect in the 1-D group (Fig. 4.4B). Circulating FFA were not affected by DEMED or ADREX as evident in air-exposed rats. FFA tended to increase in SHAM rats ($p=0.15$) when exposed to ozone on 1-D; however, this increase was not observed in DEMED or ADREX rats (Fig. 4.4C).

Air-exposed ADREX group showed significant increases in circulating BCAA on 1-D relative to SHAM rats. On day 1, BCAA also tended to increase ($p=0.13$) in the SHAM ozone-exposed group but the difference remained insignificant. On day 2, however, BCAA levels significantly increased in the SHAM rats exposed to ozone. Importantly, this ozone-induced increase in BCAA did not occur in the ADREX group (Fig. 4.4D).

4.3.5 Ozone-induced ventilatory changes are reduced in ADREX rats

Whole body plethysmography was used to assess breathing parameters. DEMED or ADREX did not significantly affect breathing frequency, tidal volume (data not shown), minute volume or PenH as determined prior to exposure in all rats or in the air groups post exposure (Fig. 4.5). Ozone exposure did not change body weight-normalized minute volume in any group (Fig. 4.5A). Immediately following 1-D of ozone exposure, PenH was markedly increased in SHAM and DEMED rats. This ozone-induced increase in PenH was significantly lower in ADREX rats compared to SHAM rats (Fig. 4.5B). The morning after the first ozone exposure, the ozone-induced increases in PenH were still apparent in SHAM and DEMED rats but the levels were much lower compared to the measurement taken immediately after the first ozone exposure. In the morning after the first ozone exposure, the

ADREX ozone-exposed group did not present significant increases in PenH compared with surgery-matched air control.

4.3.6 Ozone-induced lung injury and inflammation are diminished by DEMED and ADREX

Ozone exposure for 1-D increased markers of vascular protein leakage including BALF total protein and albumin in SHAM and DEMED groups compared to surgery-matched air controls (Fig. 4.6A and B). Ozone-induced protein and albumin increases in the SHAM rats were more pronounced on 2-D when compared to 1-D. The ADREX group did not display any ozone-induced increases in protein or albumin following 1-D ozone exposure. Likewise, ozone exposure did not increase BALF protein or albumin in DEMED and ADREX rats on 2-D (Fig. 4.6A and B). Ozone exposure led to increases in BALF LDH activity (a marker of lung cell injury) on day 1 in SHAM rats (Fig. 4.6C). Ozone-exposed DEMED and ADREX rats did not show any increase in BALF LDH activity.

Lung inflammatory responses to ozone were assessed by determining BALF cell differentials. The number of macrophages in BALF did not change significantly as a result of DEMED or ADREX in air exposed rats. Ozone exposure also did not change the number of BALF macrophages (data not shown). The baseline levels of neutrophils were slightly higher in ADREX rats exposed to air when compared to other surgery groups (Fig. 4.7A). Significant increases in BALF neutrophils occurred on 1-D and 2-D in SHAM rats after ozone exposure. This ozone-induced increase in neutrophils was much more pronounced on day 2 when compared to day 1 in SHAM rats (Fig. 4.7A). The increase in neutrophils following ozone exposure was significantly reduced in DEMED (SHAM>DEMED) rats. Surprisingly, the effect of ozone on neutrophils was nearly abolished in ADREX rats, especially on 2-D. On day 1, only the ADREX rats exposed to ozone showed increases in

BALF eosinophils compared to air controls (Fig. 4.7B). On day 2, eosinophil levels in ADREX rats exposed to ozone remained higher than the air group.

To determine systemic inflammatory response, hematological parameters were assessed. The circulating white blood cells (WBC) were significantly decreased after ozone exposure on 1-D and 2-D in SHAM rats (Fig. 4.7C). Ozone exposure led to a small decrease in WBC on day 1 also in DEMED rats, but did not decrease WBC in ADREX rats at any time point. ADREX and DEMED caused an increase in circulating platelets (Fig. 4.7D). Ozone exposure led to increases in circulating platelets in SHAM and DEMED rats but not in ADREX rats on 1-D.

4.4 Discussion

We have recently shown that acute ozone-induced peripheral metabolic alterations are associated with elevated adrenal-derived stress hormones in humans and animals (Bass et al., 2013; Miller et al., 2015, 2016). The goal of the current study was to evaluate if the adrenal-derived stress hormones are essential for mediating acute ozone-induced metabolic and pulmonary effects in rats. We used surgical DEMED to remove the medulla, which produces catecholamines in response to sympathetic stimulation, and ADREX where the medulla as well as the cortex are removed in rats. The cortex produces corticosteroids in response to pituitary-derived ACTH via the HPA axis, when exposed to ozone, SHAM-surgery rats produced typical systemic metabolic effects and lung injury similar to that observed in our previous study (Miller et al., 2015). These effects were characterized by hyperglycemia, glucose intolerance, increased levels of epinephrine, corticosterone, circulating FFA, BCAA and decreased circulating WBC, together with increased pulmonary ventilatory changes, vascular leakage and neutrophilic inflammation. All these ozone effects were partially or fully blocked in rats with bilateral DEMED or ADREX. Thus, we demonstrate that not only ozone-induced metabolic impairment, but also pulmonary injury and inflammation are largely modulated through adrenal-derived stress hormones likely through sympathetic stimulation and activation of the HPA axis.

Ozone produces nociceptive stimuli and activates pulmonary vagal C-fibers that trigger various cardiopulmonary reflexes (Jimba et al., 1995; Schelegle et al., 1993). Ozone activates the nucleus tractus solitarius (NTS) and also stimulates various hypothalamic stress–responsive regions-including the PVN and LC (Gackiere et al., 2011). Consequently, activation of these limbic regions could lead to changes in heart rate, peripheral

vasoconstriction, pulmonary arterial hypertension and increased capillary permeability (Kubin et al., 2006; Nicolaides et al., 2015). However, these neurohormonal pathways have not been linked to ozone-induced pulmonary injury and systemic metabolic effects.

Diminution of circulating epinephrine and corticosterone confirmed successful removal of adrenals. As expected, ozone-induced increases in epinephrine and corticosterone in SHAM rats were not apparent in either DEMED and ADREX rats. This confirms our earlier findings that acute ozone increases the release of stress hormones likely through activation of sympathetic adrenomedullary and the HPA axis (Bass et al., 2013; Miller et al., 2015). Since ~80% of circulating noradrenaline is released from peripheral sympathetic nerve terminals and only 20% produced by the adrenal medulla (Patel et al., 2000; Esler et al., 1990), DEMED or ADREX did not affect those levels. The lack of ozone effect on noradrenaline raises the question as to the role of efferent sympathetic stimulation in mediating systemic metabolic and pulmonary effects. Since it is technically not feasible to remove the cortex and leave the medulla intact, the question about the contribution of cortex-derived hormones in response to the release of medullary epinephrine remains.

During the stress response, epinephrine initiates a transient increase in circulating glucose by the stimulation of hepatic glycogenolysis and gluconeogenesis, and inhibition of glucose disposal by insulin-dependent tissues (Sherwin and Sacca, 1984; Sherwin et al., 1980). Similarly, cortisol suppresses insulin-mediated glucose uptake through the glucocorticoid receptors (Steiner et al., 2014). The ozone-mediated increase in circulating epinephrine and cortisol in SHAM rats, together with the diminution of ozone-induced hyperglycemia and glucose intolerance in ADREX and DEMED rats, demonstrates that the adrenal-derived stress hormones are essential for the observed impairment in glucose

metabolism. This corroborates with previous findings where DEMED suppressed hypoxia-induced hyperglycemia and insulin secretion (Shin et al., 2014). The stress hormones are known to prompt adipose tissue lipolysis, leading to the release of FFAs in the circulation, which are known to interfere with insulin signaling and contribute to insulin resistance (Shulman et al., 2000). We show that ozone-induced increases in FFA and triglycerides were diminished in ADREX and DEMED rats, further confirming the role of stress hormones in the observed lipidemia. Activation of glucocorticoid receptors in response to increases in stress hormones results in muscle protein catabolism and release of BCAA in the circulation (Braun and Marks, 2015). An increase in serum BCAA levels were apparent in the present study in SHAM rats exposed to ozone, similar to our previous study (Miller et al., 2015), but not in ADREX and DEMED rats. These data provide further confirmation that ozone induces stress hormone-mediated muscle protein catabolism. Accumulation of BCAA have been implicated in initiating pancreatic β-cell dysfunction, inflammation, endoplasmic reticular stress and apoptosis (Lynch and Adams, 2014). Since metabolic effects of ozone were found to be reversible upon termination of exposure (Miller at al., 2015), the long-term consequences remain to be investigated.

The contribution of pulmonary neural refluxes mediated by different areas within the NTS in ozone-induced ventilatory changes has been described (Schelegle et al., 1993; Schelegle and Walby, 2012; Taylor-Clark and Undem, 2010; Chen et al., 2003). It has been shown that ozone exposure induces reflux bronchoconstriction (Schelegle and Walby, 2012). In our study, PenH, which is often linked to airflow limitation, was increased in SHAM rats exposed to ozone. This might be due to changes in airway smooth muscle contractility. β receptors, which are heavily distributed in large and small airways, have been shown to play

a major role in regulating smooth muscle tone by epinephrine and corticosterone (Bosse, 2014). DEMED had no effect on PenH responses after ozone, whereas the reduction in ozone-induced increases in PenH by ADREX suggests that corticosterone, epinephrine and other circulating factors might be directly or indirectly playing a role in mediating the ozone-induced ventilatory changes.

There are likely a number of mechanisms influencing the development of pulmonary edema after ozone exposure in SHAM rats. First and foremost, ozone exposure can directly injure capillary walls, leading to leakage of protein in the alveoli (Vella et al., 2015; Banks et al., 1990). Pulmonary edema can also occur through acute sympathetic activation (neurogenic), which may lead to baroreflex-induced bradycardia. The resulting enhancement of venous return can cause pulmonary vascular congestion and increase hydrostatic pressure, which can damage the capillary wall causing fluid leakage (Šedý et al., 2015). Increased circulating epinephrine can also cause systemic capillary pressure increase, which may contribute to pulmonary edema after ozone exposure (Krishnamoorthy et al, 2012). Increases in circulating epinephrine and corticosterone, and ozone-induced cardiac depression together might affect the protein leakage. We have previously shown that ozone induces bradycardia and hypothermia (Gordon et al., 2014). Ozone has also been shown to decrease blood pressure (Uchiyama et al., 1986) and produce cardiac depression (Wagner et al., 2014) which may contribute to lung protein leakage. The finding that pulmonary protein leakage and lung cell injury are nearly abolished in ADREX and DEMED rats suggests that release of stress hormones, likely through sympathetic and HPA-activation, were critical in ozone-induced protein leakage.

Ozone-induced extravasation of inflammatory cells into the lung through activation of signaling mechanisms involving transendothelial migration has also been well characterized (Alexis and Carlsten, 2014; Hollingsworth et al., 2007). Consistent to these studies, ozone exposure in SHAM rats caused neutrophilic lung inflammation and depletion of circulating white blood cells, likely due to their extravasation into the lungs. It is generally believed that corticosteroids inhibit inflammation (Edwards, 2012); however, numerous studies have shown that acute stress-mediated increases in corticosterone and epinephrine can stimulate leukocyte trafficking and extravasation at the site of injury in an immune cell-specific manner (Dhabhar, 2009; Dhabhar et al., 2012). The precise mechanisms by which distinct types of chronic stresses modulate inflammatory processes still remain an area of intense research. The selective diminution of ozone-induced neutrophil influx but stimulation of eosinophil influx, together with the lack of an ozone-induced decrease in circulating lymphocytes in ADREX and DEMED rats suggests contribution of the dynamic and temporal changes in corticosteroids and epinephrine (Dhabhar, 2012). Interestingly, ozone-mediated thymus atrophy was reduced in adrenalectomized mice (Dziedzic and White, 1986), suggesting that adrenal hormones might modulate ozone-induced inflammatory response through changes in thymus function. These data together provide the insights into the contribution of central neurohormonal mechanisms in modulating pulmonary inflammatory responses induced after ozone exposure.

In this study, we show that acute ozone exposure induced a classical stress-associated systemic metabolic response and pulmonary injury/inflammation in SHAM rats, similar to what was seen in our previous studies (Bass et al., 2013; Miller et al., 2015). This response is characterized by increased levels of epinephrine and corticosterone, hyperglycemia, glucose

intolerance, increased circulating FFA, increased BCAA, and decreased circulating white blood cells as has been observed in our previous study (Miller et al., 2015). Surgical removal of the adrenal medulla or whole adrenal glands resulted in nearly complete inhibition of these ozone-induced metabolic and pulmonary effects. Thus, we show adrenal-derived hormones mediate systemic metabolic impairment and acute pulmonary injury/inflammation likely through sympathetic and HPA-activation.

Table 4.1 Effect of surgery and ozone exposure on body weights in rats.

	Time Point of Body Weights (g)									
	Prior to Surgery		Day 1 exposure		Day 1 exposure		Day 2 exposure		Day 2 exposure	
Group	**Air**	**Ozone**	**Air**	**Ozone**	**Air**	**Ozone**	**Air**	**Ozone**	**Air**	**Ozone**
SHAM	321± 32	318± 18	322±39	320±13	314±31	307±15	324±30	313±25	309± 26	299±15
DEMED	318± 16	321± 17	319±14	327±15	310±12	315±9	318±14	316±17	305±13	305±12
ADREX	321± 15	321± 18	305±16	299±16†	292±13	292± 9	297±13	296±14	291±11	281±9†

SHAM=sham surgery; DEMED=bilateral adrenal demedullation; ADREX=total bilateral adrenalectomy. † Significant ($p \leq 0.05$) ADREX effect relative to sham surgery.

Figure 4.1

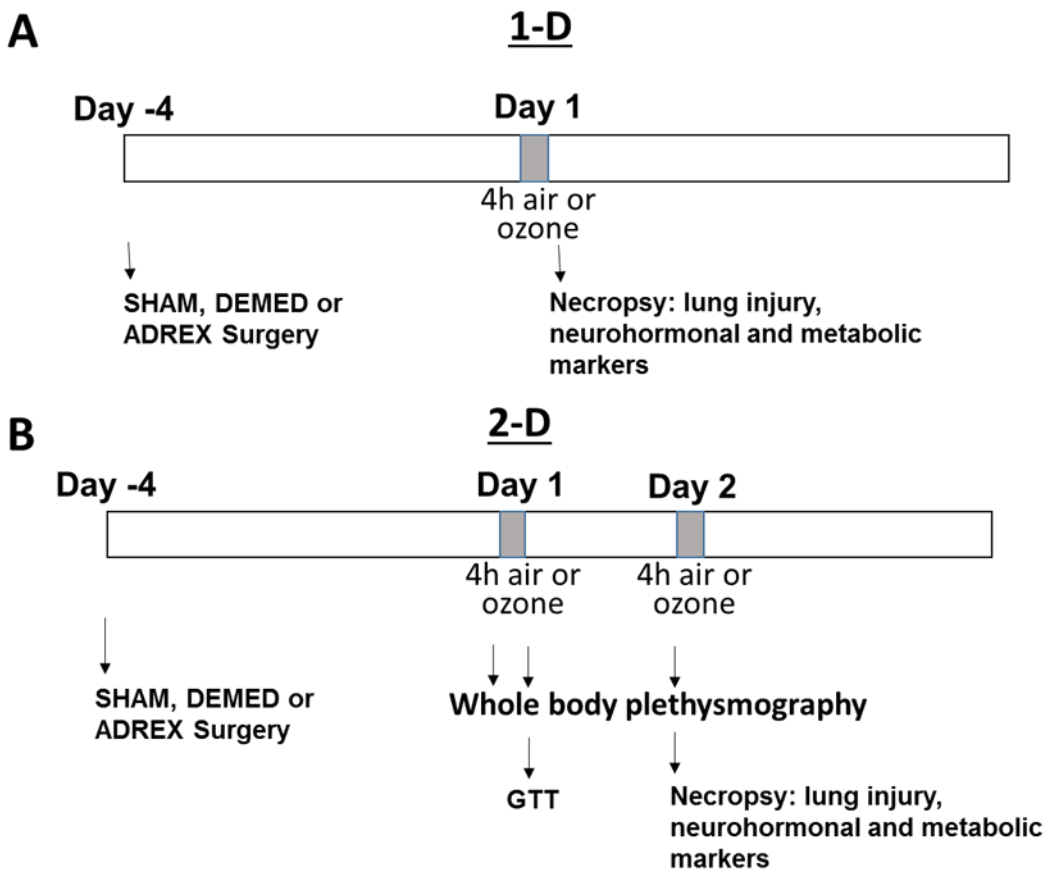

Figure 4.1. Experimental Timeline. Four days prior to air or ozone exposure, WKY rats underwent SHAM, DEMED, ADREX. After 4 days of recovery, rats were exposed to air or ozone for 4h/day for 1 day (A, 1-D) or 4h/day for 2 consecutive days (B, 2-D). GTT was performed in the 2-D exposure group only after the first day of exposure. Immediately following each exposure, rats were necropsied for analysis of lung injury and inflammation, neurohormones and metabolic biomarkers. WKY, Wistar Kyoto: SHAM, sham surgery; DEMED, bilateral adrenal demedullation; ADREX, total bilateral adrenalectomy; GTT, glucose tolerance test.

Figure 4.2

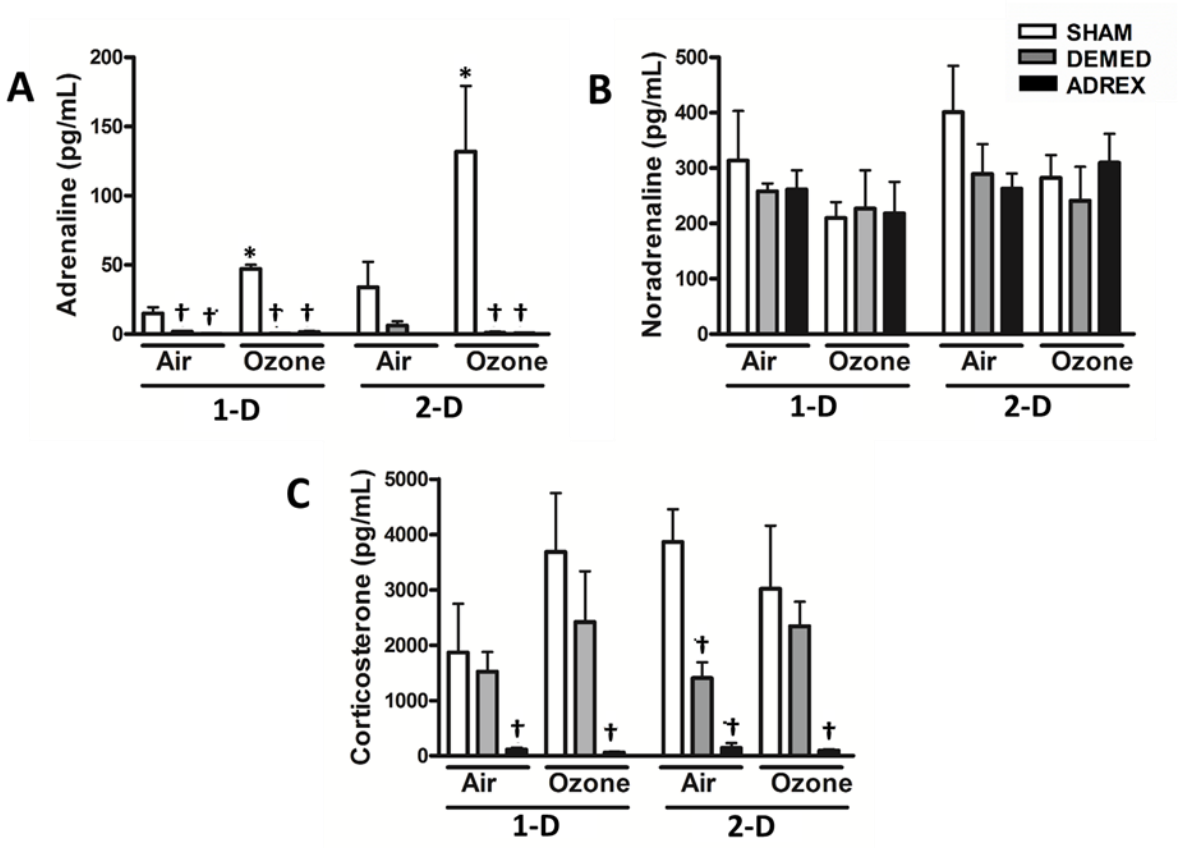

Figure 4.2 Changes in the levels of circulating hormones after air or ozone exposure in SHAM, DEMED, or ADREX rats. Adrenal cortex and medulla-derived hormones were measured in the serum and plasma, respectively, from rats exposed to air or ozone at both time points (1-D, 2-D), A, Adrenaline (Epinephrine), B, Noradrenaline (Norepinephrine), and C, Corticosterone. Values indicate mean ± standard error (n=4-6/group). *Indicates ozone effect when compared to matching air group at a given time point ($p \leq 0.05$). †Indicates surgery effect within matching exposure group at a given time point ($p \leq 0.05$).

Figure 4.3

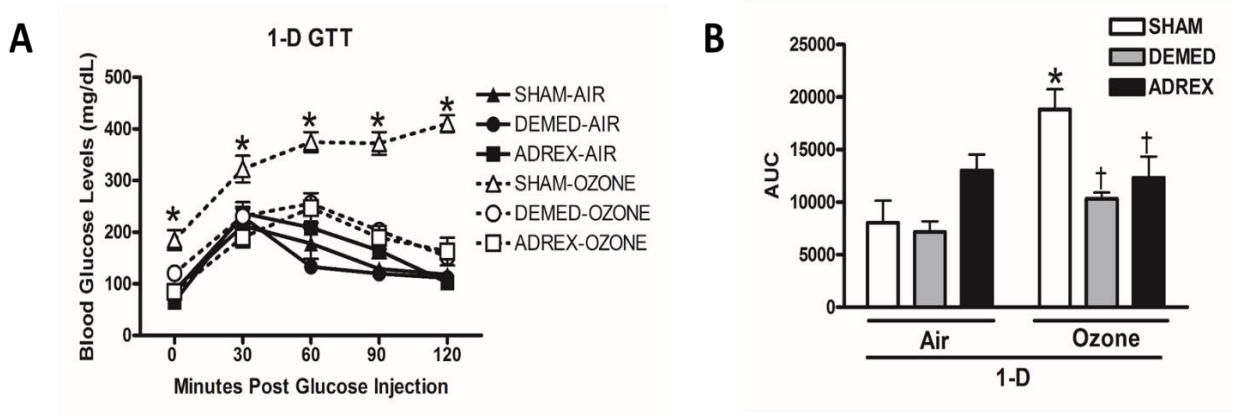

Figure 4.3 Changes in glucose tolerance after air or ozone exposure in SHAM, DEMED, or ADREX rats. GTT was performed only after the first day of exposure in the 2-D group (A). The 0 min time point shows fasting glucose levels in each group post exposure. Panel B shows the graph for the area under the curve (AUC) for the GTT data. Values indicate mean ± standard error (n=4-6/group). *Indicates ozone effect when compared to matching air group ($p \leq 0.05$).

Figure 4.4

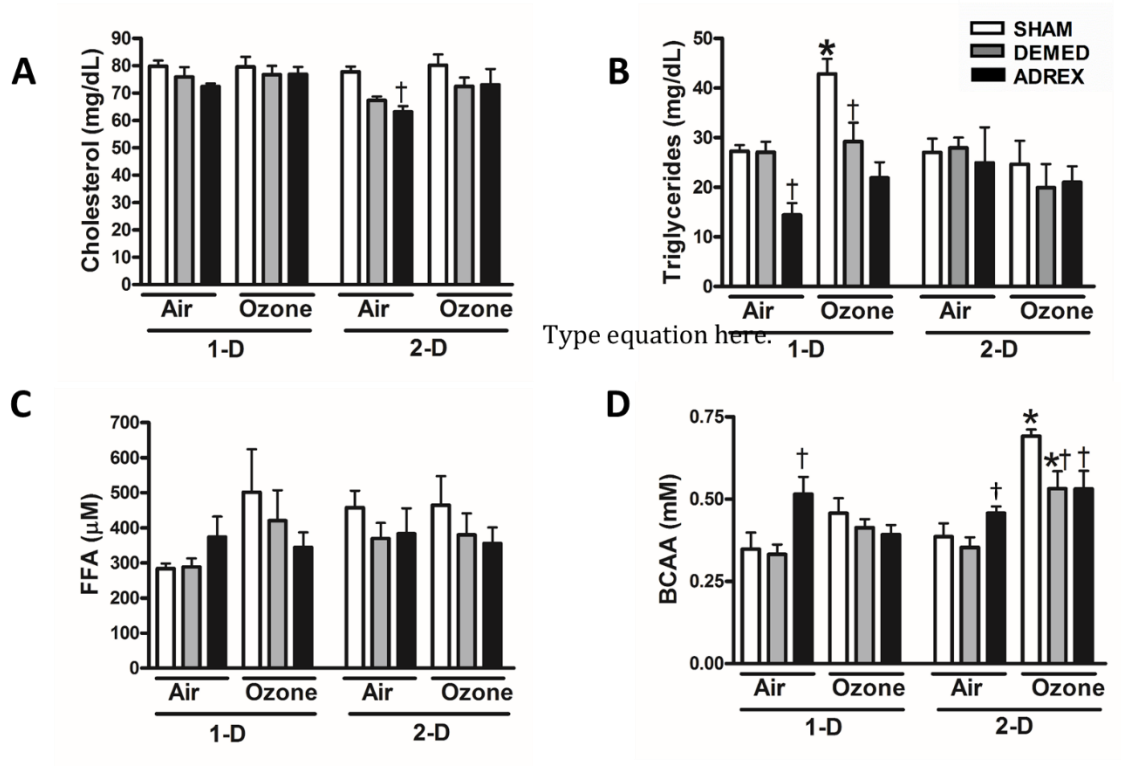

Figure 4.4 Changes in circulating metabolites after air or ozone exposure in SHAM, DEMED, or ADREX rats). Serum metabolites were measured after air or ozone exposure in rats at each time point (1-D, 2-D): A, Total Cholesterol, B, Triglycerides, C, FFA and D, BCAA. Values indicate mean ± standard error (n=4-6/group). *Indicates ozone effect when compared to matching air group at a given time point ($p \leq 0.05$). †Indicates surgery effect within matching exposure group at a given time point ($p \leq 0.05$). P=0.15 for FFA air and ozone, 1-D and p=0.13 for BCAA air and ozone 1-D in the SHAM rats. FFA, free fatty acids; BCAA, branched chain amino acids.

Figure 4.5

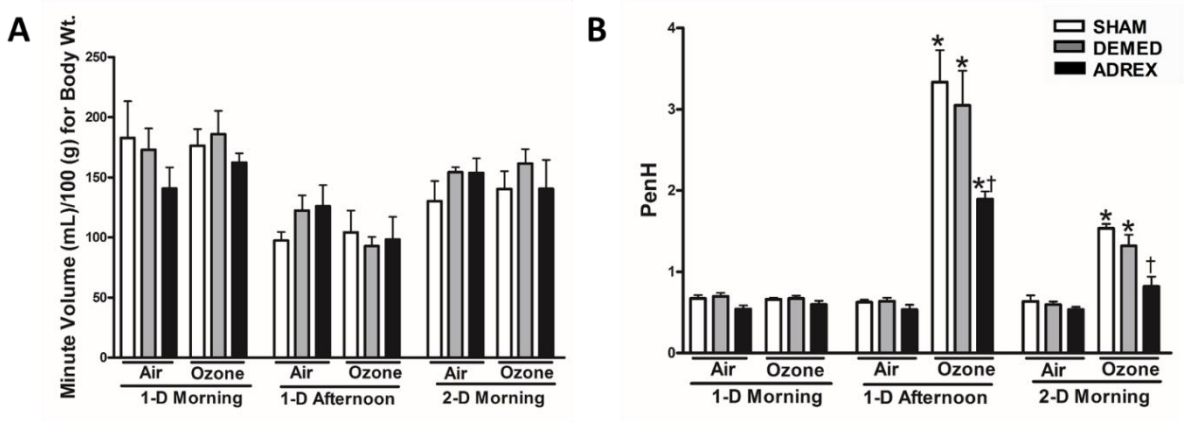

Figure 4.5 Ozone-induced changes in lung minute volume and PenH, an index of labored breathing, in SHAM, DEMED and ADREX rats. The plethysmography was performed the morning prior to first ozone exposure (1-D Morning), immediately following first ozone exposure (1-D Afternoon) and prior to the start of second exposure (2-D Morning). A, Minute volume was calculated based on breathing frequency and tidal volume and the values were normalized to body weight. B, PenH values were computed from all ventilatory parameters measured during plethysmography. Values indicate mean ± standard error (n=4-6/group). *Indicates ozone effect when compared to matching air group at a given time point $P \leq 0.05$. †Indicates surgery effect within matching exposure group at a given time point $P \leq 0.05$.

Figure 4.6

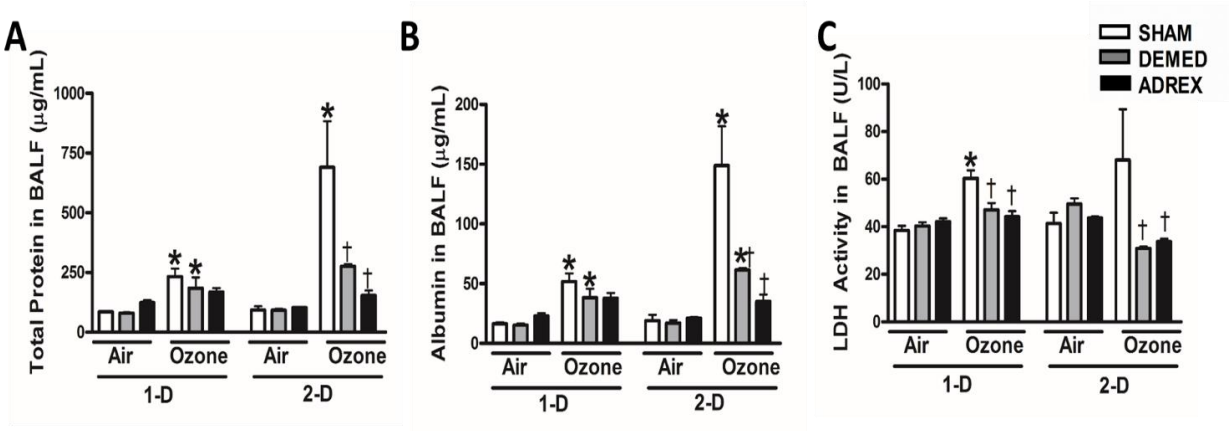

Figure 4.6 Lung injury in rats as determined by analysis of bronchoalveolar lavage fluid (BALF) after ozone exposure in SHAM, DEMED, or ADREX rats. Rats were necropsied immediately after each exposure time point (1-D, 2-D). BALF was analyzed for lung injury markers: A, Total Protein, B, Albumin, and C, LDH activity. Values indicate mean ± standard error (n=4-6/group). *Indicates ozone effect when compared to matching air group at a given time point $P \leq 0.05$. †Indicates surgery effect within matching exposure group at a given time point $P \leq 0.05$. BALF, bronchoalveolar fluid; LDH, lactate dehydrogenase.

Figure 4.7

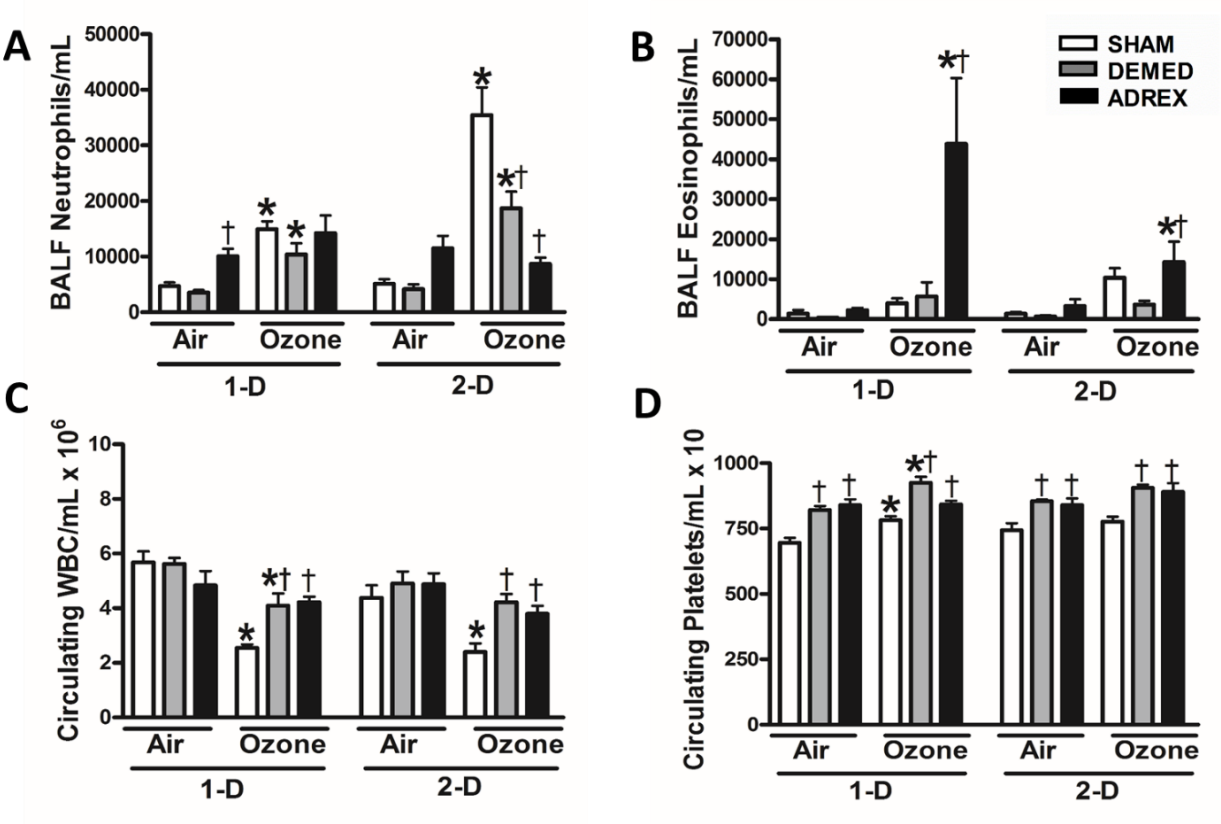

Figure 4.7 Lung inflammation as determined by analysis of cells in bronchoalveolar lavage fluid (BALF) after air or ozone exposure in SHAM, DEMED, or ADREX rats. BALF cell differentials were performed to quantify macrophages, neutrophils and eosinophils at both time points after air or ozone exposure (1-D, 2-D). A, Neutrophils and B, Eosinophils. Data for macrophages are not shown. Circulating white blood cells (WBC) and platelets were also quantified. C, WBC and D, Platelets. Values indicate mean ± standard error (n=4-6/group). *Indicates ozone effect when compared to matching air group at a given time point $P \leq 0.05$. †Indicates surgery effect within matching exposure group at a given time point $P \leq 0.05$.

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CHAPTER 5

SUBCHRONIC OZONE EXPOSURE: SYSTEMIC METABOLIC DERANGEMENT, PULMONARY EFFECTS AND INSULIN INSUFFICENCY

5.1 Introduction

In 2011, the International Diabetes Federation reported that the diabetes epidemic affected 366 million people worldwide, with 90% of the reported cases being classified as type 2 diabetes (TIID). Most mitigation efforts of TIID have focused on conventional risk factors such as sedentary lifestyle, obesity, high-fat diet, and genetic background. Recently, epidemiological studies have demonstrated a positive association between air pollutants and chronic metabolic conditions (Pearson et al., 2010; Liu et al., 2013; Thiering and Heinrich, 2015). For instance, there is an overlap of diabetes cases reported by the Center of Disease Control and high concentrations of particulate matter in the United States (Pearson, et al., 2010). Likewise, several studies have shown a positive association between ozone exposure, insulin resistance, and exacerbation of pre-existing metabolic conditions in the elderly (Stafoggia et al., 2010; Zanobetti and Schwartz, 2011; Kim and Y.C., 2012). However, the causality and underlying mechanisms for air pollutant-induced metabolic effects are unclear.

Long-term ambient particulate matter exposures are linked to the onset of metabolic risk factors for TIID such as adipose inflammation, mitochondrial dysfunction, liver endoplasmic reticulum stress and insulin resistance in mouse models of obesity (Xu et al., 2011; Mendez et al., 2013; Sun et al., 2013). It has been proposed that inhaled air pollutants may cause systemic insulin resistance by: 1) increased circulating pro-inflammatory cytokines, and/or 2) neuronal activation (Rajagopalan and Brook, 2012). While some air pollution studies have demonstrated systemic inflammation (Nurkiewicz *et al.*, 2004; Finnerty *et al.*, 2007; Mutlu *et al.*, 2007; Niwa *et al.*, 2008), many others show no increases in circulating cytokines (Campen *et al.*, 2006; Kooter *et al.*, 2006; Montero *et al.*, 2006; Gottipolu *et al.*, 2009).

Alternatively, air pollutants have been shown to cause neuroinflammation, oxidative stress and endoplasmic reticulum stress in the hypothalamus and other regions that regulate the autonomic nervous system (ANS) and hypothalamus pituitary adrenal (HPA) axis (Calderon-Garciduenas et al., 2008; Block and Calderon-Garciduenas, 2009; Levesque et al., 2011; Liu et al., 2014). Dysfunction or chronic activation of the sympathetic nervous system (SNS) and the HPA-axis, have been implicated in hyperglycemia, obesity, β-cell dysfunction and insulin resistance (Cai, 2013; Nosadini et al., 1983; Surwit and Schneider, 1993; Friedman et al., 1996; Delaunay et al., 1997; Bjorntorp et al., 1999; Rask et al., 2001). Likewise, persistent increases in stress hormones, such as epinephrine and corticosterone, are postulated to induce insulin resistance through increased circulating non-esterified free fatty acids (NEFAs) (Nonogaki, 2000). It is suggested that the continuous supply of NEFAs to the tissues could elevate intracellular ceramide and diacylglycerol levels, which can interfere with insulin signaling through increased IRS-1/2 serine phosphorylation and decreased AKT-

mediated glucose uptake (Arner and Langin, 2014; Perry et al., 2014). However, a potential link between neuronal stress response and insulin resistance has not been examined in air pollution studies.

We have recently reported that acute ozone exposure in rats induces a classical stress response characterized by increases in circulating stress hormones, glucose, long-chain free fatty acids, and branched chain amino acids (BCAA), suggesting the involvement of the SNS and activation of the HPA axis (Miller et al., 2015). The increases in circulating lipid metabolites and stress hormones were also noted in humans after an acute ozone exposure (Miller et al., 2016a). Interestingly, adrenalectomy prior to ozone exposure resulted in diminution of not only metabolic but also pulmonary effects in rats (Miller et al., 2016b). However, no insulin resistance was observed during an acute ozone exposure in our rat or human studies. This is in contrast to a study conducted by Vella *et al*. (2015), who demonstrated muscle insulin resistance in rats after 16 hr ozone exposure. It is not known if subchronic ozone exposure could lead to long-lasting elevations in circulating stress hormones lipids, or peripheral insulin resistance.

The objective of the present study is to examine if weekly episodic ozone exposure will lead to non-reversible metabolic alterations and insulin resistance in the liver and muscle tissues in rats. In this study, rats were exposed episodically to ozone 5hr/day x 3 consecutive days/week (wk) x 13wks. Metabolic alterations and hormone levels were assessed immediately after the last exposure or following a 1wk recovery. Clinical tests for glucose tolerance and insulin resistance were performed intermittently during exposure, together with assessment of stress hormones, lung injury/inflammation, and peripheral insulin signaling.

5.2 Materials and Methods

5.2.1 Animals

Heathy male Wistar Kyoto (WKY) rats (250-300g) were obtained from Charles River Laboratories Inc. (Raleigh, NC) at 10 wks of age. All rats were housed 2/cage in polycarbonate cages in an isolated animal room maintained at 21 ± 1ºC, 50 ± 5% relative humidity and on a 12hr light/dark cycle. The EPA NHEERL animal facility is approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animals received standard (5001) Purina pellet rat chow (Brentwood, MO) and water ad libitum unless otherwise stated. Animals were treated humanely and all efforts were made for alleviation of suffering. The EPA NHEERL Animal Care and Use Committee (IACUC) approved the research protocol for this study.

5.2.2 Ozone generation and animal exposures

Ozone was generated from oxygen by a silent arc discharge generator (OREC, Phoenix, AZ), and its entry into the Rochester style "Hinners" chambers was controlled by mass flow controllers. The chambers relative mean temperature was 23.3°C (74^0F) and humidity was 46%. Ozone concentrations were recorded continuously using the photometric API Model 400 ozone analyzer (Teledyne Instruments, San Diego, CA). Animals were divided into two groups: group 1 and group 2. Each group was further seperated into three exposure conditions: 1) filtered air, 2) 0.25 ppm ozone, and 3) 1.0 ppm ozone (n=8-10/exposure for each group). Both groups underwent whole body exposure to air or ozone, 5hr/day, 3 consecutive days/wk for 13wks. For group 1, necropsies were performed immediately post final exposure (13wk). For group 2, necropsies were performed after 1wk

recovery in their home cages following the 13wk exposure (13wk + 1wk recovery) to determine if ozone effects were reversible (Fig. 5.1).

5.2.3 Intraperitoneal glucose tolerance test (IPGTT)

IPGTT was conducted in group 1 animals during the 1st wk of exposure immediately following day 1 (1wk-D1) or day 3 (1wk-D3) of exposure, and during the 12th wk of exposure immediately following day 1 of exposure (12wk-D1). IPGTT was also performed in group 2 following 1wk recovery. Animals were fasted ~6hr in air or ozone exposure chambers and during loading/unloading, except for the recovery group where food was withheld for 6hr prior to IPGTT. For this test, baseline blood glucose levels were measured by pricking the tip of the tail using a sterile 25 gauge needle and Bayer Contour Glucometer (Leverkusen, Germany). Animals were then injected I.P. with pharmaceutical grade glucose solution (20% D-glucose; Sigma-Aldrich, St. Louis. MI) at 2g/10 mL/kg body weight. Glucose levels were measured at 30, 60, 90, and 120 min post glucose injection as reported in our previous studies (Miller et al., 2015; 2016b).

5.2.4 Blood collection for insulin measurement during IPGTT

During IPGTT at 12wk-D1, blood samples were collected from the lateral tail vein for insulin measurement before injecting glucose (baseline) and 30 min post glucose injection. Rats were placed in nose-only inhalation restraining tubes that were mounted on a lab bench device during blood collection. In order to reduce tube stress, rats were acclimatized for 5 min to restrainer tubes for three consecutive days prior to the start of the blood collection. The tails were warmed for 1 min using a damp warm cloth and wiped clean. A heparinized 20 gauge sterile needle was inserted into the tail vein, and after discarding the first two drops, ~200 μL of blood sample was collected into serum seperator tubes.

Hemostasis was then achieved by pressure using clean sterile gauze. These samples were spun at 3500 x g for 10 min, serum aliquots were separated, and stored at -80^{o}C until insulin analysis. For analysis of acute ozone exposure effects on glucose-mediated β-cell insulin secretion, a separate group of animals were exposed to air or 1.0 ppm ozone for 4hr. Immediately post exposure, ~50-100 μL blood samples were collected prior to and 30 min post glucose injection from the tail prick. These samples were spun and serum aliquots were stored at -80^{o}C for insulin measurement as stated above.

5.2.5 Intraperitoneal insulin tolerance test (IPITT)

Group 1 underwent IPITT at wk 1, day 2 (1wk-D2), and group 2 underwent IPITT at wk 12, day 1 (12wk-D1). As in the case with IPGTT, animals were fasted during air or ozone exposure and chamber loading/unloading for ~6hr. Immediately post air or ozone exposure in each group, baseline glucose levels were measured from the tail prick as in the case of IPGTT. HumulinR (Lilly USA, LLC, Indianapolis, IN) was injected I.P. (1.0 IU diluted in 1 mL saline/kg body weight) and blood glucose was measured at 30, 60, 90 and 120 min post insulin injection.

5.2.6 Intraperitoneal pyruvate tolerance test (IPPTT)

PPTT was conducted in group 2 at 1wk-D1, 13wk-D1 and at 13wk-D3. This procedure indirectly measures hepatic gluconeogenesis in conscious animals. As in the case with IPGTT and IPITT, immediately following exposure, fasting baseline glucose levels were measured using a glucometer through a tail prick (0 min). Pharmaceutical grade sodium pyruvate (Sigma-Aldrich, St. Louis. MI, diluted in saline (1g/2mL/kg body weight), was then injected I.P. Blood glucose levels were measured at 30, 60, 90, and 120 min following injection of pyruvate.

5.2.7 Necropsy and sample collection

Group 1 animals were necropsied immediately following the 13wk of exposure (13wk), while group 2 animals were necropsied after a 1wk recovery period (13wk + 1wk recovery)**.** 10-15 min prior to necropsy, half of the animals (n=4-5) in each group were injected I.P. with 1.0 IU/kg body weight dose of Humulin^{R} to accelerate insulin-mediated glucose uptake through AKT phosphorylation. Rats were euthanized with an I.P. injection of ~200 mg/kg sodium pentobarbital (Fatal-Plus diluted 1:1 with saline; Vortech Pharmaceuticals, Ltd., Dearborn, MI). All animals in both groups were fasted 6hr prior to necropsy.

5.2.8 Blood collection, complete blood count (CBC), and tissue collection

When animals were completely non-responsive to hind paw pinch after Fetal-Plus injection, blood samples were collected through the abdominal aorta. CBC was performed from EDTA containing blood tubes using a Beckman-Coulter AcT blood analyzer (Beckman-Coulter Inc., Fullerton, CA). All blood tubes were then centrifuged at 3500 rpm, at 4^{o}C for 10 min. Plasma and serum samples were stored at -80°C until further analysis. Liver lobe and gracilis leg muscle tissues were collected and stored at -80^{o}C for later assessment of markers of the insulin signaling pathway.

5.2.9 Bronchoalveolar lavage and bronchoalveolar lavage fluid (BALF) processing for pulmonary injury and inflammation assessment

The trachea was cannulated and then a suture was used to tie the left lung. The right lung was lavaged using Ca^{2+}/Mg^{2+} free phosphate buffered saline (pH 7.4) equal to 28 ml/kg body weight (total lung capacity) x 0.6 (right lung is ~60% of total lung weight). Three in-and-out washes were performed using the same aliquot of buffer and BALF was transfered to ice

until processing. Aliquots of BALF were used to determine total cell counts with a Z1 Coulter Counter (Coulter, Inc., Miami, FL) and cell differentials as described previously (Bass et al., 2013). Cell-free BALF aliquots, stored at -80^{o}C, were used to analyze total protein, albumin, γ-glutamyl transpeptidase (GGT) activity, and n-acetyl glucosaminidase (NAG) activity as described previously (Bass et al., 2013).

5.2.10 Serum and plasma analysis

The catecholamines adrenaline and noradrenaline in the EDTA plasma samples were measured using kits from Rocky Mountain Diagnostics (Colorado Springs, CO) per the manufacturer's protocol. Serum corticosterone concentrations were analyzed employing a rat-specific immunoassay kit from Arbor Assays (Ann Arbor, MI) per the manufacturer's protocol. Total cholesterol and triglycerides were measured in serum samples using kits from TECO Diagnostics (Anaheim, CA), while high density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured using kits from Thermo Fisher Scientific, Inc (Middletown, VA). All cholesterol kit protocols were modified for use on the Konelab Arena 30 system (Thermo LabSystems, Espoo, Finland). Serum NEFAs were measured by a coupled enzymatic reaction and the resultant hydrogen peroxide detection using a colorimetric probe as per the manufacturer's protocol (Cell Biolabs, Inc, San Diego, CA). BCAA were measured in serum using an ELISA kit and protocol based on chemiluminescence detection (Abcam, Cambridge, MA). Insulin serum levels were detected using rat-specific chemiluminescence assay kit (Millipore, Billerica, MA) via manufacturer's instructions. Serum levels of inflammatory cytokines were measured in selected groups of animals (13wk; air and 1.00 ppm ozone groups) using rat-specific V-Plex Proinflammatory

Panel 2 kit based on electrochemiluminescence detection (Meso Scale Discovery, Gaithersburg, MD) via manufacturer's instructions.

5.2.11 Insulin signaling assessment in liver and muscle

Liver and muscle tissues from 13wk and 13wk + 1wk recovery air and ozone groups were homogenized in lysis buffer containing protease and phosphatase inhibitors using a polytrone-type homogenizer. Homogenates were centrifuged at 14,000 x g at 4^{o}C for 10 min. The supernatants were analyzed for protein concentrations using Coomassie Plus protein assay kit (Pierce, Rockford, IL) and aliquots were stored at -80^{o}C for total and phosphoprotein assessment. Phosphorylated AKT (pAKT) and non-phosphorylated AKT were analyzed using a rat-specific electrochemiluminescence assay (Meso Scale Discovery, Gaithersburg, MD) via manufacturer's instructions. The pAKT level for each sample was normalized to total AKT (pAKT + non-phosphorylated AKT), and the relative fold change was calculated based on the air control for non-insulin injected animals for their respective time point.

5.2.12 General Statistics

Graph Pad Prism (6.0) was used for all data analysis. IPGTT, IPITT and IPPTT data were analyzed using a two-way repeated measure multivariate analysis of variance (MANOVA). Each of these time-dependent metabolic measures were also analyzed for area under the curve by the trapezoidal method. All other measurements had two independent variables (exposure and time) and therefore, these parameters were analyzed by two way analysis of variance (ANOVA) followed by Duncan's multiple range test. Pairwise comparisons were performed on all data and the level of significance was set at $p < 0.05$.

5.3 Results

5.3.1 Acute and subchronic episodic ozone exposure induces glucose intolerance without impacting insulin tolerance.

IPGTT was conducted immediately following exposure at 1wk-D1, 1wk-D3, 12wk-D1 and 13wk + 1wk recovery to determine if ozone-induced glucose metabolic impairment would persist during subchronic exposure and if these effects were reversible following a recovery period. Ozone exposure at 1wk-D1 induced fasting hyperglycemia and glucose intolerance at the 1.00 ppm dose (Fig. 5.2A), as observed in previous studies (Miller et al., 2015). Ozone-induced hyperglycemia and glucose intolerance appeared to be reduced at 1wk-D3 compared to the 1wk-D1 (Fig. 5.2B). Ozone exposure led to marked increases in fasting glucose and glucose intolerance at 1.00 ppm at 12wk-D1 (Fig.5.2C) which appeared to be more pronounced relative to the 1wk-D1 time point. One week recovery resulted in a complete reversal of ozone-induced fasting hyperglycemia and glucose intolerance (Fig. 5.2D).

IPITT was conducted to determine if subchronic ozone-induced glucose intolerance was accompanied with peripheral insulin resistance. Neither 1wk-D2 (Fig. 5.2E) nor 12wk-D1 (Fig. 5.2F) assessment indicated impairment in peripheral insulin-mediated glucose clearance. Ozone-induced hyperglycemia and glucose intolerance were reversed after insulin injection suggesting effective insulin-mediated glucose uptake.

5.3.2 Acute ozone increases hepatic gluconeogenesis

One of the potential contribution to hyperglycemia after ozone exposure could be increased hepatic gluconeogenesis. IPPTT allows one to examine the stimulation of gluconeogenesis since pyruvate is a gluconeogenic precursor that is converted to glucose in

the liver when injected. To determine if ozone-induced hyperglycemia resulted from stimulated gluconeogenesis, IPPTT was conducted in animals at 1wk-D1 and 13wk-D1. During 1wk-D1, 1.00 ppm ozone-exposed animals were already hyperglycemic and showed a significant increase in blood glucose levels following pyruvate injection when compared to air or 0.25 ppm groups (Fig. 5.3A). The AUC further confirmed the stimulation of gluconeogenesis at this time point. In contrast, after a 13wk subchronic ozone exposure, no increases in gluconeogenesis were observed (Fig. 5.3B & 3C).

5.3.3 Acute and subchronic episodic ozone exposure attenuates glucose-activated pancreatic β-cell insulin secretion

Impairment of pancreatic β-cell insulin secretion is another potential mechanism by which ozone could induce glucose intolerance. We evaluated the effect of acute and subchronic ozone exposure on β-cell function in rats by determining baseline and glucose-stimulated insulin release in to the circulation. No differences were observed in serum insulin levels between air and 1.00 ppm ozone at baseline prior to I.P. glucose injection at 1wk-D1 (Fig. 5.4A). However, 30 min after glucose injection, insulin levels were significantly increased in air-exposed animals, whereas ozone-exposed rats showed no insulin increase in response to glucose (Fig. 5.4A). Neither baseline nor glucose-stimulated insulin release in the 0.25 ppm ozone-exposed rats were determined during the wk 1 of exposure (Fig. 5.4A). Subchronic air and ozone exposures showed similar insulin responses compared to acute exposure responses. At 12wk-D1, baseline serum insulin levels tended to decrease with 1.00 ppm ozone exposure relative to air group ($p=0.1018$; Fig. 5.4B). Bolus glucose injection led to marked increases in serum insulin in air and 0.25 ppm ozone-exposed rats, suggesting a robust response of pancreatic β-cells to release insulin when circulating glucose levels were

high. This glucose-induced increase in insulin levels was completely abolished in rats exposed to 1.00 ppm ozone (Fig. 5.4B). Glucose-stimulated insulin secretion was not determined in rats after the 1wk recovery period following 13wks of ozone exposure. However at necropsy, we measured basal insulin levels at the 13wk and the 13wk + 1wk recovery to determine ozone effects. Although the insulin levels in 1.00 ppm exposed rats were markedly lower relative to air or 0.25 ppm groups at 13wk, this difference did not reach statistical significance using a 2-way ANOVA method due to large intragroup variability and small group size (n=3-5; Fig 5.4C). However, when insulin mean values for the air and 1.00 ppm ozone were compared with an unpaired t-test, there was a significant (p=.0007) 3-fold decrease. This ozone-induced decrease was less pronounced after a 1wk recovery period (Fig 5.4C).

5.3.4 Subchronic episodic ozone exposure does not produce liver or muscle insulin resistance

Although there was no significant change in insulin tolerance between the air- and ozone-exposed rats, we presumed that tissue-specific insulin sensitivity might be altered, specifically in the liver and muscle following subchronic ozone exposure. We measured non-phosphorylated AKT and pAKT, which mediates insulin-induced cellular glucose uptake at 13wk and after a 13wk + 1wk recovery in the liver and muscle from air and 1.00 ppm ozone-exposed rats. In a selected number of rats within a given exposure condition, insulin was injected 10-15 min prior to necropsy to increase the sensitivity of detecting levels of pAKT in response to high insulin. No changes were noted in the pAKT levels in the liver (Fig. 5.5A) nor muscle (Fig. 5.5B) following ozone exposure in rats with or without insulin injection, suggesting that insulin-mediated glucose uptake was not altered by ozone exposure

in these tissues. The validity of the pAKT measurements was confirmed by significant increases in the levels of pAKT in rats injected with insulin prior to necropsy.

5.3.5 Subchronic episodic ozone exposure alters circulating stress hormone levels

We have shown that circulating adrenaline is elevated during acute ozone exposure (Miller et al., 2015). We have also noted increases in circulating corticosterone following a 2hr ozone exposure in rats (unpublished data) and humans (Miller et al., 2016a). To determine if these stress hormones are still elevated following a subchronic episodic exposure, adrenaline, noradrenaline, and corticosterone plasma levels were measured in rats exposed to air or ozone for 13wks and after a 1wk recovery period. Serum adrenaline levels were significantly increased at 13wk following 1.00 ppm ozone with levels returning to baseline following a recovery period (Fig. 5.6A). Noradrenaline plasma levels tended to be high in the 1.00 ppm ozone-exposed rats at both time points, however, these increases were statistically insignificant (Fig. 5.6.B). The level of corticosterone tended to be low in the 1.00 ppm ozone-exposed rats relative to air controls at 13wk, however, this difference was not significant (Fig. 5.6C). No ozone effects were observed in the recovery group.

5.3.6 The effect of subchronic ozone exposure on circulating proinflammatory cytokines and metabolic biomarkers

Although circulating cytokines are postulated to be involved in peripheral metabolic effects of air pollutants, our prior studies show no significant increases in IL-6 or TNF-α after acute or subchronic ozone exposure in Brown Norway rats (Bass et al., 2013; Gordon et al., 2013). To determine if pro-inflammatory cytokines might be increased after subchronic exposure in WKY rats, we examined serum levels of cytokines at 13wks. No significant

increases were observed in IL-6, TNF-α or IL-1β however, small increases were observed in serum IFN-γ, IL-4, and IL-10 after subchronic ozone exposure (Table 5.1).

We have previously shown that acute ozone exposure elevated circulating total cholesterol, FFA and BCAA in rats (Miller *et al*., 2015). To determine if these changes would persist during a subchronic exposure, we evaluated the serum levels of these metabolic biomarkers. At 13wk, 1.00 ppm ozone significantly increased total cholesterol (Fig. 5.7A). However, after a 1wk recovery, serum cholesterol levels were decreased in both ozone-exposed groups when compared to air control (Fig. 5.7A). LDL was not significantly changed at the 13wk time point after ozone exposure, however, these levels were significantly decreased in ozone-exposed rats after the recovery period (Fig. 5.7B). HDL was significantly elevated in the 1.00 ppm ozone-exposed rats at 13wk (Fig. 5.7C). In the recovery group, HDL serum levels were significantly lower in the ozone-exposed rats compared to the air (Fig. 5.7C). Triglycerides were not significantly affected by ozone at 13wk but were found to be decreased in the 1.00 ppm ozone group after recovery (Fig. 5.7D). Neither the levels of FFA (Fig. 5.7E) nor BCAA (Fig. 5. 7F) were not significantly changed by ozone at any time point. Contrary to the marked increases in leptin following an acute 1 day ozone exposure (Miller *et al.*, 2015), there were no significant increases in leptin at 13wk, with levels decreased after recovery in rats exposed to 1.00 ppm ozone (Fig. 5.7G).

5.3.7 Subchronic episodic ozone exposure induces lung injury and inflammation

Many studies have shown that acute ozone exposure causes lung injury and inflammation in animals and humans. To determine if subchronic episodic exposure would cause persistent lung changes, BALF was evaluated for injury and inflammation biomarkers. Both total protein and albumin, markers of lung permeability, were significantly elevated at

1.00 ppm ozone when compared to air at 13wk but this effect was largely reversed following recovery (Fig. 5.8A & 8B). Rats exposed to 1.00 ppm ozone showed significant increases in BALF GGT activity at 13wk, which persisted when the animals were allowed a 1wk recovery period (Fig. 5.8C). NAG activity was also increased in rats exposed to 1.00 ppm ozone at the 13wk, however, this effect was attenuated after recovery (Fig. 5.8D).

Lung neutrophilic inflammation was increased after 13wk of ozone exposure compared to air and 0.25 ppm groups. This ozone effect on neutrophils was not observed in recovery group (Fig. 5.9A). The number of BALF alveolar macrophages were also increased in rats exposed to 1.00 ppm ozone relative to air at 13wk with levels returning to baseline following 1wk recovery (Fig. 5.9B). Interestingly, circulating white blood cells were not significantly decreased by the subchronic ozone exposure as they are following an acute exposure (Miller *et al.*, 2016b) (Fig. 5.9C). 1.00 ppm ozone exposure for 13wk increased circulating platelets, but this effect was reversed following the recovery (Fig. 5.9D).

5.4 Discussion

We have previously shown that acute ozone exposure induces a systemic stress response characterized by hyperglycemia, glucose intolerance, lipidemia, and elevations in BCAA, FFA, and circulating stress hormones (Miller *et al.*, 2015; 2016b). Humans are exposed episodically to ozone throughout their lifetime, which may cause chronic stress response activation. Chronic elevations of stress hormones have been implicated in metabolic dysfunction and postulated to lead to insulin resistance. However, the persistence of these stress and metabolic effects, and their contribution to insulin resistance have not been examined after subchronic ozone exposure. The main objectives of this study were to determine: (1) if ozone-induced acute metabolic and stress effects would persist after subchronic episodic exposure, and (2) if subchronic ozone exposure will lead to liver and muscle insulin resistance.

Our data show that, as observed after an acute exposure, 3 days/wk of subchronic episodic ozone exposure for 13wks induced fasting hyperglycemia, glucose intolerance, and increases gluconeogenesis, circulating cholesterols and epinephrine levels, but these effects are reversible upon a 1wk recovery period. More importantly, basal circulating insulin levels were decreased and glucose-stimulated insulin release was abolished after subchronic ozone exposure. No insulin resistance was noted in liver or muscle tissues after 13 week ozone exposure. In addition, no ozone-induced changes were observed in serum FFA and BCAA, suggesting the likely contribution of adaptation. Increases in lung injury and inflammation were still present at 13wk but these effects were reversible after a 1wk recovery period. Overall, our data demonstrate that ozone-induced pulmonary, metabolic and stress effects persist through 13wk episodic ozone exposure however, these effects are reversible upon a

short recovery of 1wk. Further, subchronic ozone exposure diminishes glucose-stimulated insulin release, while decreasing the baseline levels over time, without the impairment of peripheral insulin resistance.

Three potential mechanisms may explain hyperglycemia and glucose intolerance after acute and subchronic episodic ozone exposure: 1) increased glucose release from the liver through gluconeogenesis, 2) decreased glucose uptake by peripheral tissues, and 3) decreased release of insulin from pancreatic β-cells. We observed that acute ozone exposure increased hepatic gluconeogenesis but did not impair insulin-mediated glucose clearance from the circulation. In contrast, hepatic gluconeogenesis was not altered during subchronic exposure. More importantly, acute and subchronic ozone exposures abolished glucose-mediated β-cell insulin secretion.

Hepatic gluconeogenesis is activated by different physiological conditions, such as fasting or exercise, and is tightly regulated by the availability of gluconeogenic precursors and phosphorylation/expression of gluconeogenic enzymes. Moreover, gluconeogenesis is further regulated by the availability of insulin (Girard, 2006) and stress hormones (Altuna *et al*., 2006). It is postulated that insulin can directly or indirectly inhibit gluconeogenesis by specifically targeting hepatic glucose production or suppressing the release of glucagon from pancreatic α-cells, the main stimulator of gluconeogenesis. Insulin can also suppress the formation of gluconeogenic precursors through the inhibition of adipose lipolysis and skeletal muscle proteolysis (Girard, 2006). Adrenaline, through β2-adrenergic receptors, can enhance hepatic glucose output by increasing endogenous cyclic AMP (cAMP), which subsequently activates protein kinase A that stimulates fructose bisphosphatase 2 and cAMP response element binding dependent activation of phosphoenolpyruvate

carboxykinase and inhibits phosphofructokinase 2 (Valera *et al*., 1994; Burgess *et al.*, 2004). The ozone-induced increases in gluconeogenesis thus, could be explained by the increased levels of epinephrine together with decreased insulin levels and glucose-mediated insulin release.

Pancreatic β-cell insulin secretion is regulated at many levels (central and local) by factors including endocannabinoids (Jourdan *et al*., 2016), glucocorticoids (Kuo *et al.*, 2015), circulating glucose and fatty acids (Oh, 2015), micro RNAs (Esguerra *et al*., 2014), epinephrine (Iwanir and Reuveny, 2008; Gibson *et al*., 2006), and many more. We observed that acute and subchronic ozone exposure diminished glucose-mediated pancreatic β-cell insulin secretion even though circulating glucose levels remained elevated in rats. Since this effect is associated with increased circulating epinephrine during acute (Miller *et al*., 2015) and chronic exposure to ozone as seen in this study, it is conceivable that diminished insulin secretion in the present study could be due to the effect of epinephrine on pancreatic β-cells. Catecholamines have been proposed to act through different members of the α-adrenergic family of receptors and impair insulin secretion through changes in cAMP and/or Ca^{2+} levels, hyperpolarization of the pancreatic β-cells or glucagon release from pancreatic α-cells (Peterhoff *et al.*, 2003). It has been shown that glucose-induced ERK1/2 activation and subsequent insulin secretion are inhibited by epinephrine through the α2-adrenergic receptors (Gibson *et al*., 2006). However, it cannot be ascertained from our study if other factors played a role in inhibiting insulin secretion during ozone exposure. Since basal levels of insulin were not affected after acute exposure, but were decreased after subchronic ozone exposure, it is likely that the diminished insulin release could contribute to the observed persistent hyperglycemia and glucose intolerance following the 13wk subchronic exposure.

We have previously shown that subchronic ozone exposure is associated with increased levels of insulin in 24 month but not 4 month old Brown Norway rats (Gordon *et al.*, 2013), however, peripheral insulin signaling was not examined in that study. As observed before, in this study too, acute or subchronic ozone exposure did not elevate insulin levels in young WKY rats. On the contrary, serum insulin levels decreased in ozone-exposed rats relative to air group (Fig. 5.4), suggesting that there might be a tendency to develop insulin deficiency if the exposures continued in young rats. More importantly, our data show no peripheral insulin resistance in the muscle or liver after ozone exposure. Incidentally, exposure to ozone has been associated with type 1 diabetes (T1D) in pregnancy and in children (Hathout *et al*., 2002; 2006; Beyerlein *et al.*, 2015; Malmqvist *et al*., 2015). Our data show that insulin resistance is not the cause of ozone-induced hyperglycemia, glucose intolerance, or the activation of gluconeogenesis observed in our study. These findings contradict the Vella *et al.* (2015) study involving a 16hr ozone inhalation, which showed insulin resistance in the muscle involving the c-Jun N-terminal kinase pathway. This discrepancy could be due to the very long single exposure in the Vella paper as opposed to the episodic subchronic exposure in our study. The possibility that ozone may contribute to the susceptibility of T1D by producing β-cell dysfunction requires further investigation.

Since long-term particulate matter exposures have been shown to induce systemic inflammation and peripheral insulin resistance (Rajagopalan and Brook, 2012) , we examined a number of circulating cytokines after ozone exposure. No increases were observed in IL-6, TNF-α or IL-1β. Small increases were observed in serum IFN-γ, IL-4, and IL-10 after subchronic ozone exposure in our study (Table 1). Our previous studies involving long-term ozone exposure have also failed to demonstrate increases in circulating proinflammatory

cytokines such as IL-6 and TNF-α (Gordon et al., 2013; Bass et al., 2013). Since ozone-exposure was not associated with insulin resistance, the contribution of systemic inflammation in insulin resistance after particulate matter exposure remains to be examined. Although we have shown that acute ozone exposure increases circulating FFA and BCAA (Miller, et al., 2015), elevations in these circulating metabolites were not noted after subchronic ozone exposure. Total cholesterol and HDL, however, were higher in rats after subchronic ozone exposure. As in the case with acute exposure, increased epinephrine could alter cholesterol metabolism (Kunihara and Oshima, 1983) leading to increases in cholesterol levels. The increases and decreases noted after the 13wk ozone exposure and recovery in cholesterols and triglycerides may result in lipid redistribution in peripheral tissues, which will be important to examine in future studies.

Our exposure protocol differed somewhat from previous subchronic studies we have done using ozone (Kodavanti et al., 2011; Gordon et al., 2013; 2014; Bass et al., 2013) in that this study involved 3 days/wk exposure as opposed to 1 or 2 days/wk exposure. This was done with the understanding that while the 1st 2 days of weekly ozone could produce pronounced pulmonary injury and systemic response (Miller et al., 2015), 3 consecutive days of exposure was expected to attenuate these ozone-induced effects. Adaptation to ozone-induced pulmonary injury, inflammation, and function after daily repeated exposure is fairly well demonstrated in humans (JÖRres et al., 2000; Schelegle et al., 2003) and in animals (Kirschvink et al., 2002; van Bree et al., 2002). The degree of adaptation depends on the ozone concentration and the temporality of exposure, and it has been shown that the adaptation to weekly 2-3 day ozone exposure is lost within 1wk upon re-exposure (Gordon et al., 2013; 2014). Rats exposed to ozone for 6hr/day, 2 days/wk led to partial adaptation

relative to acute exposure in pulmonary and systemic metabolic effects when determined after 13wk exposures (Bass *et al.,* 2013), suggesting that weekly exposure for several wks will also dampen the degree of pulmonary and systemic effects observed after the 1st wk. In this study, we wanted to determine if the weekly adaptation would start following 3 consecutive days of exposure, and if so, whether repeated induction of adaptation over 13wk would lead to failure in this response. We noted that IPGTT and IPPTT when performed on the third day in a given wk showed marked reduction in ozone-induced hyperglycemia, glucose intolerance as well as gluconeogenesis, however, the pulmonary injury inflammation determined after day 3 post 13wks exposure were nearly as robust as what is reported after acute exposure (Miller *et al*,. 2015). This suggests an adaptation in some systemic effects following weekly ozone exposure with less robust adaptation in pulmonary outcomes.

The phenomenon of adaptation might involve many factors, the degree of injury/inflammation at a given concentration being the most critical, and thus the interpretation of any findings becomes highly complex. Nevertheless, one could speculate that central mechanisms might be playing an important role in ozone adaptation phenomenon. Since we have shown that catecholamines and glucocorticoids are potentially involved in these ozone-induced pulmonary and metabolic effects (Miller *et al*., 2016b), it is likely that adaptive mechanisms may involve altered stress response signaling during subchronic exposure. Chronic stress activation can lead to the adaptive response "habituation", involving neural plasticity and downregulation of glucocorticoid and β-adrenergic receptors in the central nervous system, and in the periphery leading to reduced stress-mediated responses (Herman, 2013).

One of the critical questions we wanted to address is the reversibility of effects upon discontinuation of ozone exposure. The persistence of the effects, including insulin insufficiency, can have implications in chronic disease outcomes. As noted, pulmonary and systemic effects observed immediately following the 13wk exposure were largely reversed following the 1wk recovery. However, the circulating lipids showed a reversed pattern of change, suggesting that while most effects are not persistent upon discontinuation of exposure, lipid redistribution might remain an important outcome of chronic episodic exposure and needs to be further examined in light of liver and muscle diseases and their link to pulmonary complications.

In conclusion, we demonstrate that subchronic weekly episodic ozone exposure induces pulmonary injury/inflammation, hyperglycemia, glucose intolerance, and increase cholesterols as observed after acute exposures. We further show that while peripheral insulin resistance did not occur by subchronic ozone, it had a major impact on β-cell insulin secretion in response to circulating glucose, likely mediated by increased circulating epinephrine levels. This, together with reduced circulating insulin levels after subchronic ozone exposure, supports a link between chronic ozone and TID. Despite the fact that most subchronic ozone-induced changes are reversible upon termination exposure, the question remains about lipid redistribution in peripheral tissues and chronic episodic pollution effects. Overall, these data provide further insight into the mechanism(s) of how subchronic ozone exposure may contribute to insulin insufficiency and produce other systemic impairments.

Table 5.1 Serum levels of proinflammatory cytokine biomarkers in WKY rats after subchronic exposure to air or 1.00 ppm ozone

Biomarker	Exposure	
	Air	Ozone
IL-6 (pg/ml)	210.52 ±9.01	229.62 ± 4.68
TNF-α (pg/ml)	3.88 ± 0.35	4.57 ± 0.45
IL-1β (pg/ml)	63.37 ± 4.58	70.19 ± 7.74
IFN-γ (pg/ml)	26.99 ±0.92	29.48 ± 0.49*
IL-10 (pg/ml)	81.00 ±2.45	88.91 ± 1.38**
IL-4 (pg/ml)	6.85 ±0.15	7.32 ± 0.12**
IL-5 (pg/ml)	23.96 ±5.65	16.25 ± 2.28
KC-GRO (pg/ml)	191.21 ±23.36	195.66 ± 34.85

Serum samples obtained after 13wk air and 1.00 ppm ozone (mean ± SEM; n=8) were analyzed using a Mesoscale Discovery Inc. cytokine multiplex panel kit. *Indicates significant ozone effect when compared to matching air group (*$p \leq 0.05$, **$p \leq 0.01$). Note that half of rats per group were injected with 1U/kg insulin, 10-15 min prior to necropsy.

Figure 5.1

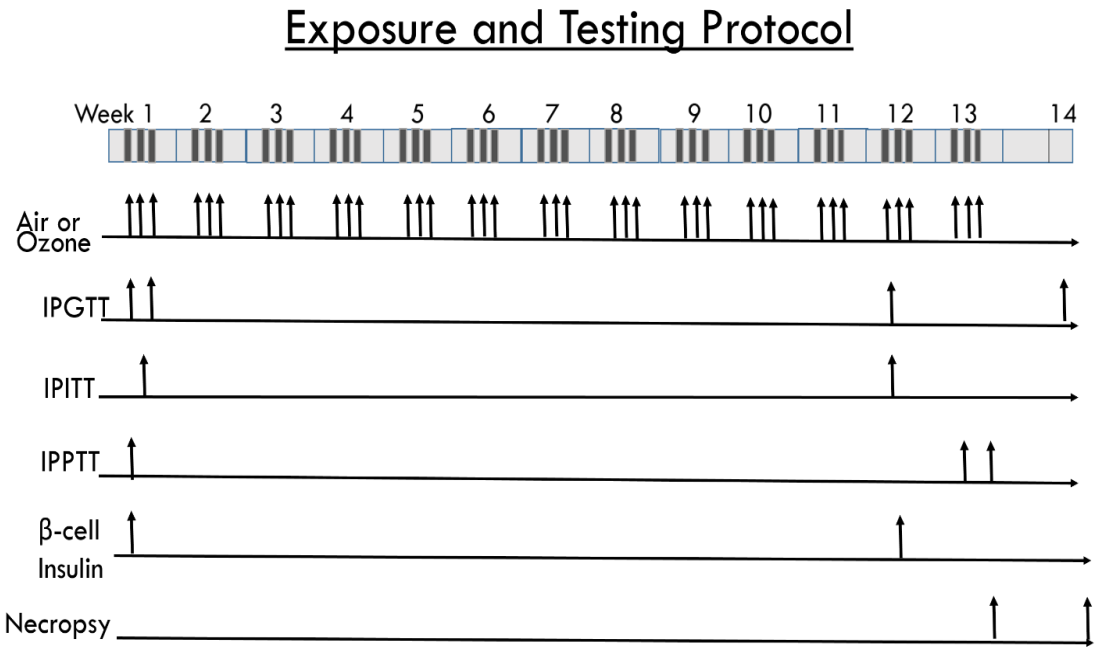

Figure 5.1 Experimental design. Two groups of animals were exposed to: 1) filtered air, 2) 0.25 ppm or 3) 1.00 ppm ozone for 5h/d, 3d/wk for 13wks (n=8-10 rats/exposure/ group). Group 1 rats were necropsied immediately after the final exposure. Group 2 animals were necropsied after a 1wk recovery period following 13wk of episodic exposure (13wk + 1wk recovery). Metabolic testing time points are indicated by arrows for each test: intraperitoneal glucose tolerance test (IPGTT: 1wk-D1; 1wk-D3; 12wk-D1; 13wk + 1wk recovery; the final test conducted six days following final exposure), intraperitoneal insulin tolerance test (IPITT:1wk-D2; 12wk-D1) intraperitoneal pyruvate tolerance test (IPPTT:1wk-D1; 13wk-D1; 13wk-D3). All IPGTT, IPITT, IPPTT were conducted immediately following exposure time points. β-cell insulin secretion test was conducted on 1wk-D1 and 12wk-D1.

Figure 5.2

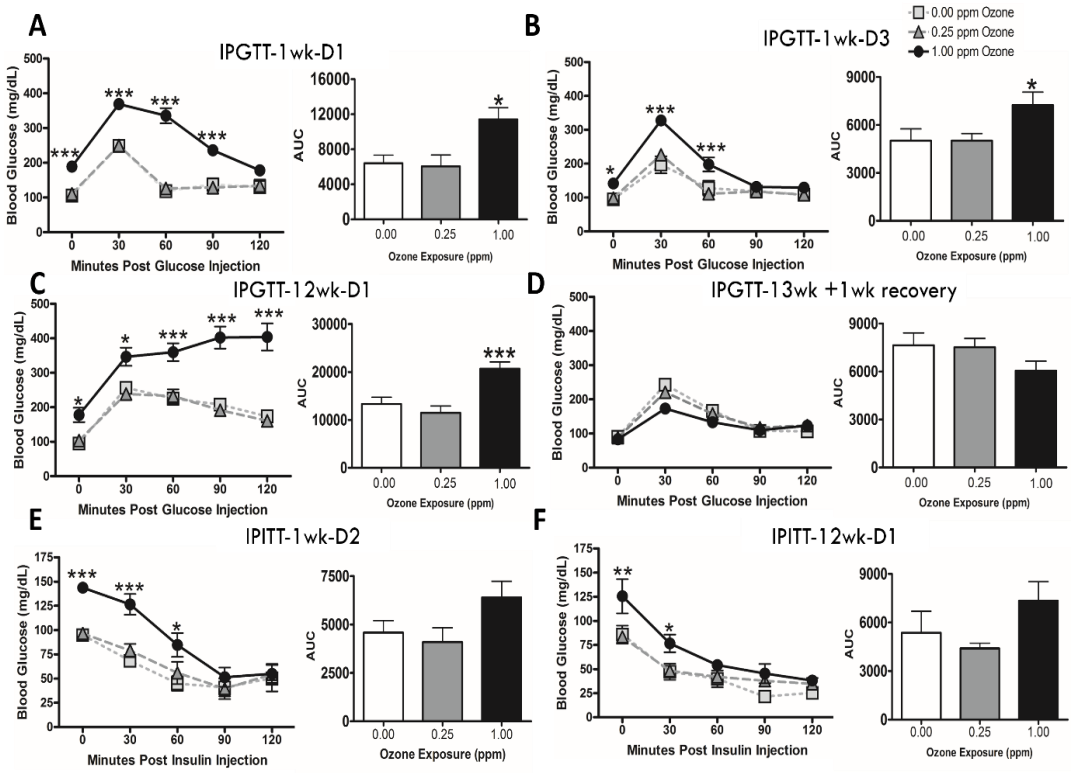

Figure 5.2 Acute and subchronic ozone exposures induce hyperglycemia and glucose intolerance without impairing insulin tolerance. IPGTT was conducted in animals at (A) week 1, day 1 (1wk-D1), (B) week 1, day 3 (13wk-D3), (C) week 12, day 1 (12wk-D1), and (D) six days after the 13wk final exposure (13wk + 1wk recovery). IPITT was conducted at (E) week 1, day 2 (1wk-D2) and (F) week 12, day 1 (12wk-D1). Bar graphs show respective area under the curve (AUC) values for each test. Values indicate mean ± SEM (n=8-10). *Indicate ozone effect when compared to matching air group (*p≤0.05, **p≤0.01, ***p≤0.001).

Figure 5.3

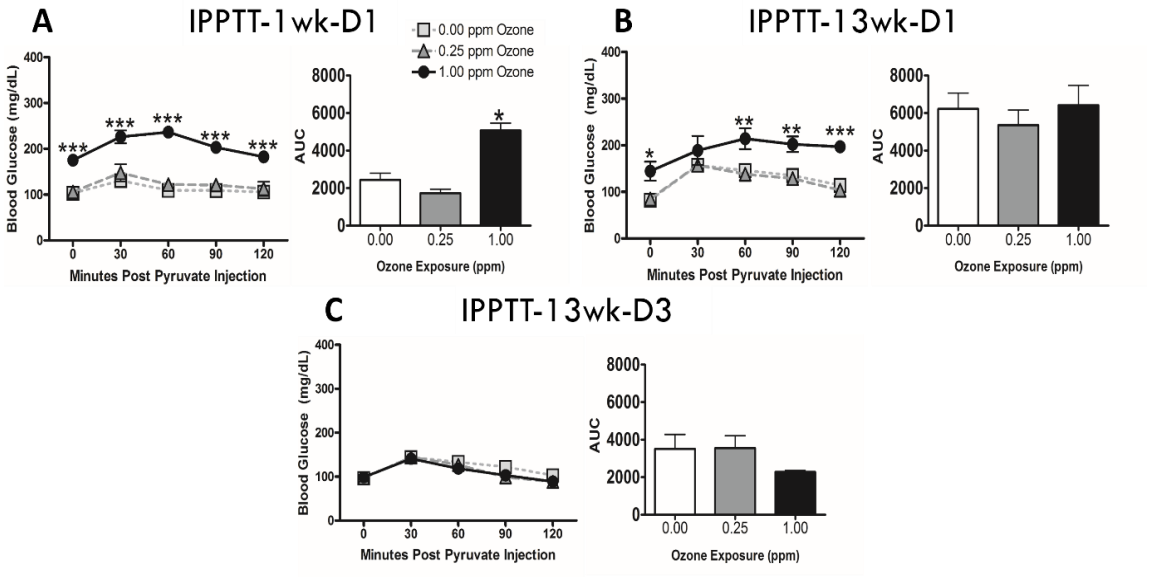

Figure 5.3 Acute and subchronic ozone exposures induce hepatic gluconeogenesis. IPPTT was conducted in animals at (A) week 1, day 1 (1wk-D1), (B) week 13, day 1 (13wk-D1) and (C) week 13, day 3 (13wk-D3). Bar graphs show respective area under the curve (AUC) values for each test. Values indicate mean ± SEM (n=8-10). *Indicate significant ozone effect when compared to matching air group (*p≤0.05, **p≤0.01, ***p≤0.001).

Figure 5.4

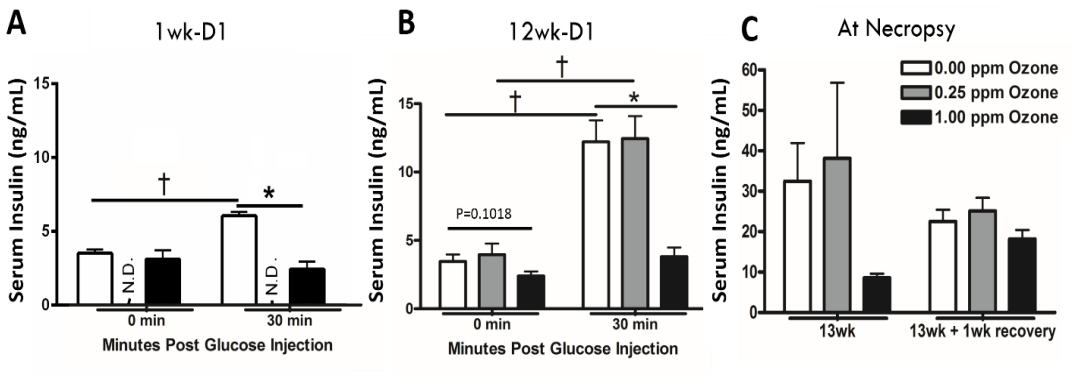

Figure 5.4 Acute and subchronic ozone exposure inhibits glucose-stimulated β-cell insulin secretion. Serum insulin levels were measured immediately following 1 day air or 1.00 ppm ozone exposure (A; 1wk-D1; a separate group of rats was used for this testing and air or ozone exposures was performed only for 4hr) and at 12wk-D1 (B). Insulin levels were measured at baseline (0 min) and 30 min after I.P. injection of glucose (20% D-glucose; 2g/kg). (C) Insulin levels were also determined immediately after necropsy at 13wk and 13wk + 1wk recovery. Insulin levels were not determined (N.D.) in 0.25 ppm ozone group during acute exposure. Values for A and B indicate mean ± SEM (n=6-10) while values for C indicate mean ± SEM (n=3-5). *Indicate significant ozone effect when compared to matching air group (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). † Indicate significant effect of glucose injection compared to baseline levels.

Figure 5.5

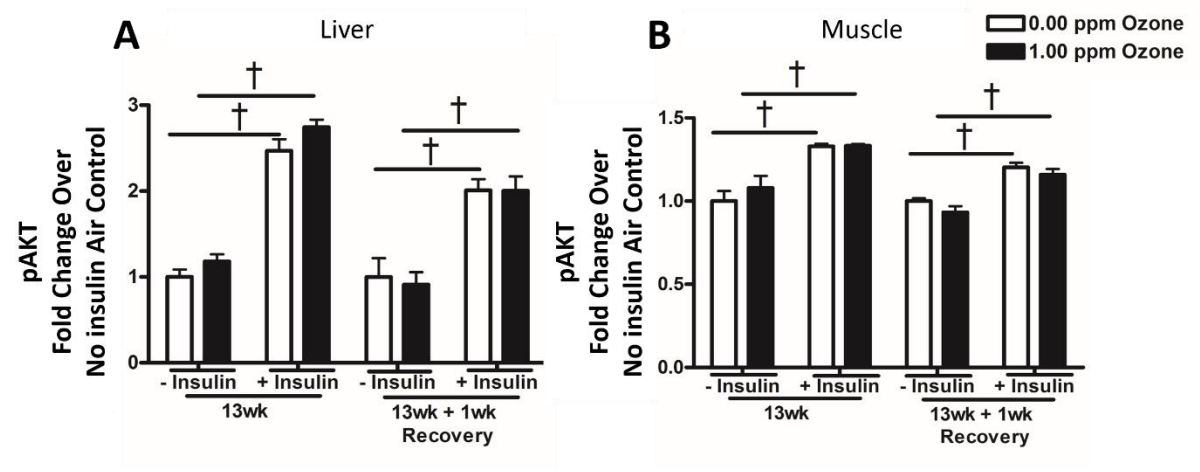

Figure 5.5 The effect of subchronic ozone exposure and 1wk recovery on insulin signaling in liver and muscle tissue. Half of the rats from each group were I.P. injected with 1.0 IU insulin/kg body weight 10-15 min prior to blood collection (necropsy) to enhance phosphorylation of AKT, which mediates glucose uptake through insulin receptors. The levels of non-phosphorylated and phosphorylated AKT (pAKT) were measured in the (A) liver and (B) muscle extracts, and the relative pAKT levels were normalized to non-insulin injected air control group. Values indicate mean ± SEM (n=4-5). †Indicates significance between insulin injected and non-insulin injected rats ($p \leq 0.05$).

Figure 5.6

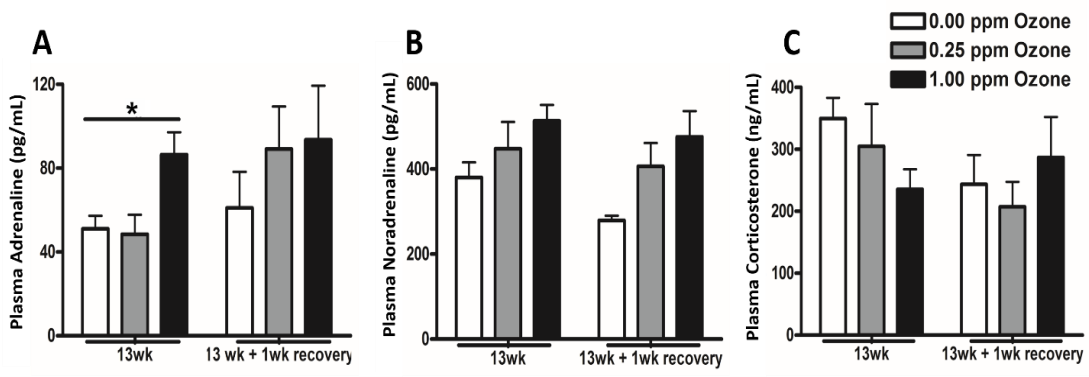

Figure 5.6 Subchronic ozone exposure changes circulating stress hormones. Stress hormones were measured in plasma samples collected using EDTA as an anticoagulant in rats exposed to air, or ozone at 0.25 ppm, or 1.00 pm immediately after final exposure at (13wk) and immediately after 1wk recovery. Stress hormones included (A) adrenaline, (B) noradrenaline and (C) corticosterone. Values indicate mean ± SEM (n=8-10). *Indicates significant ozone effect when compared to matching air group (*$p \leq 0.05$).

Figure 5.7

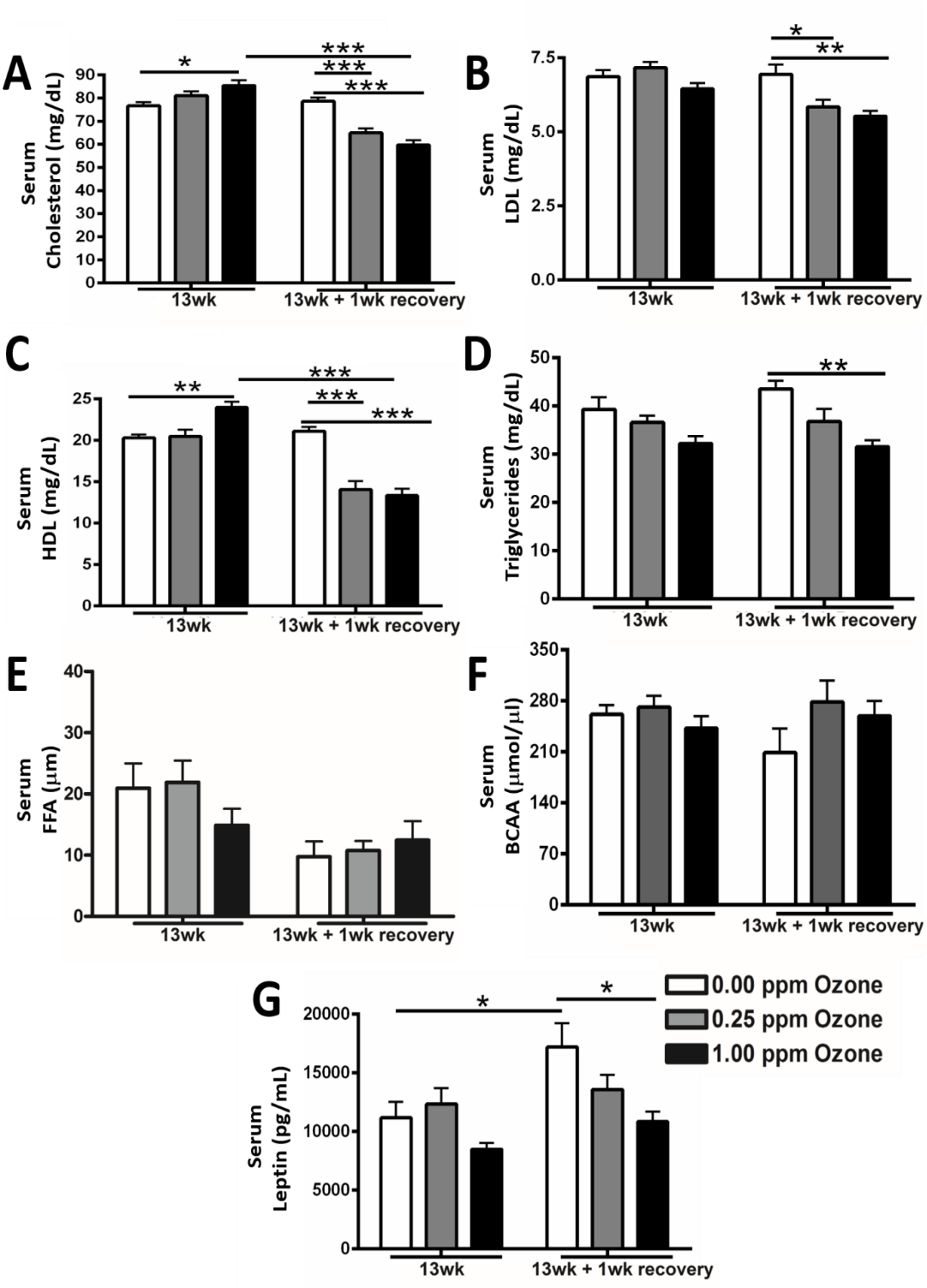
A
Serum Cholesterol (mg/dL)
90
80
70
60
50
40
30
20
10
0
*
***
***
***
13wk
13wk + 1wk recovery
B
Serum LDL (mg/dL)
7.5
5.0
2.5
0.0
*
**
13wk
13wk + 1wk recovery
C
Serum HDL (mg/dL)
25
20
15
10
5
0
**
***
***
***
13wk
13wk + 1wk recovery
D
Serum Triglycerides (mg/dL)
50
40
30
20
10
0
**
13wk
13wk + 1wk recovery
E
Serum FFA (μm)
40
30
20
10
0
13wk
13wk + 1wk recovery
F
Serum BCAA (μmol/μl)
350
280
210
140
70
0
13wk
13wk + 1wk recovery
G
Serum Leptin (pg/mL)
20000
15000
10000
5000
0
*
*
13wk
13wk + 1wk recovery
0.00 ppm Ozone
0.25 ppm Ozone
1.00 ppm Ozone

Figure 5.7 Subchronic ozone-induced changes in serum metabolic markers. Changes in circulating metabolites and leptin were measured in rats exposed to air, 0.25 ppm, or 1.00 pm ozone at 13wk and after 1wk recovery. The biomarkers included (A) total cholesterol (Chol.), (B) low density lipoprotein (LDL), (C) high density lipoprotein (HDL), (D) triglycerides, (E) free fatty acids (FFA), (F) branched chain amino acids (BCAA) and (G) leptin. Values indicate mean ± SEM (n=8-10). *Indicates significant ozone or recovery effects (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

Figure 5.8

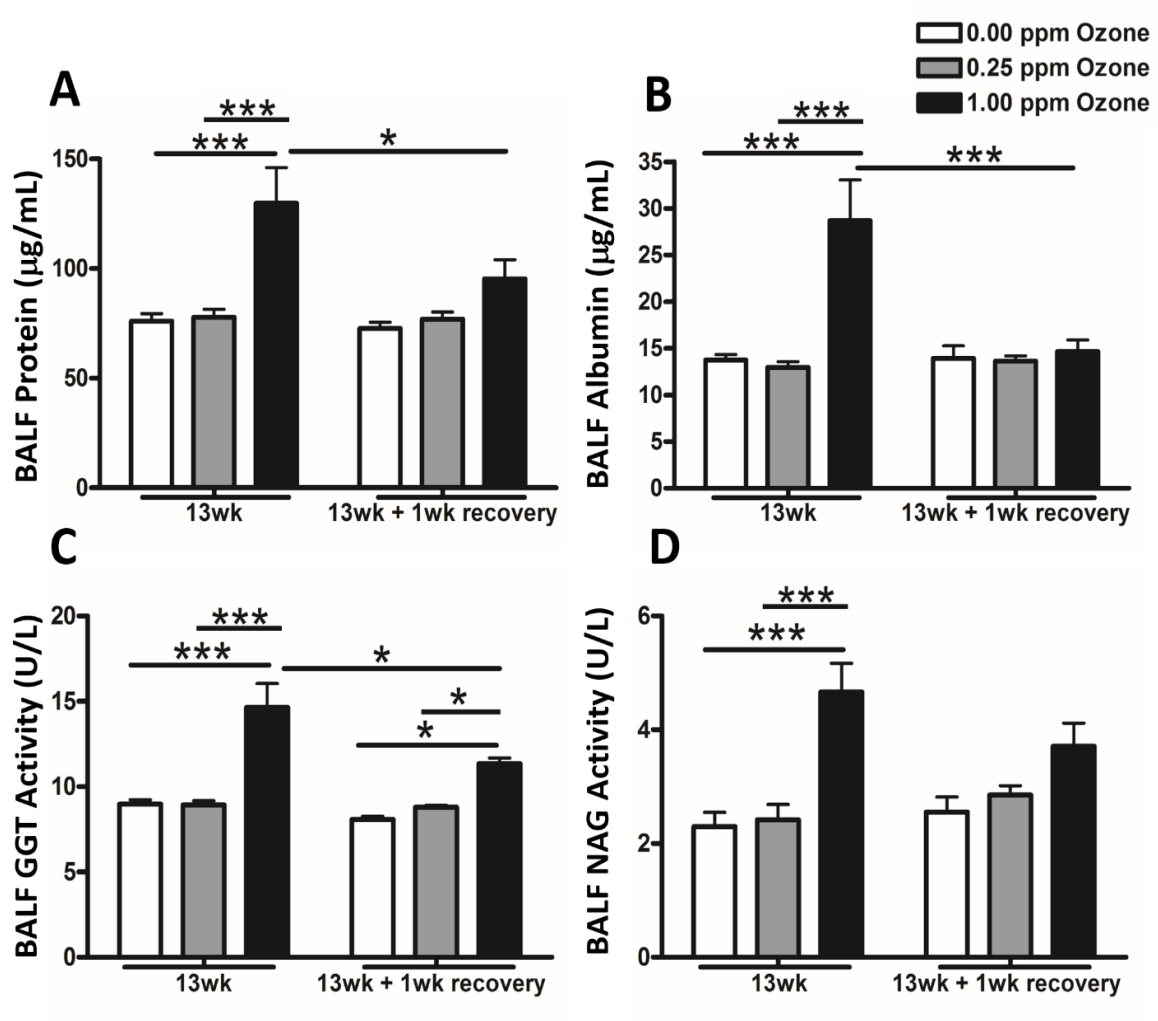

Figure 5.8 Subchronic ozone induced lung injury as determined by the assessment of bronchoalveolar lavage fluid (BALF). Lung injury markers were determined immediately after air, 0.25 ppm or 1.00 ppm ozone exposure for 13wks (13wk) and 1wk recovery following 13wk exposure. BALF was analyzed for lung injury markers: (A) total protein, (B) albumin, (C) γ-glutamyl transferase (GGT) activity and (D) N-acetyl glucosaminidase (NAG) activity. Values indicate mean ± SEM (n=8-10). *Indicates differences between groups (*p≤0.05, ***p≤0.001).

Figure 5.9

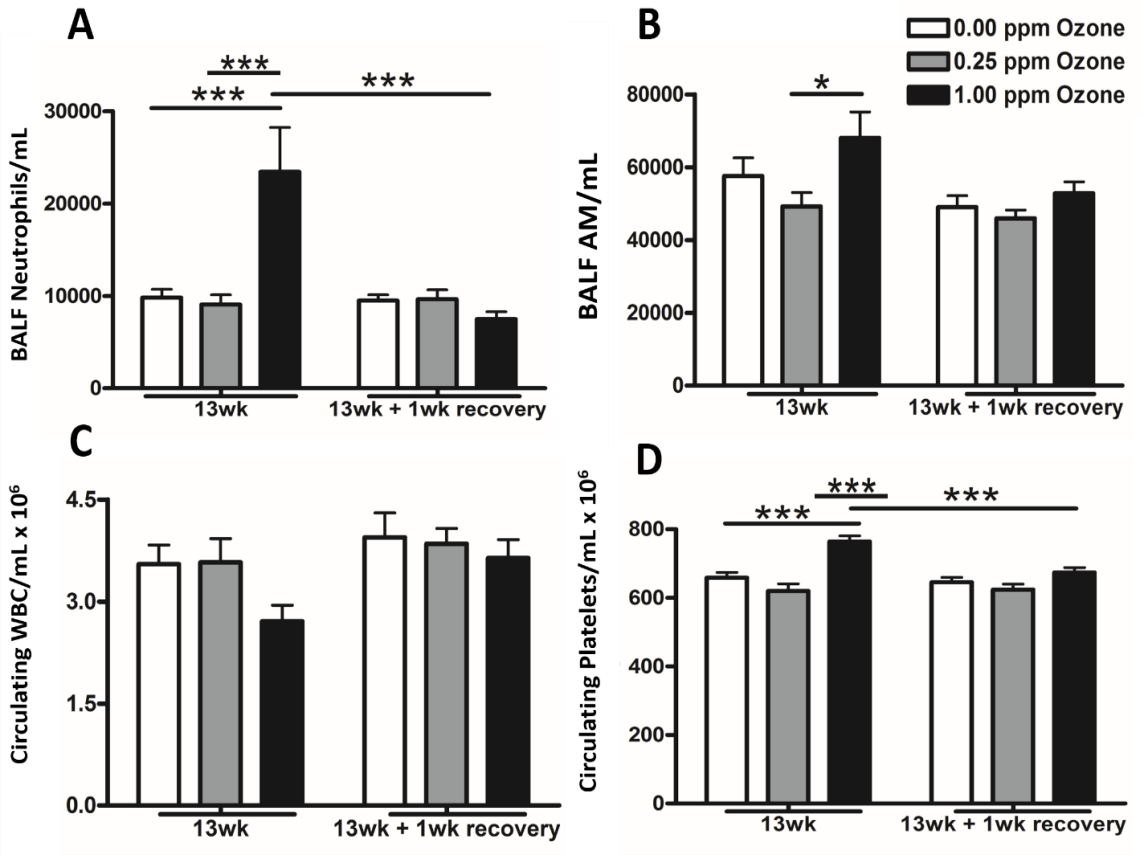

Figure 5.9 Subchronic ozone induced lung inflammation as determined by the assessment of bronchoalveolar lavage fluid (BALF). Lung inflammation was determined by analysis of cells in bronchoalveolar lavage fluid (BALF) immediately after air, 0.25 ppm or 1.00 ppm ozone exposure for 13wks (13wk) and after 1wk recovery following 13wk exposure. BALF total cell count and cell differentials were performed to quantify (A) alveolar macrophages (AM) and (B) neutrophils. Circulating white blood cells (WBC) and platelets were also quantified using a Beckman-Coulter AcT blood analyzer (C, WBC; D, platelets). Values indicate mean ± SEM (n=8-10). *Indicates significance between groups (*$p \leq 0.05$, ***$p \leq 0.001$).

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CHAPTER 6

OVERALL CONCLUSIONS AND SIGNIFICANCE

6.1 Air pollution and metabolism/diabetes

Recent epidemiological studies have implicated air pollutant exposure as a risk factor for diabetes (Pearson *et al.*, 2010; Liu *et al.*, 2013; Thiering and Heinrich, 2015). Specifically, respirable ambient particulate matter (PM) has been associated with increases in HbA1c (a marker of chronic high levels of blood glucose) (Chuang *et al.*, 2011; Tamayo *et al.*, 2014), impairment in glucose metabolism (Teichert *et al.*, 2013), elevated homeostatic model assessment of insulin resistance (HOMA-IR), and decreased insulin sensitivity in humans (Kim and Y.C., 2012; Brook *et al.*, 2013; Thiering and Heinrich, 2015). High ozone levels have been positively correlated with fasting hyperglycemia, insulin resistance and exacerbation of diabetes symptoms in humans (Stafoggia *et al.*, 2010; Zanobetti and Schwartz, 2011; Kim and Y.C., 2012). Exposure to ozone has also been associated with type 1 diabetes in pregnancy and in children (Hathout et al., 2006; Beyerlein *et al.*, 2015; Malmqvist et al., 2015). However, the question of how pollutants, specifically ozone, might contribute to these metabolic effects still remains. Mechanistic understanding of the causality of air pollutant-induced metabolic impairment will provide insight into the actual human risk for metabolic disease from pollutant exposures and the implementation of effective preventative measures.

Experimental studies on pollutant-induced metabolic effects have primarily focused on ambient respirable PM and long-term exposures in mice. Findings from these studies suggest that ambient PM exposure can impair glucose metabolism, increase adipose inflammation and induce liver insulin resistance in healthy and obese mice (Sun *et al*., 2009; Xu *et al*., 2011; Yan *et al*., 2011; Conklin et al., 2013; Sun *et al*., 2013; Laing *et al*., 2010). Regarding ozone, limited experimental studies have explored its role in inducing systemic metabolic effects. Previous ozone studies in our lab have shown that acute exposure causes hypothermia and bradycardia (Gordon *et al*., 2014) together with hyperglycemia and glucose intolerance in Brown Norway (BN) rats (Bass *et al*., 2013), while subchronic exposure increased circulating insulin in aged rats with a 1 day per week exposure protocol (Gordon *et al.*, 2013), suggesting that like PM, ozone can induce glucose metabolic effects. A recent study has demonstrated that a single 16 hour ozone exposure in rats increased oxidative stress biomarkers and reduced insulin sensitivity in the muscle (Vella *et al*., 2015). However, the mechanisms by which an acute ozone exposure may induce systemic metabolic effects and over a longer period of time lead to insulin resistance are not clearly understood.

6.2 Proposed mechanisms of pollutant-induced metabolic dysfunction and insulin resistance

A number of mechanisms have been proposed on how air pollutants may trigger metabolic dysfunction. Based on their physicochemical properties, inhaled pollutants might induce systemic effects by: (1) translocation of pollutant components from the airway to the periphery, (2) increases in circulating secondary oxidative byproducts and pro-inflammatory mediators and/or (3) effects on the CNS that regulate peripheral organ functions (Rajagopalan and Brook, 2012). It is postulated that these mechanisms can work

independently or synergistically with each other and target peripheral organs in a tissue-specific manner to perturb biochemical processes.

Ozone, an oxidizing agent, once inhaled immediately reacts with airway lining components, such as proteins and lipids, and is not likely to translocate systemically. Thus, ozone-mediated peripheral effects are likely to be caused by other proposed mechanisms. While PM exposure studies have implicated chronic systemic inflammation and oxidative stress in the mechanisms for metabolic dysfunction and the onset of insulin resistance, our data show no increases in circulating cytokines acutely or after subchronic ozone exposure, although acute phase proteins are increased (Bass *et al.*, 2013; Gordon *et al.*, 2013; Miller *et al.*, 2015). Some PM exposure studies have shown increases in circulating pro-inflammatory mediators (Nurkiewicz *et al.*, 2004; Finnerty *et al.*, 2007; Mutlu *et al.*, 2007; Niwa *et al.*, 2008), but other inhalation studies present no increases (Campen *et al.*, 2006; Kooter *et al.*, 2006; Montero *et al.*, 2006; Gottipolu *et al.*, 2009). Thus, systemic inflammation as an initiating and causal factor for the inhaled pollutant-induced metabolic effects is not consistently supported by the published studies, especially those involving ozone.

6.3 The potential involvement of the CNS and stress-response pathway

It has been shown that ozone can induce alterations in pulmonary and cardiovascular physiology through the autonomic nervous system, which includes sympathetic and parasympathetic arms. We have previously shown that acute ozone exposure causes hypothermia and bradycardia within 1hr, suggesting the involvement of the CNS (Gordon *et al.*, 2014). Ozone is thought to initiate changes in the autonomic nervous system through the activation of the transient receptor potential cation channel, member A1 (TRPA1) present on pulmonary C-fibers that terminate in the CNS (Taylor-Clark and Undem, 2010). Ozone has

been shown to activate the nucleus tractus solitarius (NTS) in the brain stem and different hypothalamic stress-responsive regions, such as the paraventricular nucleus (PVN) and the locus coeruleus (LC) (Gackiere *et al.*, 2011) that are linked to the autonomic nervous system and the hypothalamus pituitary adrenal (HPA) axis (Tsigos and Chrousos, 2002; Smith, 2006). These hypothalamic regions stimulated by ozone exposure might regulate adaptive stress responses, such as the regulation of thermogenesis, immune response, and peripheral tissue-specific metabolic alterations, and suggest a potential link between the nervous system and ozone-induced systemic effects.

Generally, when presented with a physical injury or psychological threat, alarm signals in the body are sent to hypothalamic stress-responsive regions that activate the integrated responses of the sympathetic nervous system (SNS) and HPA axis to release neuroendocrine hormones that initiate temporally precise peripheral metabolic and immune changes to counter the threat and restore homeostasis (McEwen, 2007). This classical stress response involves tissue specific changes in processes to mobilize and provide energy substrates to affected tissues (McEwen, 2007). The stress hormones, catecholamines (norepinephrine or noradrenaline and epinephrine or adrenaline) and glucocorticoids (corticosterone) regulate biochemical processes involving glucose, fat and protein metabolism through their action on stress hormone receptors (adrenergic for catecholamines and glucocorticoid for corticosterone), which show tissue-specific distribution in peripheral organs and centrally. This classical neuronally-mediated hormonal stress response and its implications for chronic diseases has been well studied for decades and still remains an area of interest. Persistent increases in these stress hormones and associated metabolic changes have been implicated in chronic metabolic dysfunction and insulin resistance through a

number of mechanisms involving the role of systemic inflammation and lipids. However, this stress response has not been linked to air pollution-induced systemic effects and metabolic dysfunction.

We have shown that circulating catecholamines increased after ozone exposure in rats (Bass *et al.*, 2013). Other investigators has also noted increases in circulating corticosterone and adrenocorticotropic hormone (ACTH) after ozone exposure (Thomson *et al.*, 2013). The adrenal gland is one of the main sources of stress hormones, where catecholamines are released from the adrenal medulla and glucocorticoids are released from the adrenal cortex, and play a central role in stress-mediated changes in metabolic homeostasis. The adrenal cortex can be activated through the HPA axis, which involves the activation of the hypothalamus and the secretion of corticotrophin releasing hormone (CRH). CRH activates the anterior pituitary gland to release ACTH that directly stimulates the adrenal cortex to secrete glucocorticoids (cortisol in humans and corticosterone in rats) and other hormones (Smith, 2006). In contrast, the adrenal medulla can be directly stimulated by sympathetic efferent nerves, which results in the secretion of epinephrine and/or norepinephrine (Smith, 2006). Therefore, based on the evidence of acute ozone-induced increases in stress hormones and hyperglycemia, it can be postulated that the initiating event for metabolic alterations might involve the activation of a neurohormonal stress response, leading to effects in multiple organs. No prior air pollution studies have examined the contribution of the neurohormonal stress response and subsequent release of epinephrine and corticosterone in mediating air pollution-induced systemic effects. In addition, no prior air pollution studies have reported in detail the types of systemic metabolic alterations or linked these effects to a neuronal stress response.

6.4 Project goals

The goals of this dissertation were to address the critical missing link between ozone exposure and peripheral metabolic alterations involving neuronal stress response mechanisms, and the potential of these effects to cause insulin resistance after subchronic exposure. As depicted in the second chapter, we wanted to characterize the nature of extra-pulmonary metabolic effects of acute ozone exposure in rats using a metabolomics approach and examine the levels of circulating cytokines and hormones. We also wanted to examine the peripheral metabolic changes, especially in the liver as it is the major metabolic organ, using global gene expression assessment to understand the mechanisms of ozone-induced systemic alterations. The temporal assessment of ozone-induced systemic metabolic derangement was important in understanding the role of the SNS and HPA axis. To assess the relevancy of our findings to humans, as depicted in the third chapter, we examined the profile of circulating metabolites using a metabolomics approach applied to serum samples from a clinical study involving human exposure to air or ozone. This was done to establish coherence between humans and rats with respect to ozone-induced systemic alterations. Moreover, we also presumed that using a metabolomic approach would lead to the identification of novel non-protein biomarkers of acute ozone exposure and inform the regulatory decision for ozone. In the fourth chapter, we evaluated if adrenal-derived stress hormones were essential in ozone-induced systemic and pulmonary effects using the surgical interventions of adrenal demedullation and total bilateral adrenalectomy followed by the exposure of rats to ozone. We also wanted to determine if the activation of TRPA1 and TRPV1 receptors were essential in mediating systemic and pulmonary effects of ozone (as discussed in this chapter). Lastly, as described in the fifth chapter, we determined if acute

ozone-induced metabolic and stress effects would persist during subchronic episodic ozone exposure, and lead to insulin resistance in the liver and muscle. Below, I have summarized the major findings from the dissertation project, stated limitations and made some suggestions for future research.

6.5 Acute ozone exposure induces peripheral metabolic alterations in rats

We show that acute ozone exposure produces fasting hyperglycemia, glucose intolerance and increases hepatic gluconeogenesis in Wistar Kyoto (WKY) rats in a concentration and time-dependent manner (Chapter 2) as observed previously in BN rats (Bass et al., 2013). We further sought to evaluate the mechanism of glucose intolerance to get a better understanding of the contributing mechanisms. Acute ozone exposure increased gluconeogenesis as evident by the pyruvate tolerance test. However, acute ozone exposure did not induce peripheral insulin resistance as determined by the insulin tolerance test. Instead, ozone attenuated glucose-mediated pancreatic β-cell insulin secretion (Chapter 5). These results indicate that ozone-induced hyperglycemia and glucose intolerance might not originate from peripheral tissue insulin insensitivity but might occur as a result of insulin insufficiency. Altogether, our ozone studies implicate the pancreas as a potential tissue target in ozone-induced metabolic alterations. More research evaluating the mechanism of the pancreatic alterations after ozone exposure would provide insight into how ozone may alter β-cell insulin secretion.

To further characterize ozone-induced global metabolic effects, we employed a serum metabolomics approach since this emerging technology allows for unbiased, comprehensive identification and relative quantification of various biochemical metabolites. This approach allowed us to identify systemic changes that have not been previously reported

in any air pollution study. Serum metabolomics analysis in rats revealed significant ozone-induced changes in hundreds of metabolites in the circulation. These significantly-changed metabolites consisted of numerous long- and medium-chain FFA, carnitines, bile acids, cholesterols, glucose, and BCAA, and revealed alterations in metabolic processes in the liver, muscle and adipose tissues. We noted that the pattern of metabolite changes after ozone exposure reflected those that are induced in a classical flight-or-fight hormonal stress response, revealing adipose lipolysis, muscle protein catabolism and a number of changes in liver reflecting increased gluconeogenesis and major changes in lipid metabolism. These metabolic effects were further associated with increases in circulating catecholamines and glucocorticoids as observed in our prior study (Bass *et al.*, 2013), which collectively supports the hypothesis that ozone induces systemic metabolic alterations through the activation of a classical neurohormonal stress response.

It is important to note that this stress response is well established with other physical and psychological stressors (injury, surgery, infection, fear etc.) (Schneiderman *et al*., 2005). However, these systemic changes and activation of stress mechanisms have not been studied with air pollution. Our data demonstrate that ozone, a prototypic air pollutant, can elicit a stress response and produce a myriad of extra-pulmonary effects. These data also allow us to postulate that a variety of environmental and social stressors might involve one common mechanism that can be linked to chronic disease conditions. Future studies could include the evaluation of the metabolomic profile of the liver, adipose and muscle tissue directly to provide additional insight in to the mechanism and identification of biochemical pathways affected by ozone exposure. Since these metabolic effects may persist during longer

exposure, metabolic profiling could also be used to examine the temporality and long-term effects of ozone exposure.

6.6 Acute ozone exposure increases circulating stress hormones and induces metabolic alterations in humans

To determine whether humans would present a similar pattern of metabolomic alterations and stress response as observed in rats when exposed to ozone, a metabolomics approach was used to assess human serum samples obtained from a clinical study involving air or ozone exposure. We hypothesized that since stress response-mediated mechanisms and metabolic processes are conserved across species, ozone exposure in humans would produce comparable metabolic dysfunction as in rats. In this study, serum samples from ozone-exposed humans showed a similar pattern of change in long-chain FFA and glycerols, indicating adipose lipolysis as observed in rats. This study also depicted increases in circulating cortisol, supporting the role of a neuronally-mediated stress response in systemic metabolic effects of ozone. Since humans were performing intermittent exercise during air or ozone exposure, more wide-spread changes in lipid metabolites were noted, such as increases in lysolipids a variety of phospholipid metabolites and β-oxidation byproducts, which were not evident in rats exposed during inactivity. Exercise alone is known to increase metabolic responses and thus, might have influenced some of the effects of ozone in humans. However because our study did not include non-exercising subjects, it is not possible to accurately separate ozone and exercise effects. A future study where one evaluates the differences between resting-state versus exercise-state in ozone-induced metabolic effects would further enhance our understanding of whether air pollutants might have more detrimental effects from ozone where humans are generally exposed outdoors during high

level activity. Future analysis to determine coherence between animals and humans' metabolic effects should repeat the current study with a higher number of subjects so that data can be stratified based on different characteristics, such as diet and exercise, and statistical power can be increased. The future studies may also include clinical assessments such as glucose tolerance testing for comparisons to the acute ozone-induced glucose intolerance observed in rodents. Additionally, metabolic profiling can be used to profile effects of ozone exposure with other contributing factors that may predispose humans to additional metabolic dysfunction (diet, pre-existing disease, BMI, etc.), however, we did not see any correlation with BMI and the effects induced by ozone on lipid metabolites (Chapter 3).

6.7 Adrenal-derived stress hormones are required for acute ozone-induced metabolic effects

To determine the contribution of adrenal-derived glucocorticoids and catecholamines in ozone-induced metabolic effects, we performed a comparative assessment of ozone metabolic and pulmonary effects in rats that underwent total adrenalectomy (to determine overall influence of sympathetic and HPA-mediated hormone release) or adrenal demedullation (to determine the influence of direct sympathetic stimulation on adrenal to release epinephrine). Moreover, the adrenalectomy and demedullation allowed for a more direct approach to control for circulating stress hormones levels in contrast to pharmacological interventions, which can have non-specific off-target effects. These surgical interventions have been utilized in many studies to evaluate HPA integrity and the assessment stress hormones effects (Kaminski and Watts, 2012; Weidenfeld and Feldman, 2000; Helmreich *et al*., 1996). However, this approach is sparingly used in air pollution

studies, and no studies have implicated HPA axis activation in air pollutant-induced systemic effects.

Overall, we demonstrated that the surgical removal of adrenal glands eliminated both corticosterone and epinephrine, while adrenal demedullation led to nearly complete elimination of epinephrine with a small reduction in corticosterone. This allowed us to examine the role of these hormones in ozone-induced systemic and pulmonary effects. Ozone-induced hyperglycemia, glucose intolerance, and increases in circulating adrenaline, FFA and BCAA were completely abolished by adrenalectomy and partially diminished by adrenal demedullation. Thus, it was clear that ozone-induced metabolic effects were produced from circulating catecholamines and corticosterone. Overexpression of the glucocorticoid receptors or increased levels of glucocorticoids *in vivo* has been shown to induce glucose intolerance and decrease insulin secretion, which results in insulinopenia, as observed in Cushing's disease patients (Delaunay *et al.*, 1997; Pivonello *et al.*, 2010; Kuo *et al.*, 2015). To further support if these stress hormones are involved in ozone-induced metabolic effects, a future experimental design should incorporate re-supplementation of the stress hormones at various concentrations in adrenalectomized rats to determine if the ozone-induced effects would resurge.

6.8 Adrenal-derived stress hormones are required for acute ozone-induced pulmonary injury and inflammation

The mechanism of ozone-induced lung injury and inflammation has been widely studied for decades. Upon inhalation, it has been demonstrated that the oxidant ozone can injure lung epithelial cells and mediate subsequent recruitment of inflammatory cells at the site of injury. It is believed that ozone-induced lung injury is a local event. No prior studies

have implicated the role of circulating stress hormones in mediating lung effects of ozone *in vivo* or *in vitro*. We were intrigued with our findings that ozone-induced lung protein leakage, lung cell injury and neutrophilic inflammation were partially inhibited in rats lacking adrenal medulla and nearly completely abolished in rats lacking whole adrenal glands. These results suggest that adrenal-derived hormones are not only involved in ozone-induced peripheral metabolic effects but also the inflammation and vascular leakage in the lung.

Our data show that in adrenalectomized rats, ozone-induced protein leakage was abolished. One potential mechanism of vascular leakage might relate to ozone-induced bradycardia and generalized cardiac depression (Wagner *et al*., 2015), which can increase hydrostatic pressure in respiratory capillary walls and increase the vascular leakage (Šedý *et al*., 2015). Another study showed that sympathetically-induced increased systemic blood pressure can also contribute to pulmonary vasculature pressure and injury (Krishnamoorthy *et al*, 2012), however, ozone exposure is known to decrease systolic blood pressure (Uchiyama et al., 1986; Wagner et al., 2014). β-adrenergic stimulation by epinephrine can increase right ventricular output and generalized vasodilation, which can increase the blood volume in pulmonary capillary bed and increase capillary pressure leading to edema (Rassler, 2012). As indicated above, ozone exposure leads to decreased blood pressure and systemic vasodilation. The ozone-induced increase in epinephrine, but not norepinephrine, together with decreased blood pressure-related systemic vasodilation, supports the role of β-adrenergic mechanisms in inducing lung protein leakage after ozone exposure and the decrease of edema when epinephrine is removed from circulation via adrenelectomy or demedullation.

Ozone-induced lung neutrophilia is associated with a decrease in circulating white blood cells (lymphocytes) as observed in SHAM rats exposed to ozone. Supplementing cortisol-mimic to patients with adrenal insufficiency leads to depletion of lymphocytes in the circulation (Geigen et al., 2015). Generally, stress hormones, such as glucocorticoids, are known to produce immuno-suppression and reduce inflammatory responses in chronic diseases (Edwards, 2012), however, in more acute inflammatory conditions, glucocorticoids can initiate leukocyte trafficking and extravasation at the site of injury in an immune cell-specific manner (Dhabhar, 2009; Dhabhar et al., 2012). For instance, circulating lymphocytes can decrease, as observed following ozone exposure, and neutrophils can increase in blood, which was not separately quantified in our study. Catecholamines initiate recruitment of neutrophils to the airways though initiation of IL-8 production from monocytes (Linden, 1996; Kavelaars *et al.*, 1997). These findings support our results where removal of the adrenal medulla and the total adrenalectomy diminished ozone-induced increases in neutrophils in the lung while not affecting circulating lymphocytes. Instead, the removal of stress hormones appeared to increase eosinophils, which might suggest either the differential influence of catecholamines versus corticosterone in an immune cell-specific manner or the time-related differences in the type of cells extravasated. Identification of different types of immune cells and their maturation stages will need to be evaluated in a temporal manner using flow cytometry to understand the precise contribution of stress hormones to the homeostatic innate immune response after ozone exposure. These studies should evaluate the role of specific stress hormones in selective recruitment of neutrophils, monocytes, and eosinophils to the lung from their marginated pool within bone marrow, spleen, circulation or other depots within the lung (i.e. lung-associated lymph nodes).

6.9 The role of pulmonary afferent C-fibers in mediating ozone induced activation of neuronal stress response through TRPA1 and TRPV1

It was hypothesized that ozone activation of brain stress responsive regions through TRPA1 and transient receptor potential-member V1 (TRPV1) receptors (Lee and Pisarri, 2001), co-localized on pulmonary C-fibers, were involved in peripheral metabolic effects. These TRP receptors on C-fibers are activated in the presence of internal and/or external chemical and thermal stimuli and send neural impulses to the CNS regions, including the NTS, which regulate respiratory mechanics and its synchronization/coordination with cardiovascular function (Lee and Pisarri, 2001). The activation of TRPA1 on bronchial vagal C-fibers by ozone has been implicated in bradycardia and arrhythmias in rats (Hazari *et al.*, 2011), and is likely to be involved in ozone-induced metabolic effects through stress hormones. To evaluate the role of TRP receptors in ozone-induced systemic and pulmonary effects, we pretreated rats with selective pharmacological antagonists of TRPA1 (HC030031) and TRPV1 (AMG 9810), separately or in combination, and then assessed ozone-induced metabolic impairment. The results from this study showed that antagonism of the TRPA1 and TRPV1 separately or in combination did not show significant protection in ozone-induced metabolic or pulmonary effects (Fig. 6.1.). Although the contribution of TRPA1 has been reported in the literature using same antagonists and similar treatment protocols, our data raises a concern as to the availability of the sufficient quantity of antagonists at the airway surface lining after intraperitoneal treatment, and potential contribution of TRPs in mediating neuronal activation. We did not determine the circulating levels of these pharmacological antagonists, therefore, it will be important to evaluate the bioavailability, efficacy, and the pharmacokinetics of the TRPA1 and TRPV1 receptor antagonists used in our study. Another

limitation is that we did not assess receptor activation to make sure the drug effectively inhibited the respective receptors. We presumed that the protocols reported to effectively antagonize TRP receptors would be adequate to modulate their effect in our experimental setting. It is also possible that other potential mechanisms might be involved for neuronally-mediated stress response activation by ozone. The roles of other neurons with rapidly adapting receptors and slowly adapting receptors that terminate in the CNS will also need to be clarified since high specificity exists in which neurons are activated by distinct stimuli (Taylor-Clark and Undem, 2010).

6.10 Long-term episodic subchronic ozone exposure effects on stress and metabolic outcomes

To determine if persistent stress and metabolic effects induced by long-term ozone exposure are linked to peripheral insulin resistance, we conducted a weekly episodic subchronic ozone exposure study in rats (Chapter 5). In this study, we showed that subchronic ozone exposure is associated with persistent pulmonary injury and inflammation, fasting hyperglycemia, glucose intolerance, and elevated circulating adrenaline and cholesterol levels when determined at the end of the last exposure, however, these responses are of lesser magnitude than the effects noted after 1 or 2 days of ozone exposure. Moreover, the increases in FFA and BCAA that were noted after an acute ozone exposure were not apparent following a subchronic exposure. More importantly, if rats were allowed a one week recovery, these effects were largely reversed. Our data further show that subchronic ozone exposure did not significantly increase gluconeogenesis as determined after 13 weeks, but it abolished the response of β-cells to increase insulin secretion in response to hyperglycemia. Catecholamines, such as epinephrine, decrease glucose-mediated uptake and

pancreatic insulin secretion through the α-adrenergic α (2A) and α (2C)-adrenoreceptors (Iversen, 1973; Halter *et al.*, 1984; Ullrich and Wollheim, 1984; Philipson, 2002; Peterhoff *et al.*, 2003). Future analysis of the direct implication of ozone-mediated stress response and the examination of α-adrenergic and/or glucocorticoid receptors on β-cell insulin secretion using pharmacological means will provide valuable mechanistic information on how air pollutants may impair the function of the pancreatic β-cells. Our data indicating ozone-induced impairment in insulin secretion are consistent with the finding that ozone exposure decreases circulating insulin after subchronic ozone exposure. More importantly, no peripheral insulin resistance was noted after subchronic ozone exposure, as determined by an insulin tolerance test and liver and muscle pAKT levels. Thus, our findings in young-adult rats provide insights into epidemiological studies that show a positive association between ozone exposures and T1D in children and pregnant women (Hathout *et al.*, 2002; Hathout *et al.*, 2006; Beyerlein *et al.*, 2015). It is possible that ozone-induced β-cell dysfunction may secondarily contribute to insulin resistance and a TIID phenotype in the long term due to non-regulation of glucose, lipid, and protein metabolites that have been implicated in decreased insulin sensitivity.

Repeated daily ozone exposure has been shown in many studies to induce an adaptation response, characterized by the reduction in ozone-induced lung injury and inflammation. Since, our experiment included 3 days per week exposure, we expected that the effects seen after 3 days would be smaller magnitude than the effects seen during the 1st or 2nd day. Therefore, we performed IPGTT and IPPTT on the 1st and 3rd day to determine if the effects seen on the 1st day were diminished on the 3rd day. We noted that on the 3rd day, ozone-induced glucose intolerance and gluconeogenesis response was markedly reduced

relative to the 1st day response, suggesting an induction of adaptation upon continuous exposure. This adaptation response is lost if animals were allowed 4-5 days recovery prior to the next ozone hit. The mechanisms of ozone adaptation are unknown. Since repeated stimulated stress responses have been shown to decrease c-fos staining in the medial parvocellular region of the PVN and glucocorticoid receptors in hypothalamic regions and in the periphery (Herman, 2013), we postulate that ozone adaptation is centrally regulated and might involve the HPA-axis. We believe that subchronic ozone-induced stress mechanisms are likely involved in adaptation to repeated exposure in a given week and episodic exposures over 13 weeks. To address the role of neuronal mechanisms in ozone-induced adaptation, more studies are needed with varied temporal exposure scenarios.

6.11 Summary/conclusions and implications on human health

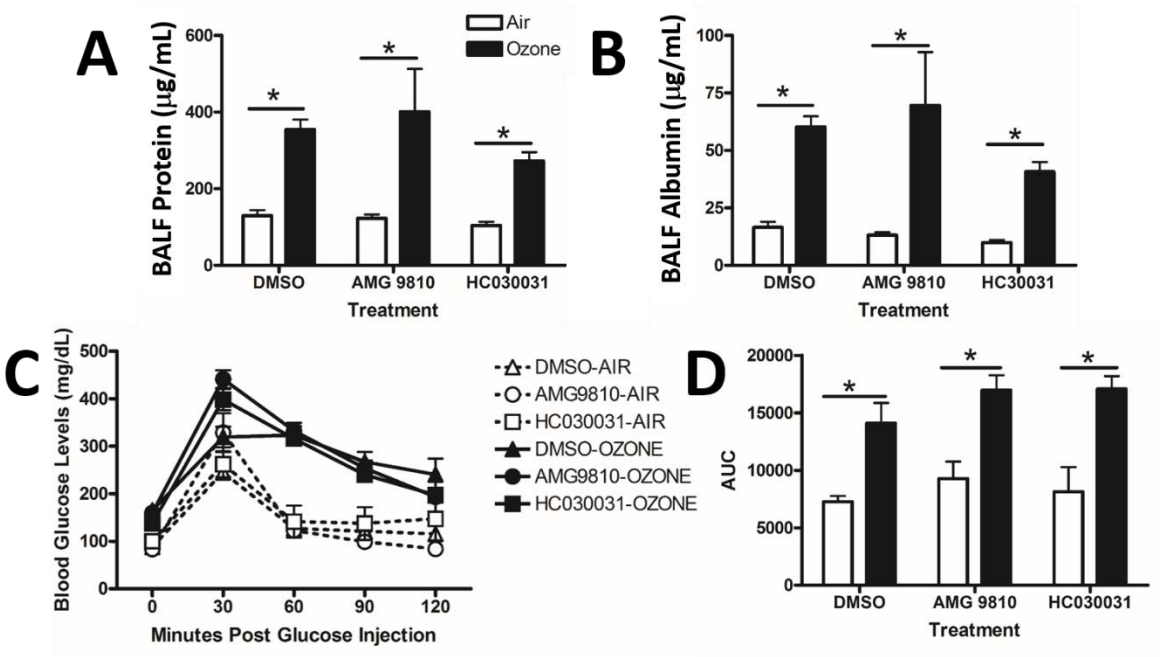
- Neurohormonal stress response has been well characterized for decades but has not been linked to air pollution effects. We show that the activation of neurohormonal stress response pathway is involved in ozone-induced systemic metabolic and pulmonary effects in rats.
- We further show that this hormonal stress response is also induced in humans after ozone exposure, and that rat and human metabolic responses to ozone are coherent. Our rat and human metabolomic data allowed us to identify novel non-protein biomarkers of acute ozone effects.
- Through the use of metabolomics, we characterized "the circulating molecular shrapnel" referred for unknown biochemical identity by others in air pollution studies. This included over 100 metabolites and other biochemicals (neurohormones,

lipids, fatty acids, cholesterols, glucose metabolites, and acute phase proteins) – most are known to have varied cellular effects (Fig. 6.2).

- Through the use of surgical intervention involving adrenalectomy that has not been used in prior air pollution studies, we demonstrated that not only systemic metabolic impairment but also pulmonary injury/inflammation are mediated through circulating stress hormones such as epinephrine and corticosterone after ozone exposure (Fig. 6.2).
- Chronic perturbation in these homeostatic pathways can lead to disturbed metabolic status, immune dysfunction, disrupted neuronal regulation, and failure to repair the injury caused by stressors such as air pollutants. We demonstrate that ozone-induced pulmonary and metabolic affects still persist during subchronic exposure but are reversible upon termination of exposure. However, it remains to be determined how pre-existing conditions or longer duration may alter adaptation and reversibility of ozone-induced metabolic and stress effects.
- Through the subchronic ozone inhalation study, we demonstrated that ozone, contrary to what was hypothesized, does not induce peripheral insulin resistance, but rather severely impairs the ability of the β-cells to secrete insulin in response to high circulating glucose, and results in lower circulating insulin upon subchronic exposure.

- We believe that this neurohormonal stress response pathway induced after ozone exposure can also be induced by other environmental insults and thus, can be used as a common mechanistic pathway to stratify risk from many environmental chemicals.
- This research emphasizes on the need to investigate the potential contribution of air pollution to systemic metabolic diseases, such as neuronal, developmental, liver, and muscular diseases, in people who have impaired stress mechanisms.

Figure 6.1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90
120
Minutes Post Glucose Injection
DMSO-AIR
AMG9810-AIR
HC030031-AIR
DMSO-OZONE
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Treatment

Figure 6.1 TRPA1 and TRPV1 antagonism does not inhibit acute ozone-induced pulmonary injury nor glucose intolerance.

Rats were intraperitoneally injected with either DMSO (control), AMG9810 (TRPV1 antagonist), or HC030031 (TRPA1 antagonist; 50 mg/kg) four times every 10-12 hours (the night before exposure, immediately before the 1st exposure, the night after the 1st exposure and the morning before the 2nd exposure). Rats were exposed to air or 1.00 ppm ozone for 4h/day for two consecutive days. Immediately after day 1, GTT was performed. Rats were necropsied immediately following the 2nd day exposure and bronchoalveolar lavage fluid (BALF) was analyzed for lung injury markers: (A) protein and (B) albumin, as indicators of vascular protein leakage. (C) GTT and (D) AUC for GTT in rats exposed to air or ozone for 4 hr, 1 day. The values in the bar graphs are displayed as mean ± SEM of n=6/exposure group. The GTT curve shows mean value ± SEM of n=6/group with repeated measures over two hours. The 0 min time point shows fasting glucose levels in each group. * Indicates significant difference from respective air-exposed groups at a given time (*=$p<.05$)

Figure 6.2

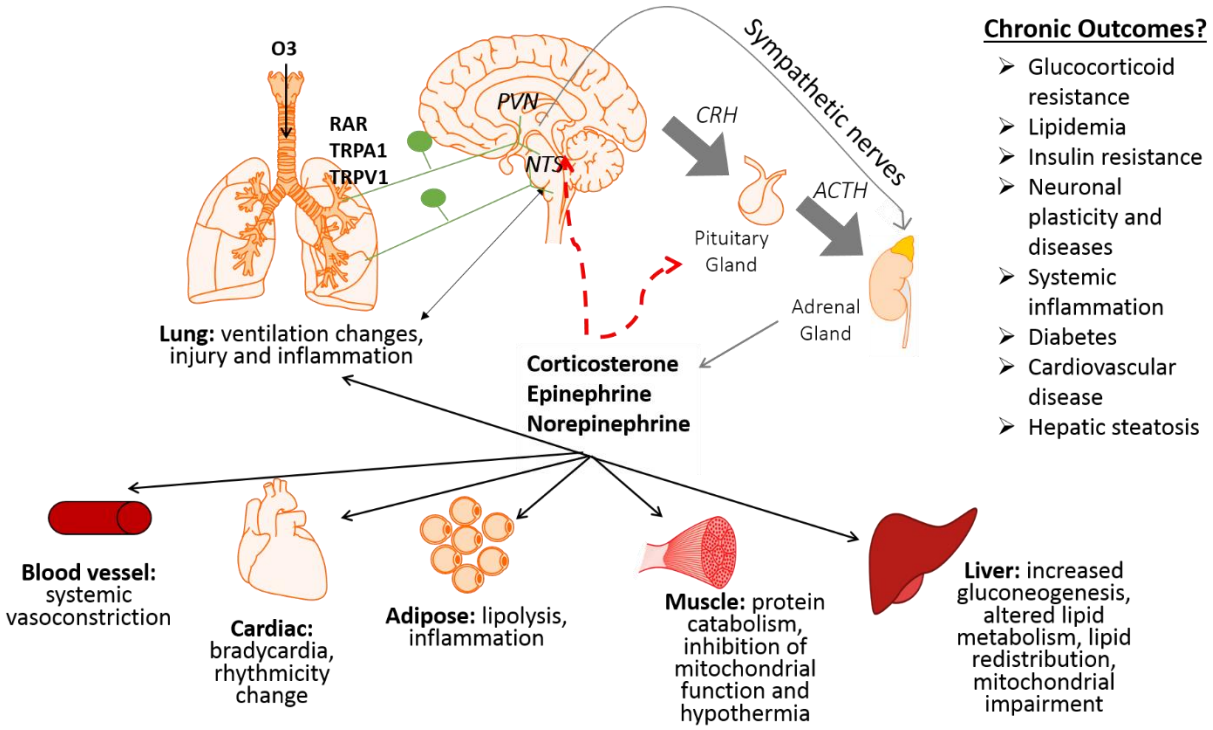

Figure 6.2 Proposed mechanism(s) of overall ozone-induced stress mediated pulmonary, cardiovascular and metabolic responses. In our studies, we show that ozone induces a classical stress response reflected by changes observed in multiple organs, including the lung, liver, adipose, and muscle, that overtime may be linked to chronic diseases. Upon inhalation, ozone may elicit these extrapulmonary responses through activation of the CNS and subsequent activation of the HPA axis, and/or SNS, resulting in changes in the periphery through neuroendocrine hormones. NTS, nucleus tractus solarius; PVN, paraventricular nucleus; HPA, hypothalamus pituitary adrenal axis; CRH, corticotropin-releasing hormone; ACTH, adrenalcorticotrophic hormone; TRPA1, Transient receptor potential-member A1; TRPV1, Transient receptor potential-member V1; RAR, rapidly adapting receptor

6.12 REFERENCES

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APPENDIX A.

CHAPTER TWO

Figure 1A.

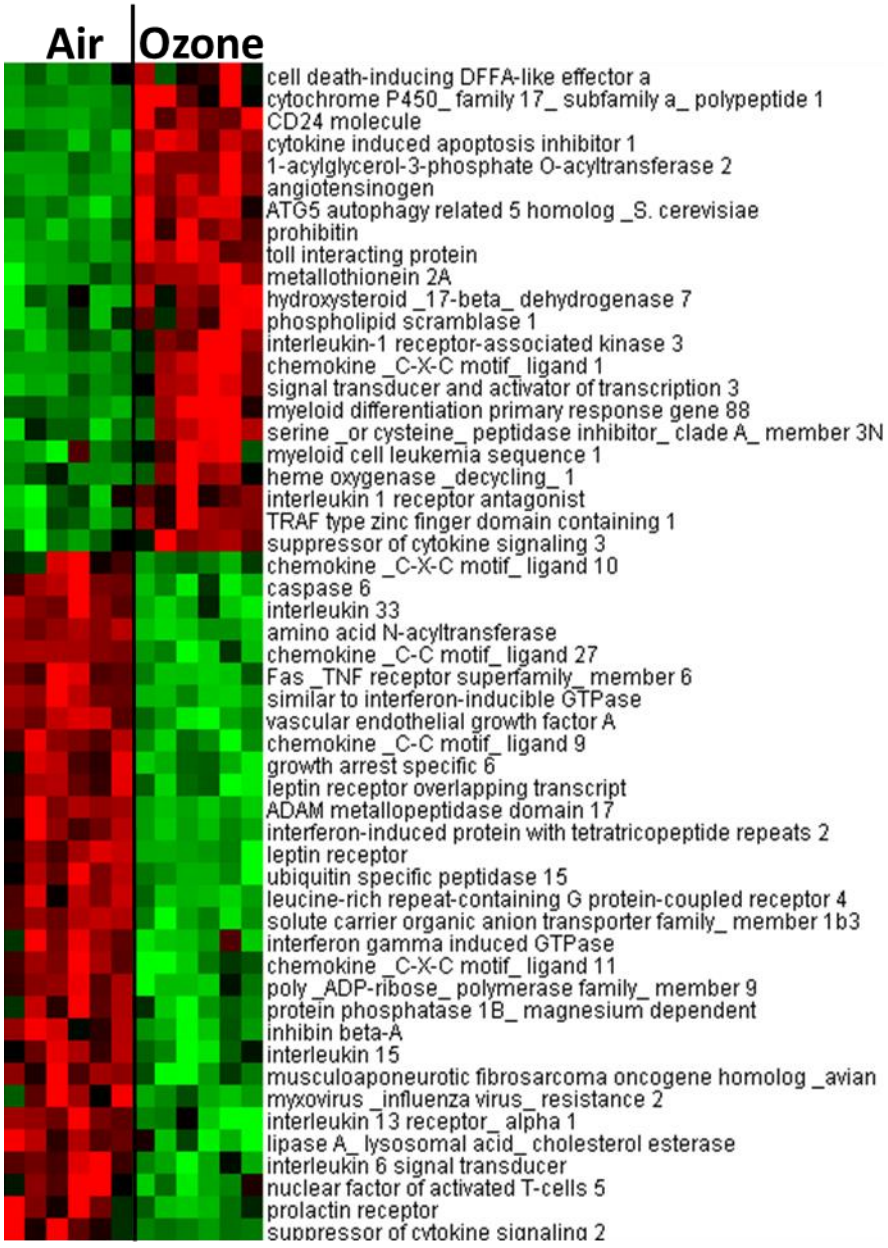


Figure 1B.

Figure 1C.

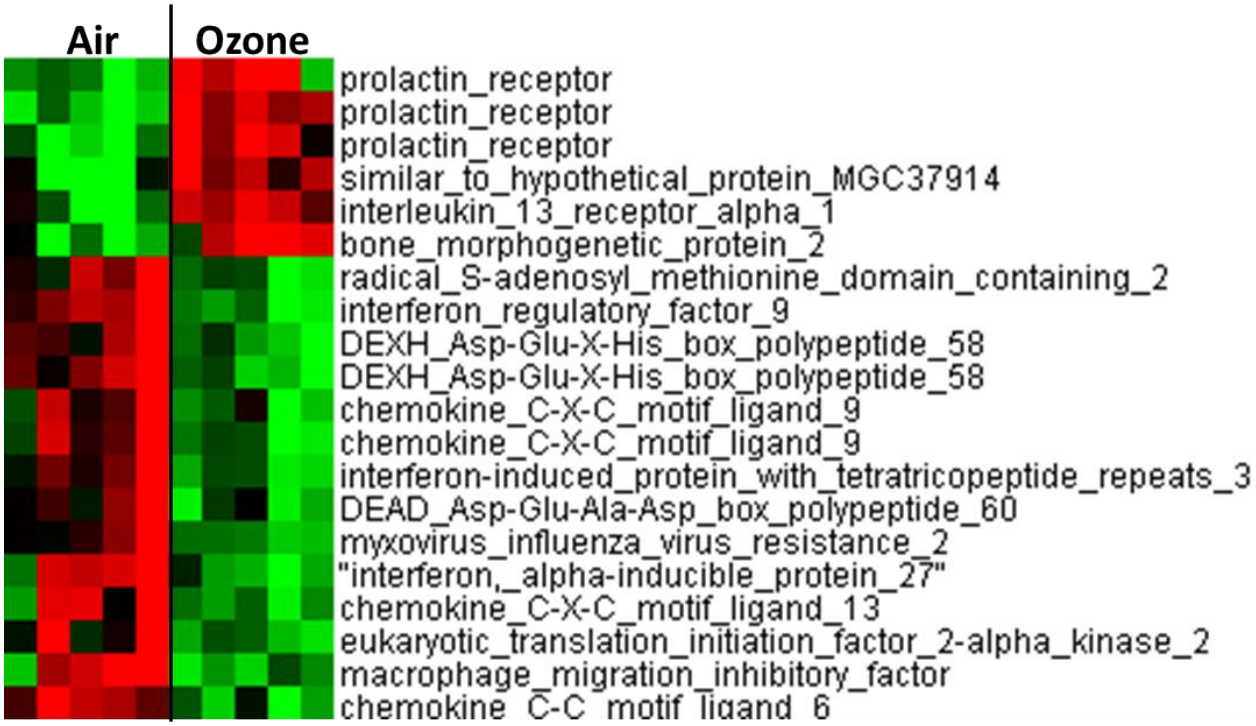
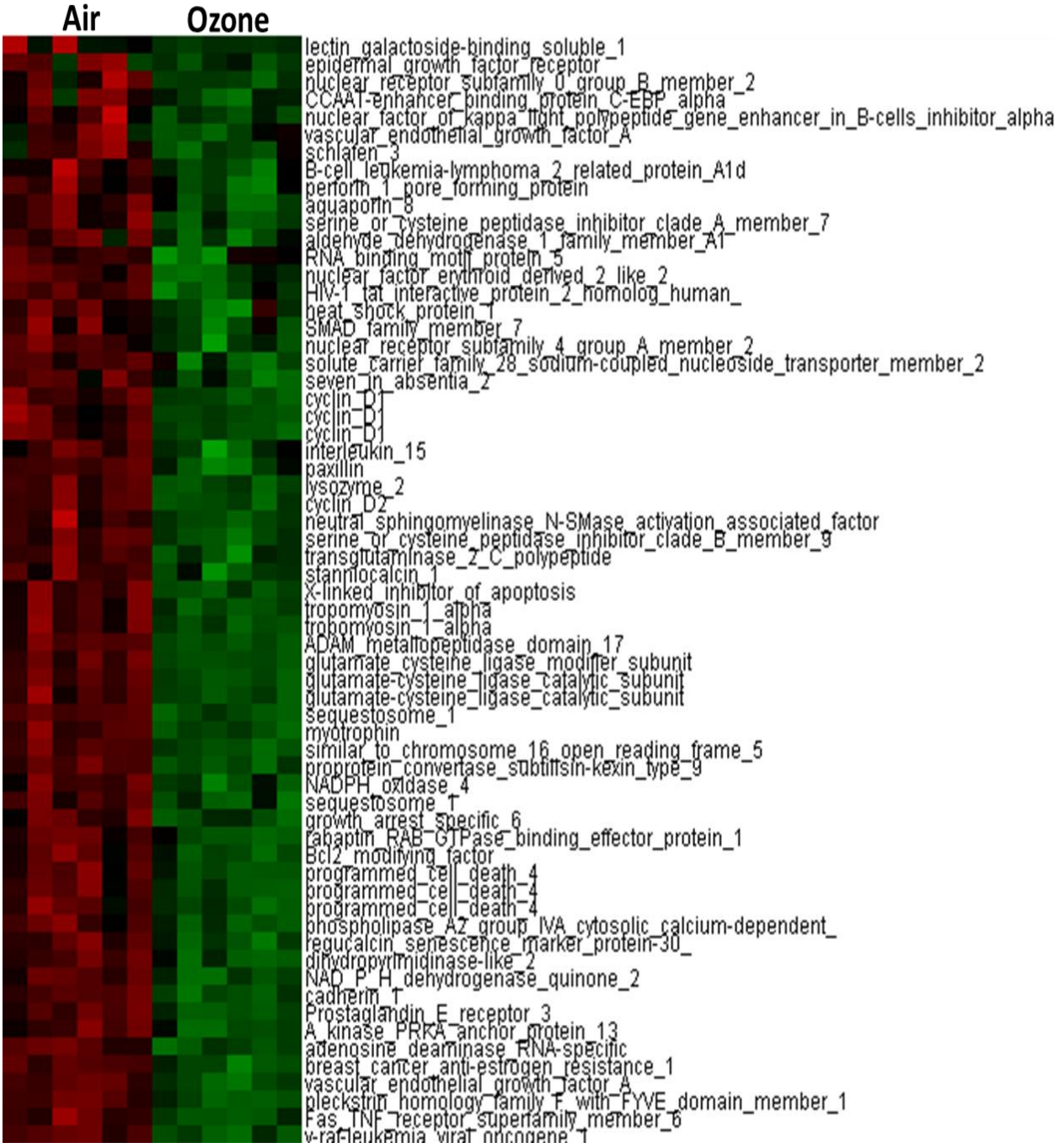


Figure 1: Heatmaps of liver gene expression for cytokine network in rats exposed to ozone (A: immediately after 1-day 6-hr air or ozone, 0-hr time point; B. immediately after 2-day 6-hr air or ozone, 2-day, 0-hr) and C. 18-hr after 2-day 6-hr air or ozone; 2-day, 18-hr) exposure. Functional gene lists were generated by NetAffx queries at the Affymetrix website (www.affymetrix.com) and identified from differentially expressed genes (DEGs) list for each time point (air vs O_3) based on the query term, "cytokines". Genes were then median centered with average linkage, hierarchically clustered using Cluster 3.0 and displayed through Java Treeview. Heat map of DEGs with significant O_3 effect were identified separately at each time point. Red indicates genes that have high expression values across both groups, green indicates genes that have low expression values across both groups, and black indicates median expression.

Figure 2A.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schlafen_3
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perforin_1_pore_forming_protein
aquaporin_8
serine_or_cysteine_peptidase_inhibitor_clade_A_member_7
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heat_shock_protein_1
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cyclin_D1
cyclin_D1
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lysozyme_2
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neutral_sphingomyelinase_N-SMase_activation_associated_factor
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transglutaminase_2_C_polypeptide
stanniocalcin_1
X-linked_inhibitor_of_apoptosis
tropomyosin_1_alpha
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glutamate-cysteine_ligase_catalytic_subunit
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myotrophin
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Prostaglandin_E_receptor_3
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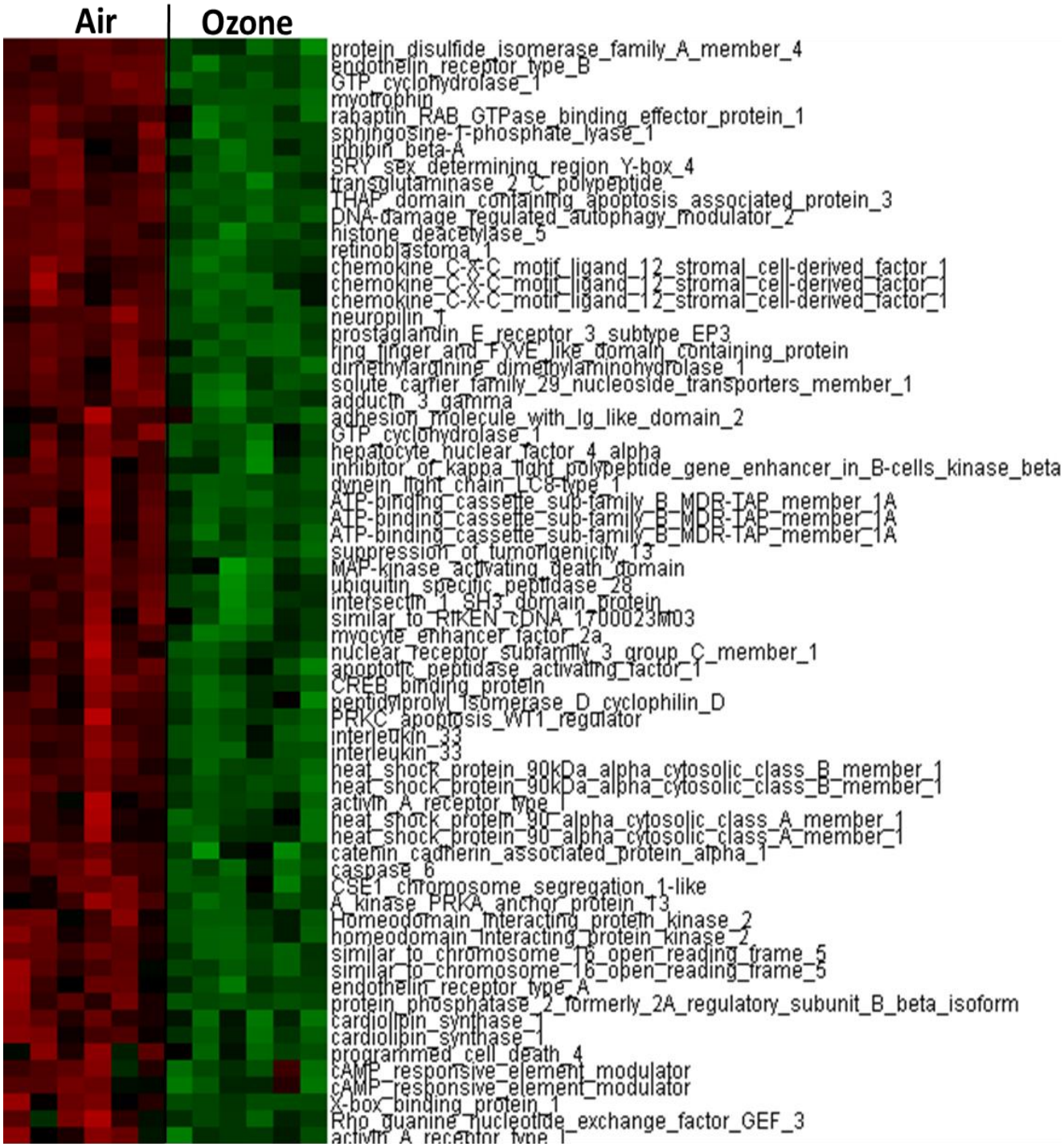
Figure 2B.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sphingosine-1-phosphate_lyase_1
inhibin_beta-A
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transglutaminase_2_C_polypeptide
THAP_domain_containing_apoptosis_associated_protein_3
DNA-damage_regulated_autophagy_modulator_2
histone_deacetylase_5
retinoblastoma_1
chemokine_C-X-C_motif_ligand_12_stromal_cell-derived_factor_1
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solute_carrier_family_29_nucleoside_transporters_member_1
adducin_3_gamma
adhesion_molecule_with_Ig_like_domain_2
GTP_cyclohydrolase_1
hepatocyte_nuclear_factor_4_alpha
inhibitor_of_kappa_light_polypeptide_gene_enhancer_in_B-cells_kinase_beta
dynein_light_chain_LC8-type_1
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ubiquitin_specific_peptidase_28
intersectin_1_SH3_domain_protein
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cardiolipin_synthase_1
programmed_cell_death_4
cAMP_responsive_element_modulator
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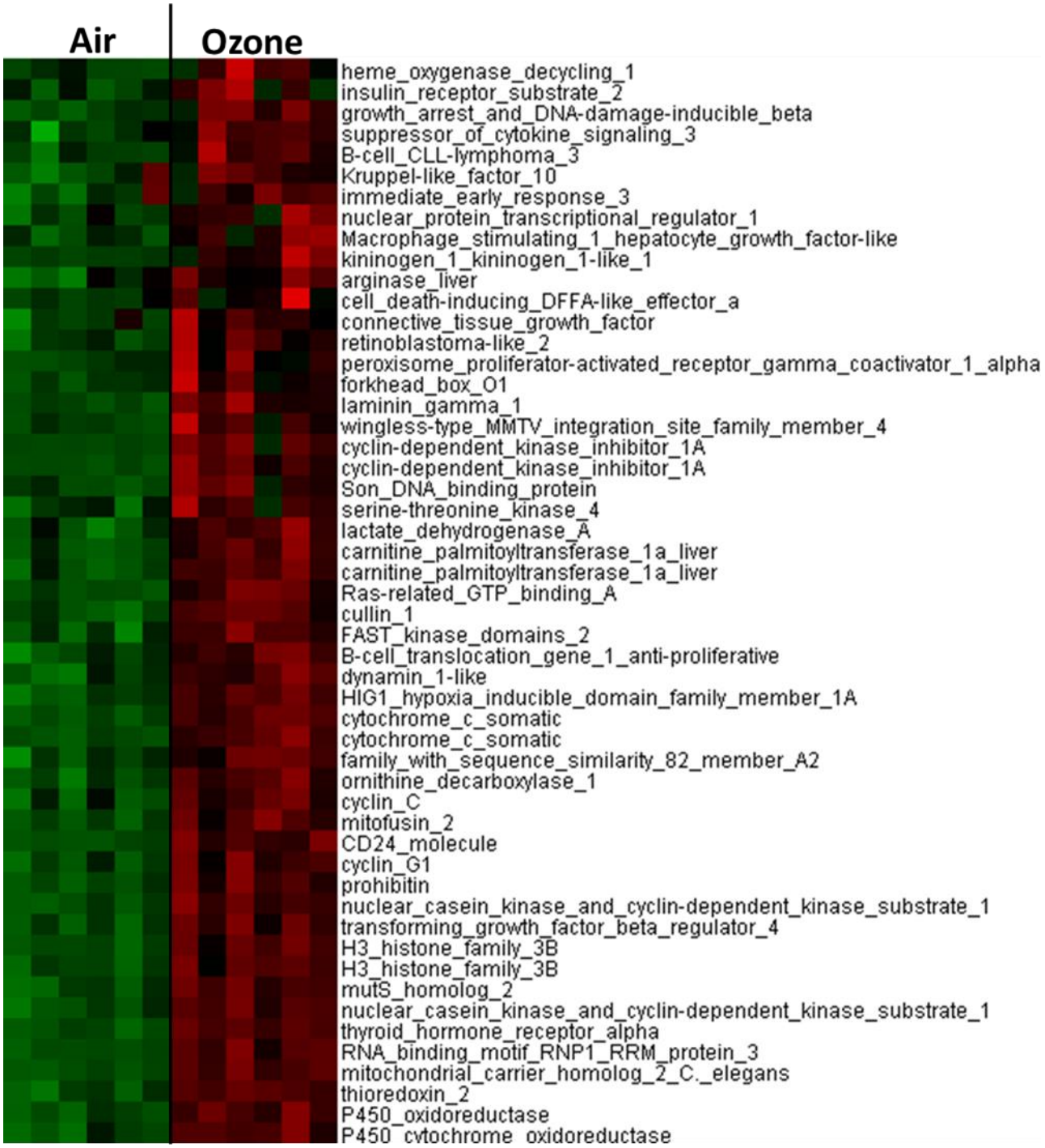
Figure 2C.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Kruppel-like_factor_10
immediate_early_response_3
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cell_death-inducing_DFFA-like_effector_a
connective_tissue_growth_factor
retinoblastoma-like_2
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forkhead_box_O1
laminin_gamma_1
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cyclin-dependent_kinase_inhibitor_1A
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lactate_dehydrogenase_A
carnitine_palmitoyltransferase_1a_liver
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Ras-related_GTP_binding_A
cullin_1
FAST_kinase_domains_2
B-cell_translocation_gene_1_anti-proliferative
dynamin_1-like
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cyclin_C
mitofusin_2
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cyclin_G1
prohibitin
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transforming_growth_factor_beta_regulator_4
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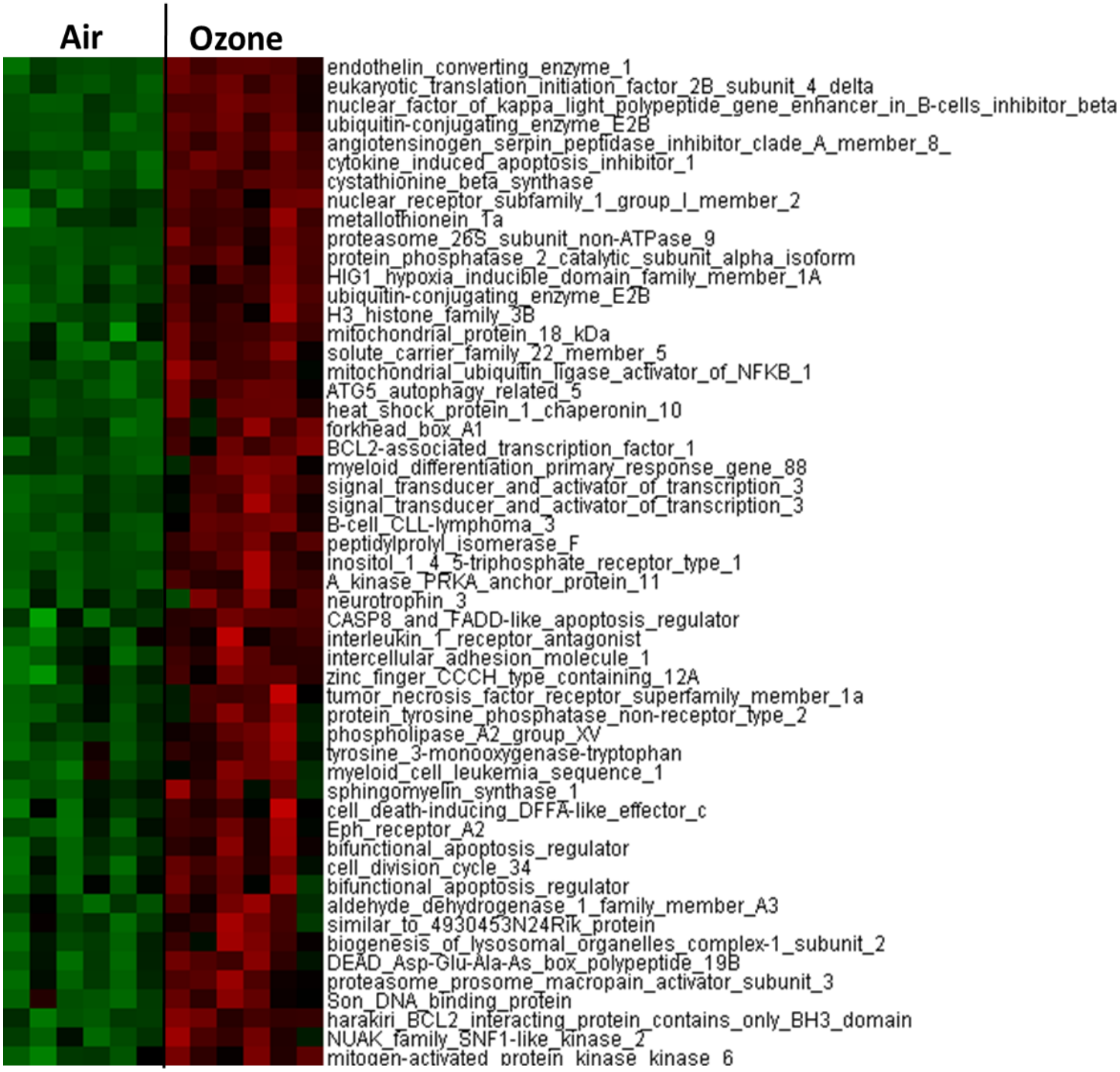
P450_cytochrome_oxidoreductase

Figure 2D.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ubiquitin-conjugating_enzyme_E2B
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ubiquitin-conjugating_enzyme_E2B
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mitochondrial_protein_18_kDa
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forkhead_box_A1
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CASP8_and_FADD-like_apoptosis_regulator
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phospholipase_A2_group_XV
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sphingomyelin_synthase_1
cell_death-inducing_DFFA-like_effector_c
Eph_receptor_A2
bifunctional_apoptosis_regulator
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biogenesis_of_lysosomal_organelles_complex-1_subunit_2
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proteasome_prosome_macropain_activator_subunit_3
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Figure 2E.

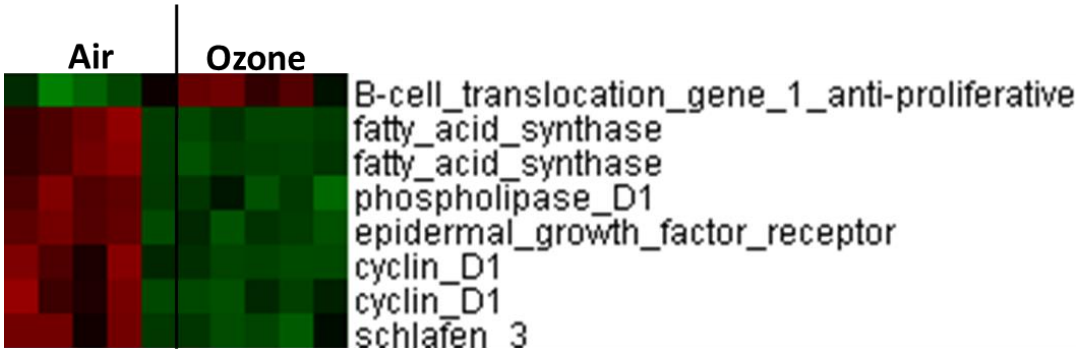


Figure 2F.

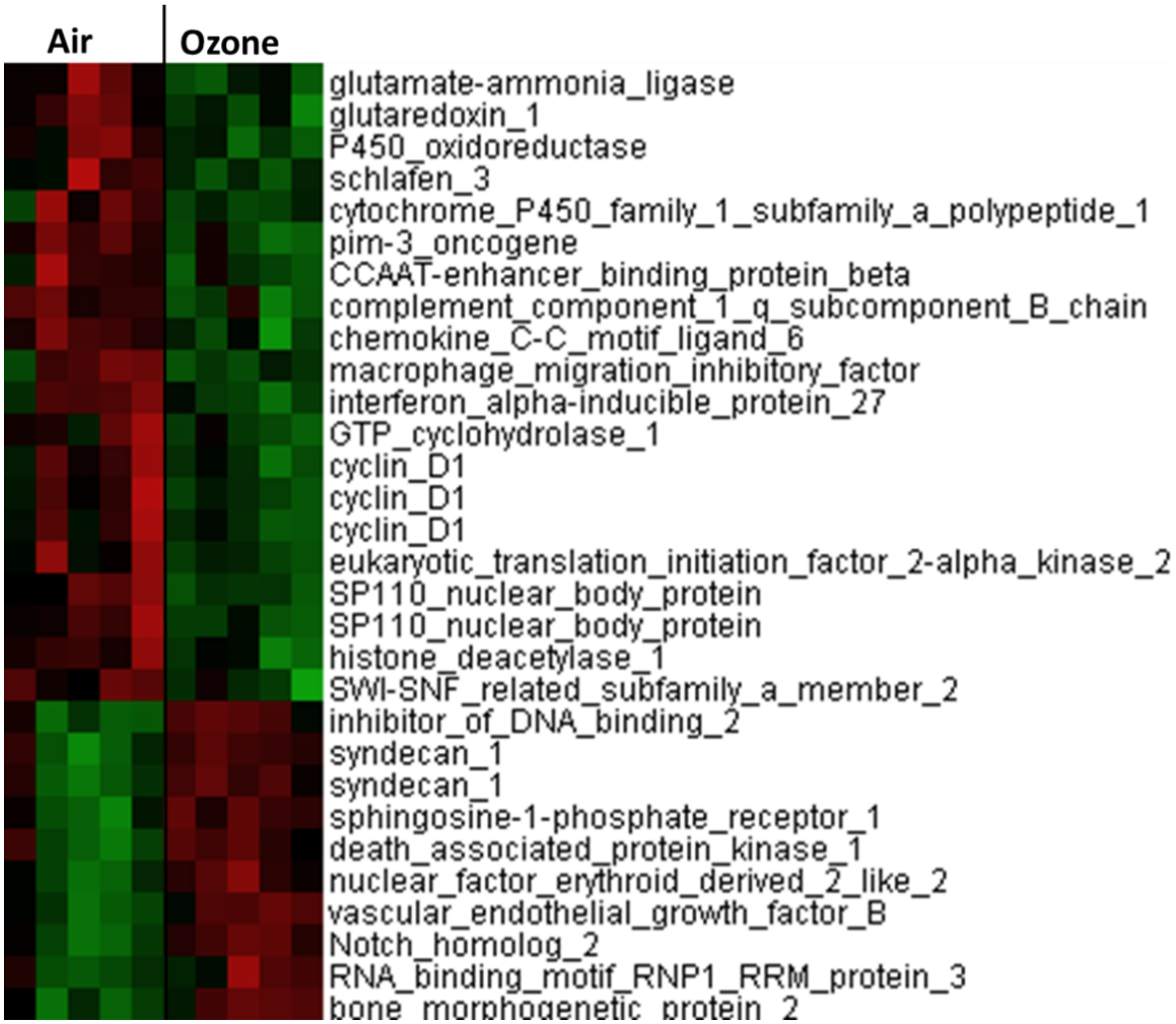


Figure 2: Heatmaps of liver gene expression showing differentially expressed genes (air versus O_3) for apoptosis network in rats exposed to O_3 (A-D, exposed for 6hr/day; immediately after 1-day; E, immediately after 2-day, F, 18-hr after 2-day time point). Functional gene lists were generated by NetAffx queries at the Affymetrix website (www.affymetrix.com) and identified from differentially expressed genes (DEGs) list based on the query term, "apoptosis". Genes were then median centered with average linkage, hierarchically clustered using Cluster 3.0 and displayed through Java Treeview. Heat map of DEGs with significant O_3 effect were identified separately at each time point. Red indicates genes that have high expression values across both groups, green indicates genes that have low expression values across both groups, and black indicates median expression.

Figure 3.

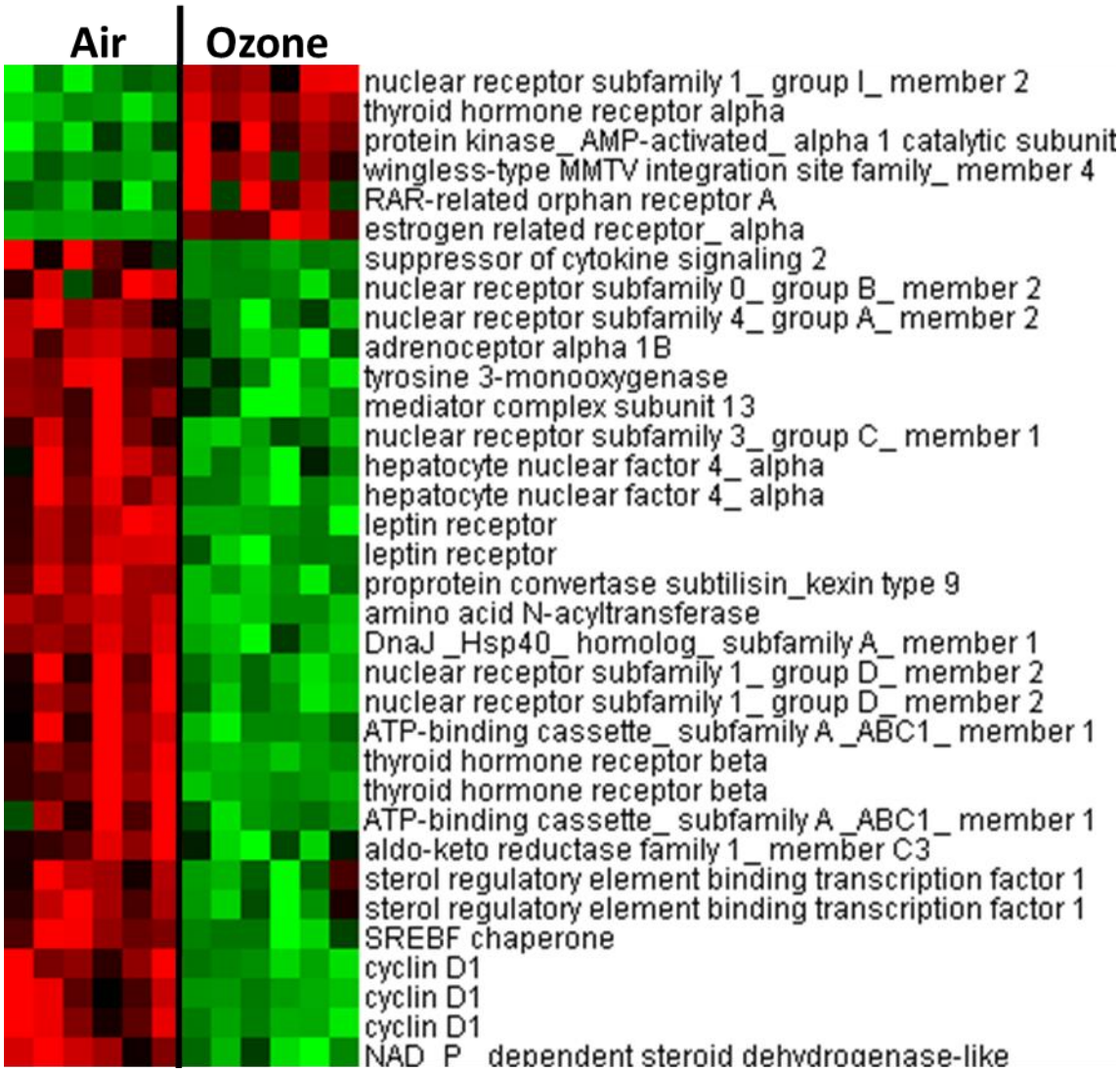


Figure 3: Heatmap of liver gene expression showing differentially expressed genes (air versus O_3) for Steroid, insulin and fatty acid receptor signaling network in rats exposed to O_3 for 6hr/day for 1-day (immediately after exposure). Functional gene lists were generated by NetAffx queries at the Affymetrix website (www.affymetrix.com) and identified from differentially expressed genes (DEGs) list based on the query term, "steroid, insulin and fatty acid signaling". Genes were then median centered with average linkage, the normalized expression values for those probesets present on both lists were converted to z values and hierarchically clustered using Cluster 3.0 with average linkage and displayed with Treeview. Red indicates genes that have high expression values across both groups, green indicates genes that have low expression values across both groups, and black indicates median expression.

Figure 4. Ingenuity pathway analysis showing ozone exposure-induced changes in liver steroid receptor (A), insulin receptor (B) and fatty acid signaling (C).

Figure 4A

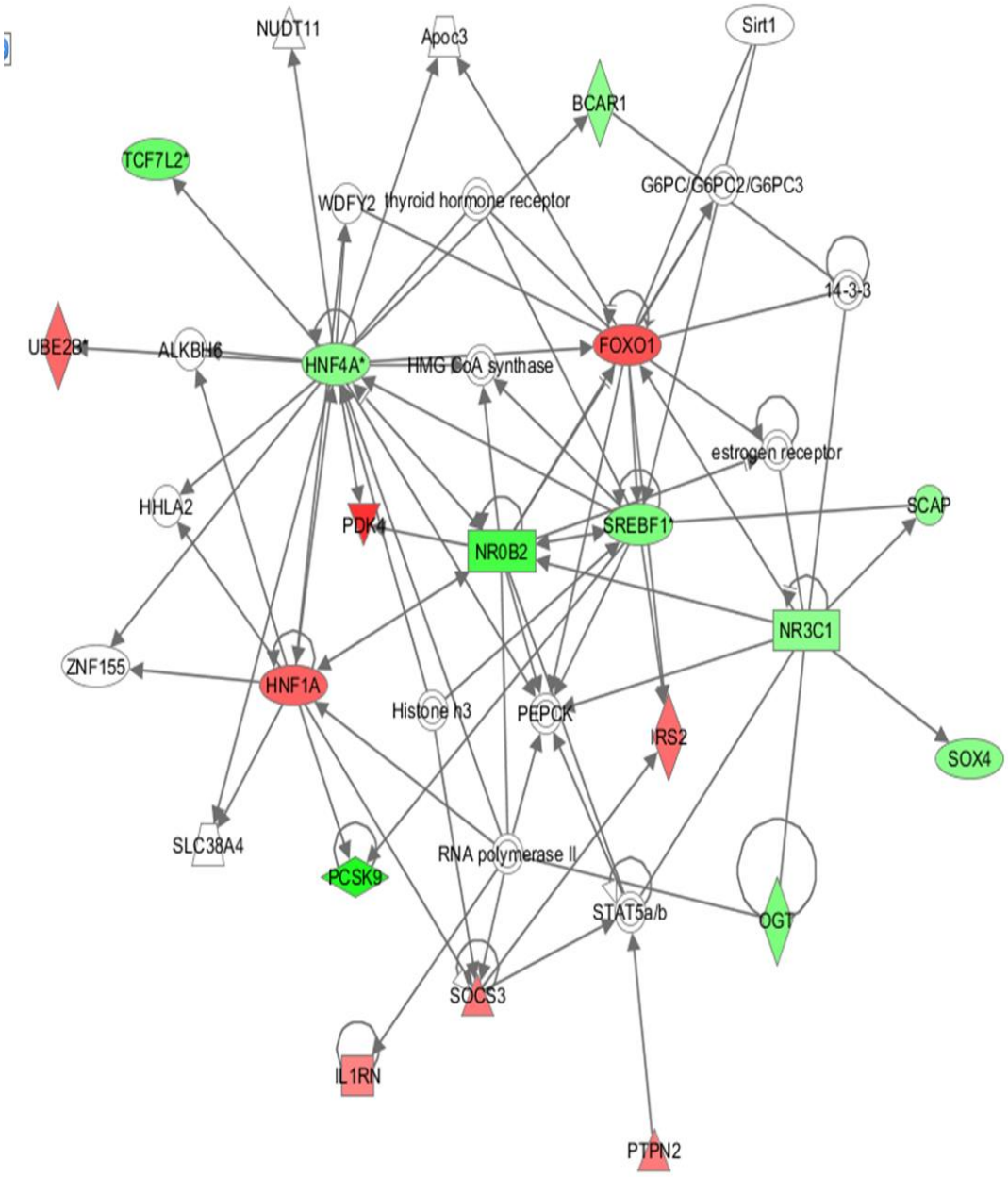
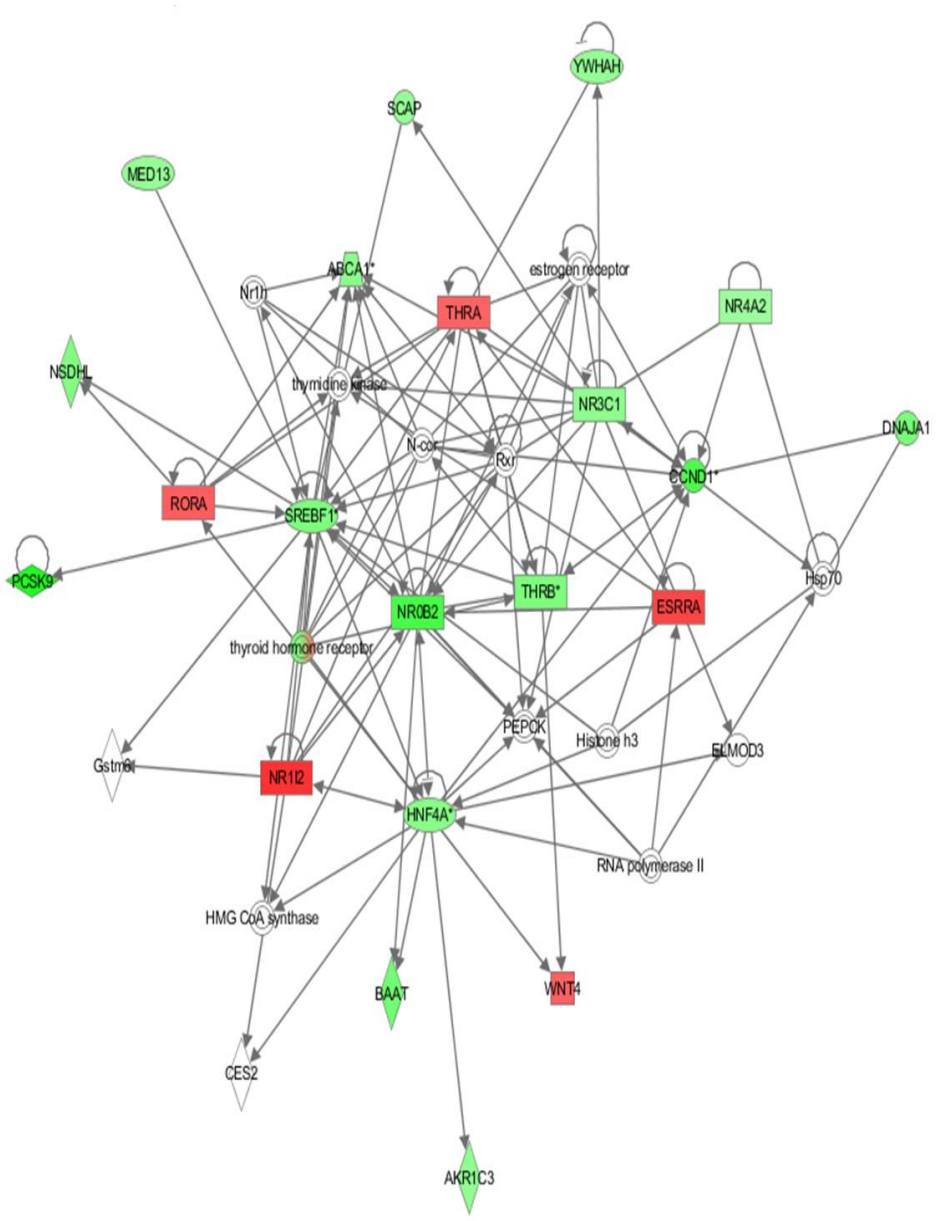


Figure 4B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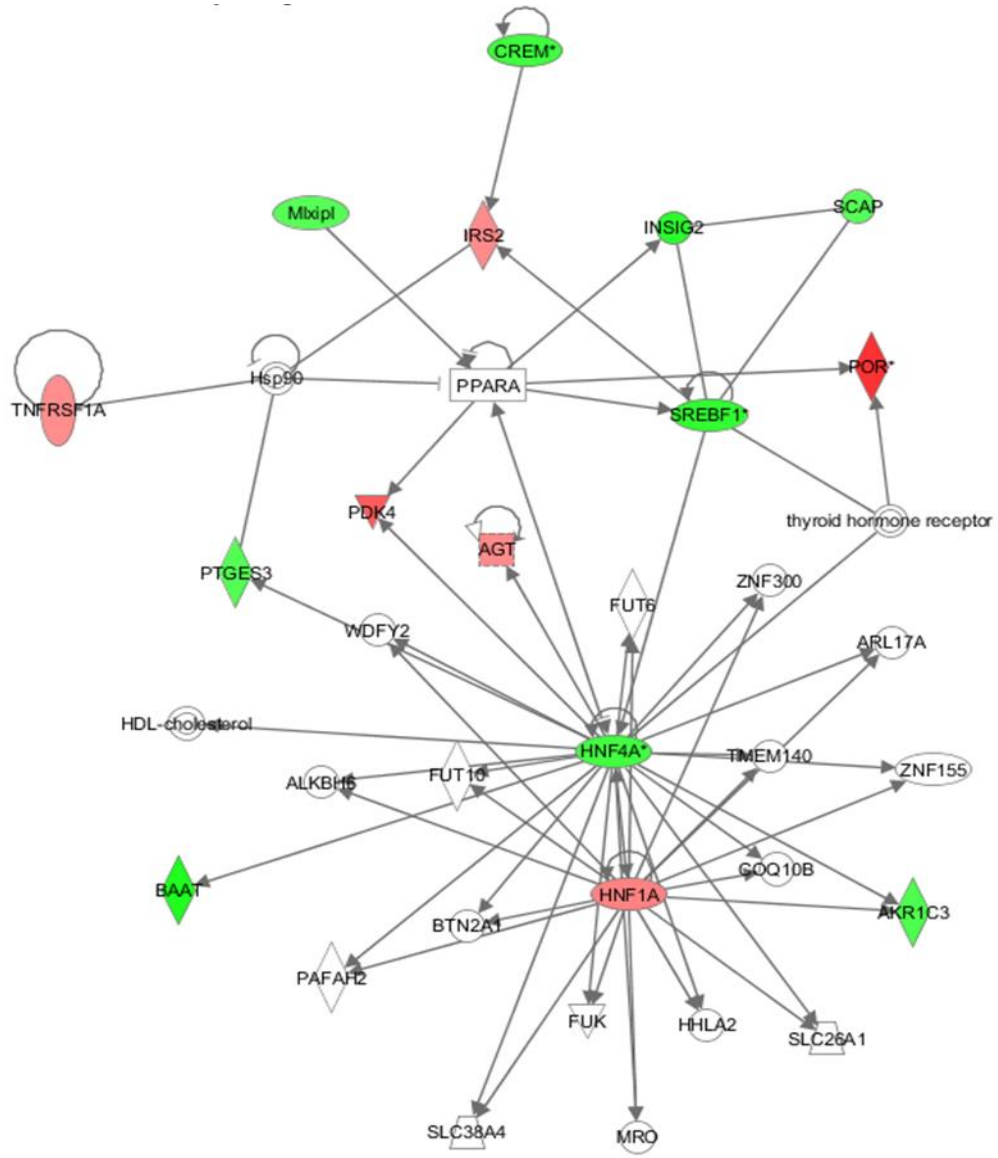
MED13
ABCA1*
Nr1h
estrogen receptor
THRA
NR4A2
NSDHL
thymidine kinase
NR3C1
DNAJA1
N-cor
Rxr
CCND1*
RORA
SREBF1
PCSK9
THRB*
Hsp70
NR0B2
ESRRA
thyroid hormone receptor
PEPCK
Histone h3
ELMOD3
Gstm6
NR1I2
HNF4A*
RNA polymerase II
HMG CoA synthase
BAAT
WNT4
CES2
AKR1C3

Figure 4C



Three functional probeset lists based on queries of "steroid receptor", "insulin receptor" or "fatty acid" were obtained from NetAffx. Each of these lists was compared to the list of differentially expressed genes (DEGs) for the one day zero hour time point to identify DEGs in each of the three functional categories. Each functional list was processed separately by Ingenuity Pathway Analysis (IPA) to produce a direct relationship graph. The graph constructed from the Ingenuity knowledgebase depicts some of the biological relationships among the probesets on the list. The shape of a gene-labeled object indicates its molecular function and its color indicates whether it is upregulated (red) or downregulated (green). The features of the connecting lines convey the nature of the molecular interaction. For a detailed legend of these IPA graphs see the ingenuity.com website.

Table 1: Effect of acute ozone exposure on the expression of probe sets belonging to different biological processes in the liver.

Process	Number of probes on array	Number of DEGS from Master List	Per Cent Master List DEGs on Functional List
Amino acid biosynthesis	4650	507	11
Apoptosis	2345	259	11
Diabetes	743	117	16
Gluconeogenesis	80	21	26
Glycolysis	114	20	18
Mitochondria	671	111	17
Steroid Metabolism	416	77	19
Tricarboxylic	29	10	34
Unfolded protein	154	26	17

Significantly different probes for specific biological processes were identified from a master differentially expressed genes list (DEGs) for air versus O_3 exposure which included genes for all three time points (Immediately after 1-day 6-hr, immediately after 2-day, 6-hr and 18 hours after 2-day, 6-hr).

APPENDIX B.

CHAPTER THREE

Methods

Details of the human clinical study where serum samples were obtained

Study population

Serum samples were obtained from a prior clinical study in which subjects were recruited under an EPA contract with Westat Corporation (Rockville, MD), Clinical Trial NCT01492517 (E1). A total of 24 volunteers were involved and completed the study. The median age was 27.9 years; there were 20 male and 4 female participants (22 whites, 2 Hispanics). Participants were free of cardiopulmonary diseases and allergies as determined by a detailed medical history questionnaire and physical examination. All subjects were nonsmokers and had normal spirometry. Subjects were informed of the procedures and potential risks and signed an informed consent. The protocol and consent forms were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects and the US Environmental Protection Agency's Institutional Review Board. The data for cardiovascular, systemic and pulmonary effects have been published by Devlin and collaborators (E1).

The original study design and ozone exposures (E1): Selected serum samples for this exploratory assessment were obtained from a previously conducted clinical study (Clinical Trial NCT01492517). This study was conducted in a randomized crossover design where two clinical visits of each individual were separated by at least two weeks (E1). During each visit, the subjects arrived in the morning. In each case, the participant was exposed to 0.3 ppm ozone

or clean air in a blinded manner for 2hr. During the 2hr exposure, each subject alternated between 15 minutes of rest and 15 minutes of exercise on a cycle ergometer. Most subjects underwent moderate to intense intermittent exercise during the 2hr period. Exercise intensity (i.e. cycle ergometer workload) was adjusted so that subjects breathed at a ventilatory rate, normalized for body surface area, of 25 L/meter^2/minute. In these subjects, this approximated to 50 L/minute (i.e. a VO_2 of approximately 1.0 L/min). Exposures were performed as previously described (E1-E2). All exposures were conducted at the U.S. EPA Human Studies Facility on the campus of the University of North Carolina, Chapel Hill. The exposure chambers were maintained at 40% relative humidity for all exposures. Ozone was generated by a silent electric discharge method (model 502; Meckenheim, Bonn, Germany) and did not deviate beyond 0.001 ppm of the target concentration (0.3 ppm). During exposure, fine particulate matter (by TEOM, DataRa, and filter weight), NO_2, NO, total hydrocarbon, SO_2, and CO were measured for the air and ozone chambers. All measurements were either zero or negligible except for total hydrocarbon, which was between 1 and 3 ppm (all of which is methane). All participants completed the entire study and no adverse events were reported. Ozone concentrations were maintained at 0.3 ppm during ozone exposure. All exposures began at the same time in the morning (~9am) to avoid having circadian fluctuations as a confounder. Prior to the start of exposure, during exposure and post exposure, blood pressure, heart rate and EKG were recorded (E1). Each individual's systolic as well as diastolic blood pressure and heart rate were recorded and the data were available to us for correlation assessment in this study.

Sample collection and analysis

Prior to the start of exposure, immediately upon completion of designated exposure (within 1hr), and during the next day's morning follow up visit, venous blood was drawn in serum separator vaccutainers. Serum samples were aliquotted and stored at -80^{0}C for further analysis. One aliquot of serum collected immediately following air or ozone exposure (two separate visits) for each individual was used for this exploratory metabolomic analysis. These serum samples were sent to Metabolon Inc. (Durham, NC) for global metabolomic analysis, as described below. Serum samples were also analyzed for insulin, leptin, interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α, using human-specific antibody-based electrochemiluminescence assays (Meso Scale Discovery, Gaithersburg, MD) via manufacturer's instructions. In addition, during this clinical study, the clinical chemistry assessment along with complete blood cell counts were completed for blood samples collected prior to and post air or ozone exposure, and during next day follow up visit. While the data for total cholesterol and blood pressure are published as group means (E1), the individual data for all clinical measurements were available for this manuscript for supporting the metabolomic exploratory assessment performed in serum samples collected post air or ozone exposure. Post exposure glucose assessment was used to derive HOMA-IR with insulin data collected from the serum aliquot used for metabolomic assessment by Metabolon Inc (Durham, NC).

Clinical and biomarker data assessment

Clinical chemistry assessment completed for serum samples collected prior to, immediately post air or ozone exposure, and during next day follow-up visit included measurement of glucose, triglycerides and cholesterols (LabCorp Inc, Durham, NC). Clinical markers data were first normalized to pre-exposure values and analyzed for differences following air or ozone exposure using a paired *t* test with each subject serving as their own control. HOMA-IR was calculated from glucose data from clinical assessment and insulin data obtained post air or ozone exposure, and analyzed using a paired *t* test for assessment of difference post air and ozone exposure. Pearson's correlation coefficient (r) was used to determine the relationship between ozone-induced changes (fold-increases in selected serum free fatty acids and stress hormones; metabolomic data) as well as serum levels of metabolites (glucose, triglycerides and different cholesterols) measured in clinical chemistry to each subject's BMI, gender, and exercise level. GraphPad Prism (GraphPad Software version 6.04, San Diego, CA) was used for statistical analyses. A p-value < 0.05 was considered statistically significant.

Metabolomic analysis

Sample accessioning: Following receipt, samples were inventoried and immediately stored at -80^{o}C. Each sample received was accessioned into the Metabolon LIMS system and was assigned by the LIMS a unique identifier that was associated with the original source identifier only. This identifier was used to track all sample handling, tasks, results, etc. The samples (and all derived aliquots) were tracked by the LIMS system. All portions of any sample were automatically assigned their own unique identifiers by the LIMS when a new task was created;

the relationship of these samples was also tracked. All samples were maintained at -80^{o}C until processed.

Sample preparation: Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. A recovery standard was added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: one for analysis by UPLC-MS/MS with positive ion mode electrospray ionization, one for analysis by UPLC-MS/MS with negative ion mode electrospray ionization, one for LC polar platform, one for analysis by GC-MS, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. For LC, the samples were stored overnight under nitrogen before preparation for analysis. For GC, each sample was dried under vacuum overnight before preparation for analysis.

QA/QC: Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample, allowed instrument performance monitoring and aided chromatographic alignment. Tables E1 and E2 describe these QC samples and standards. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to

injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections as outlined in Figure E1.

Table E1: Description of Metabolon QC Samples

Type	Description	Purpose
MTRX	Large pool of human plasma maintained by Metabolon that has been characterized extensively.	Assure that all aspects of the Metabolon process are operating within specifications.
CMTRX	Pool created by taking a small aliquot from every customer sample.	Assess the effect of a non-plasma matrix on the Metabolon process and distinguish biological variability from process variability.
PRCS	Aliquot of ultra-pure water	Process Blank used to assess the contribution to compound signals from the process.
SOLV	Aliquot of solvents used in extraction.	Solvent Blank used to segregate contamination sources in the extraction.

Table E2: Metabolon QC Standards

Type	Description	Purpose
RS	Recovery Standard	Assess variability and verify performance of extraction and instrumentation.
DS	Derivatization Standard	Assess variability of derivatization for GC-MS samples.
IS	Internal Standard	Assess variability and performance of instrument.

Figure E1.

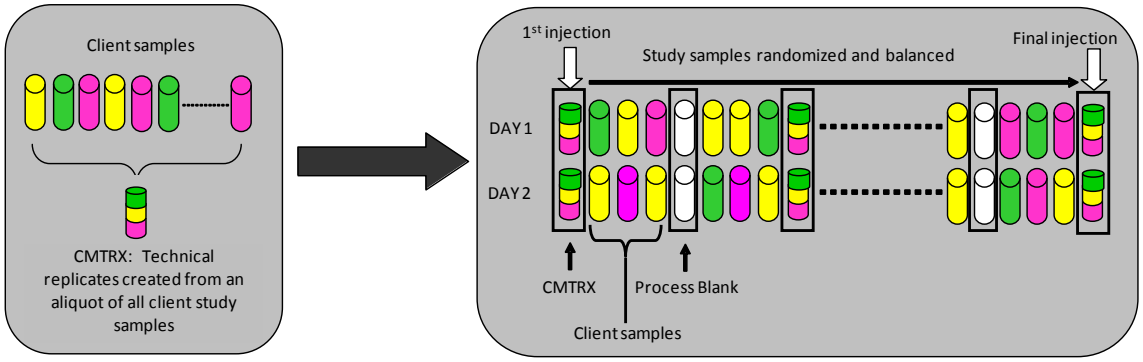


Figure E1. Preparation of client-specific technical replicates. A small aliquot of each study sample (colored cylinders) is pooled to create a CMTRX technical replicate sample (multi-colored cylinder), which is then injected periodically throughout the platform run. Variability among consistently detected biochemicals are used to calculate an estimate of overall process and platform variability.

Ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS): The LC/MS portion of the platform was based on a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution (E3). The sample extract was dried then reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm). Extracts reconstituted in acidic conditions were gradient eluted from a C18 column using water and methanol containing 0.1% formic acid. The basic extracts were similarly eluted from C18 using methanol and water, however with 6.5mM Ammonium Bicarbonate. The third aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate. The MS analysis alternated between MS and data-dependent MS2 scans using dynamic exclusion, and the scan range was from 80-1000 m/z. Raw data files are archived and extracted as described below.

Gas chromatography-mass spectroscopy (GC-MS): The samples destined for analysis by GC-MS were dried under vacuum for a minimum of 18 h prior to being derivatized under dried nitrogen using bistrimethyl-silyltrifluoroacetamide. Derivatized samples were separated on a 5% diphenyl / 95% dimethyl polysiloxane fused silica column (20 m x 0.18 mm ID; 0.18 um film thickness) with helium as carrier gas and a temperature ramp from 60° to 340°C in a 17.5 min period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization (EI) and operated at unit mass resolving power. The scan range was from 50–750 m/z. Raw data files are archived and extracted as described below.

Bioinformatics: The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

LIMS: The purpose of the Metabolon LIMS system was to enable fully auditable laboratory automation through a secure, easy to use, and highly specialized system. The scope of the Metabolon LIMS system encompasses sample accessioning, sample preparation and instrumental analysis and reporting and advanced data analysis. All of the subsequent software systems are grounded in the LIMS data structures. It has been modified to leverage and interface with the in-house information extraction and data visualization systems, as well as third party instrumentation and data analysis software.

Data extraction and compound identification: Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. These systems are built on a web-service platform utilizing Microsoft's .NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (*m/z)*, and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 0.005 amu, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals. More than 3300 commercially available purified standard compounds have been acquired and registered into LIMS for distribution to both the LC-MS and GC-MS platforms for determination of their analytical characteristics. Additional mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

Curation: A variety of curation procedures were carried out to ensure that a high quality data set was made available for statistical analysis and data interpretation. The QC and

curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, mis-assignments, and background noise. Metabolon data analysts use proprietary visualization and interpretation software to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected if necessary.

Metabolite quantification and data normalization: Peaks were quantified using area-under-the-curve. A data normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately (termed the "block correction"; Figure E2).

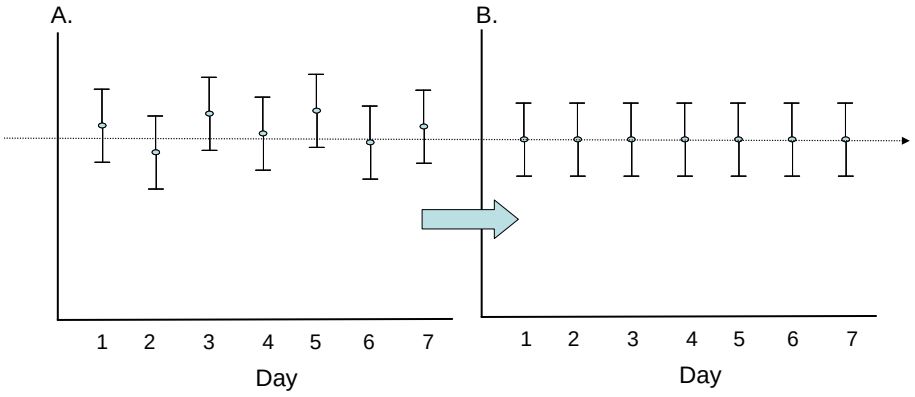


Figure E2: Visualization of data normalization steps for a multiday platform run.

Statistical analysis of metabolomic analysis: Statistical analysis included log transformation and imputation followed by one way analysis of variance (ANOVA) with repeated measures to identify biochemicals that differed significantly between experimental groups. An estimate of the false discovery rate (*q*-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. Pathways were assigned for each metabolite, allowing examination of overrepresented pathways. Significant ($p<0.05$) pathway enrichment output (cumulative hypergeometric distribution) was assessed

for each of the selected contrasts using MetaboLync (E4) to determine the metabolic processes impacted by ozone.

Results

Figure E3 shows the line plots of systolic blood pressure and heart rate data of subjects as determined prior to their air or ozone exposure, immediately after exposure (within 1hr) and during their next day morning follow-up visit to the clinic. These were no significant differences as determined by paired t-test between air and ozone exposure when compared immediately post exposure. Note that only 1 subject during clinical visit had increased heart rate prior to the start of air exposure, however, this was not apparent during their visit for ozone exposure. The heart rate and blood pressure in subjects did not appear to deviate between the three time points.

Figure E4 shows the line plots for levels of serum glucose, cholesterol, triglycerides, HDL and LDL determined prior to, immediately post air or ozone exposure and the next day follow-up visit. While the time of measurement does not appear to change HDL, LDL and cholesterol levels in subjects, the levels of glucose seem to vary among subjects when measured prior to exposure. This variability between subjects seen prior to the start of exposure was reduced for the measurements performed on the next day morning. This could be due to the fact that during the first visit, subjects were not fasted while during the second day follow up visit, subjects were fasted overnight as they underwent bronchoscopy at that time (E1). Individual subject variations between pre and post exposure times in triglycerides and cholesterols appeared to be minimal. No ozone-related effects were observed in these parameters when data were normalized to pre-exposure levels.

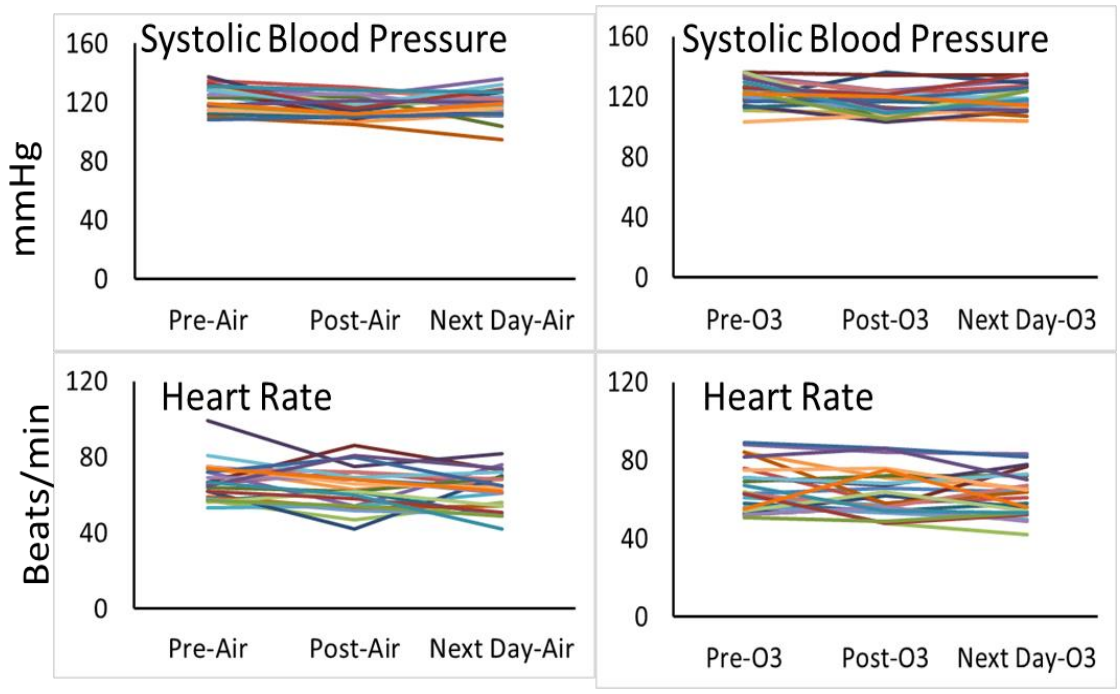
The graphs in Figure E5 shows line plots of leptin, insulin and HOMA-IR post-air and post-ozone exposure. As shown in the top panel, one individual had very high leptin levels relative to all other individuals regardless of air or ozone exposure. No significant differences were noted between air and ozone exposure. Insulin and HOMA-IR levels varied between subjects regardless of air or ozone exposure. Mostly these variations were noted in two or three individuals but all others did not appear to vary markedly between air and ozone exposure. No significant differences were evident between air and ozone HOMA-IR, insulin or leptin as determined using a paired t-test.

In Figures E6A-E, the fold increases after ozone exposure (ratios of ozone/air) for selected individual lipid metabolites and stress hormones that significantly changed (except for cortisone) were plotted against individual subject BMI to determine if high BMI could be linked to increased susceptibility to ozone-induced changes. These exploratory scatter plots appear to show no consistent relationship between the BMI value of subjects and ozone-induced changes in individual metabolites. As in the case of Figure E6, in Figure E7A-E,

ozone-induced fold changes in these same metabolites were correlated with subjects prior exercise habit. The level of exercise routinely performed by each subject was rated as mild (1), moderate (2) or heavy (3) obtained through questionnaire. The scatter plots between individuals with different exercise habits and ozone-induced changes in lipid metabolites as well as steroid hormones did not appear to be related to individual's prior exercise habit. However, these correlations were made with very few subjects in each exercise intensity group, and thus the data are limited. There were only 4 females in the study, nevertheless, we decided to explore any potential relationship between sex and significant ozone-induced fold changes in lipid metabolites and steroid hormones (Figure E8A-E). Because of the limited number of female subjects in this study, no inferences can be drawn from these data for potential sex differences in ozone-induced metabolic alterations; however, the plots suggest that some relationships are likely.

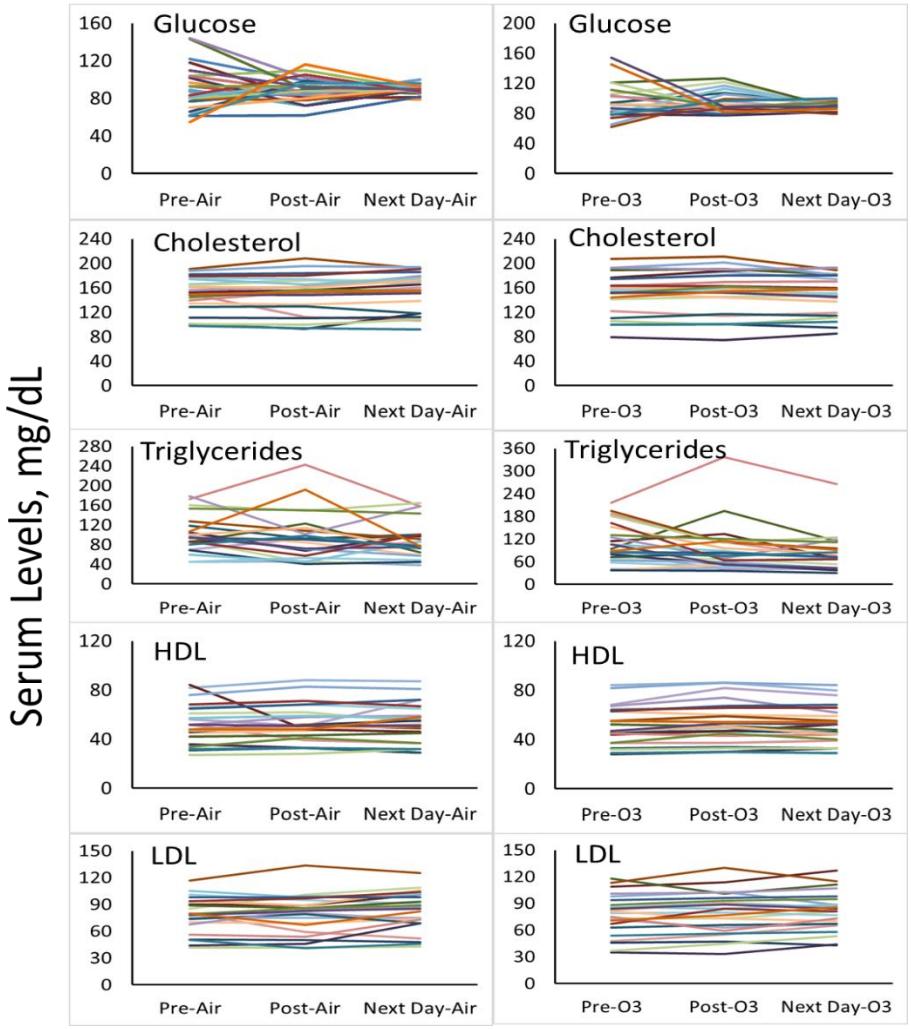
Since we had the clinical chemistry data available which included serum levels of glucose, and lipids, such as triglycerides and cholesterols, we explored the potential relationship between levels of glucose as well as these lipids post air or post ozone exposure and 1) BMI in Figure E9A-B; 2) the prior exercise level (1, 2, or 3) in Figure E10A-B; and 3) sex in Figure E11A-B using scatter plots. Surprisingly, in these healthy subjects, the BMI appear not to correlate with post exposure circulating triglycerides, cholesterols or glucose, regardless of air or ozone exposure. The correlations might exist in some measured lipids with the level of exercise and gender, however, there seem to be no differences between post air and post ozone exposure.

Figure E3: Line plots of cardiac physiological measurements taken prior to the start, post exposure, and during next day morning follow up visit (post-air, left; post-ozone, right)*.



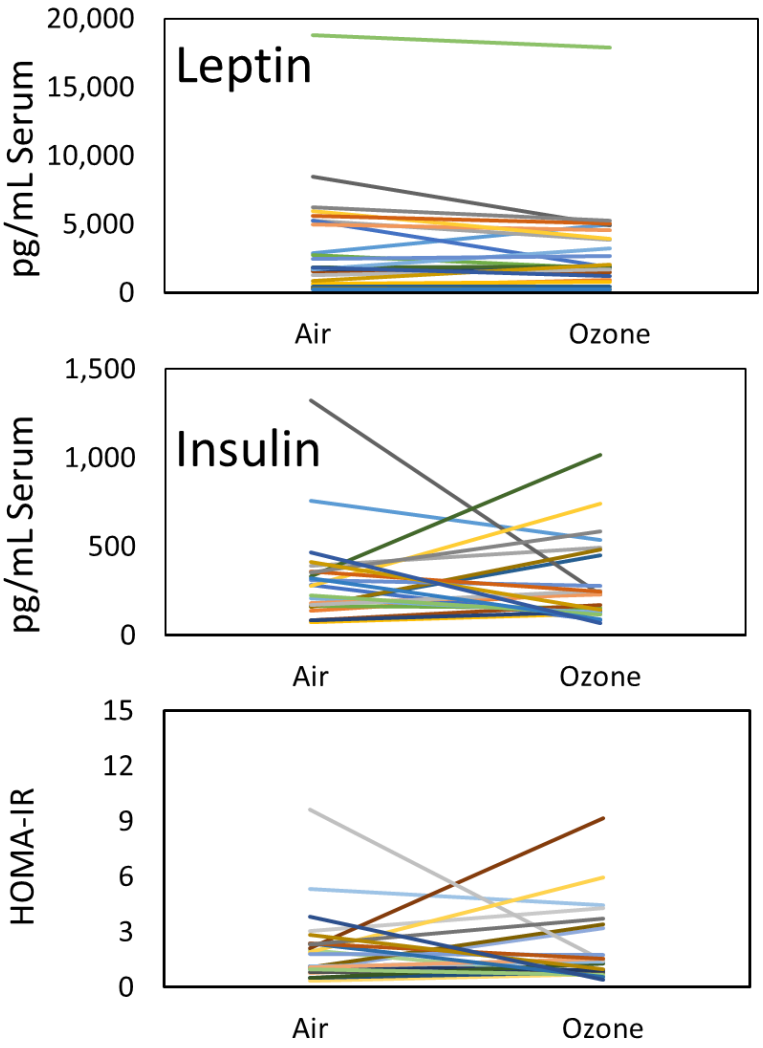
* The data for systolic blood pressure and heart rate have been published by Devlin et al (E1) as group means. We explored the time-related individual subject differences in these parameters from the clinical data available to us. This was a randomized clinical assessment where each subject arrived to the clinic at two different times (separated by at least two weeks) for either air or ozone exposure and for the follow up visit, next-day morning. Thus, each subject visited clinic 4 times (for air exposure, follow up visit next day, for ozone exposure, follow up visit next day). Subjects were exposed to either 0.3 ppm ozone or clean air for 2hr in the morning time in a blinded manner. Heart rate and blood pressure were monitored prior to the start of exposure, immediately post exposure (within 1hr) and next day morning, in addition to continuous Holter monitoring (E1). Note that subjects were fasted overnight when they arrived next day morning for a follow up visit. The plots indicate values for each subject (n=24; 20 males and 4 females).

Figure E4: Line plots of serum glucose and lipid measurements taken prior to the start, post exposure, and next day morning during follow up visit (post-air, left; post-ozone, right)*.



*The data for serum cholesterol have been published previously by Devlin et al (E1) as group means. We explored the time-related differences in these parameters from the clinical data available to us. In this clinical assessment, each subject arrived for either air or ozone exposure on separate days (each visit separated by more than two weeks) and for a follow up visit next-day morning. Thus, each subject visited clinic 4 times (for air exposure, follow up visit next day, for ozone exposure, follow up visit next day). Subjects were exposed to either 0.3 ppm ozone or clean air for 2hr in the morning time in a blinded manner. Serum samples collected prior to the start of exposure, immediately post exposure (within 1hr) and next day morning and assessed for glucose and lipids by LabCorp Inc (Durham, NC). Note subjects were fasted overnight when they arrived next day morning for a follow up visit. The plots indicate values for each subject (n=24; 20 males and 4 females).

Figure E5: Line plots of serum leptin, insulin and HOMA-IR as determined immediately post-air or post-ozone exposure.*



* Insulin and leptin levels were measure in serum samples collected post air or ozone exposure using antibody-based assays as indicated in methods within this supplement. Subjects were exposed to either 0.3 ppm ozone or clean air for 2hr in the morning time in a blinded manner prior to collecting blood samples. The data for serum glucose from the clinical data set and insulin measured from archived post air or post ozone exposure serum samples, were used to calculate HOMA-IR. HOMA-IR was calculated by multiplying glucose value (mmol/L) with insulin value (mU/L) and dividing with 22.5. The plots indicate values for each subject (n=24; 20 males and 4 females).

Figure E6A-E. Exlporatory correlation plots showing the relationship between body mass index (BMI) and ozone-induced fold change in various lipid metabolites as well as stress hormones (as determined using metabolomics).

Figure E6A

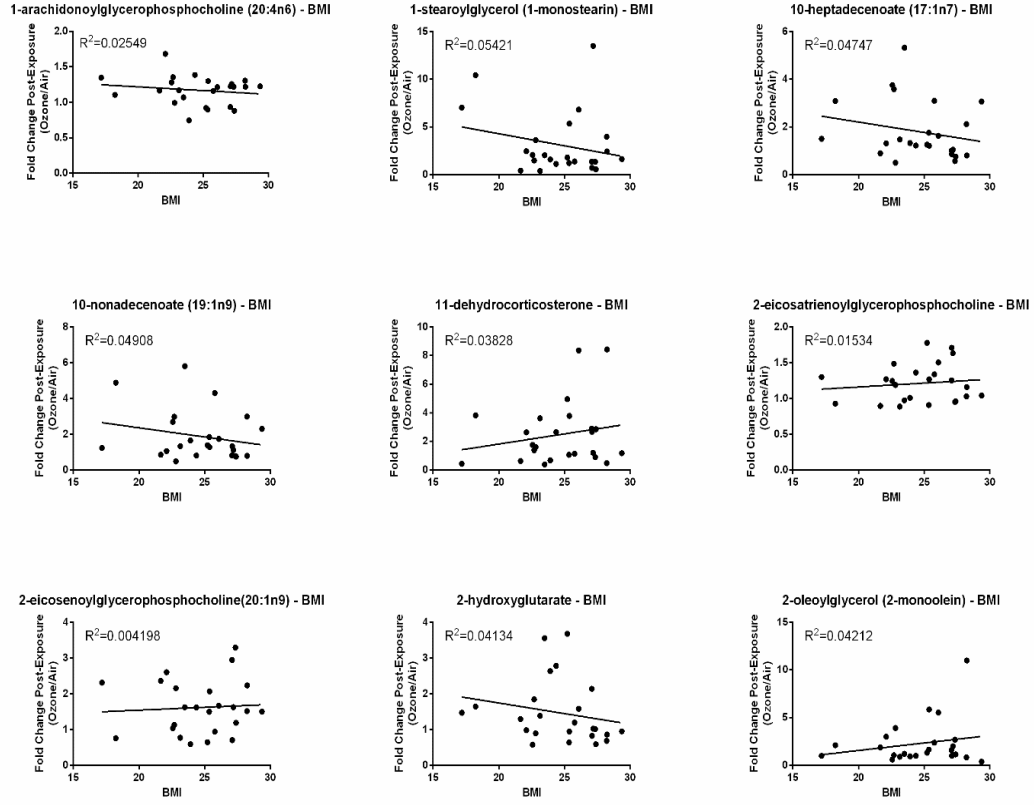


Figure E6B

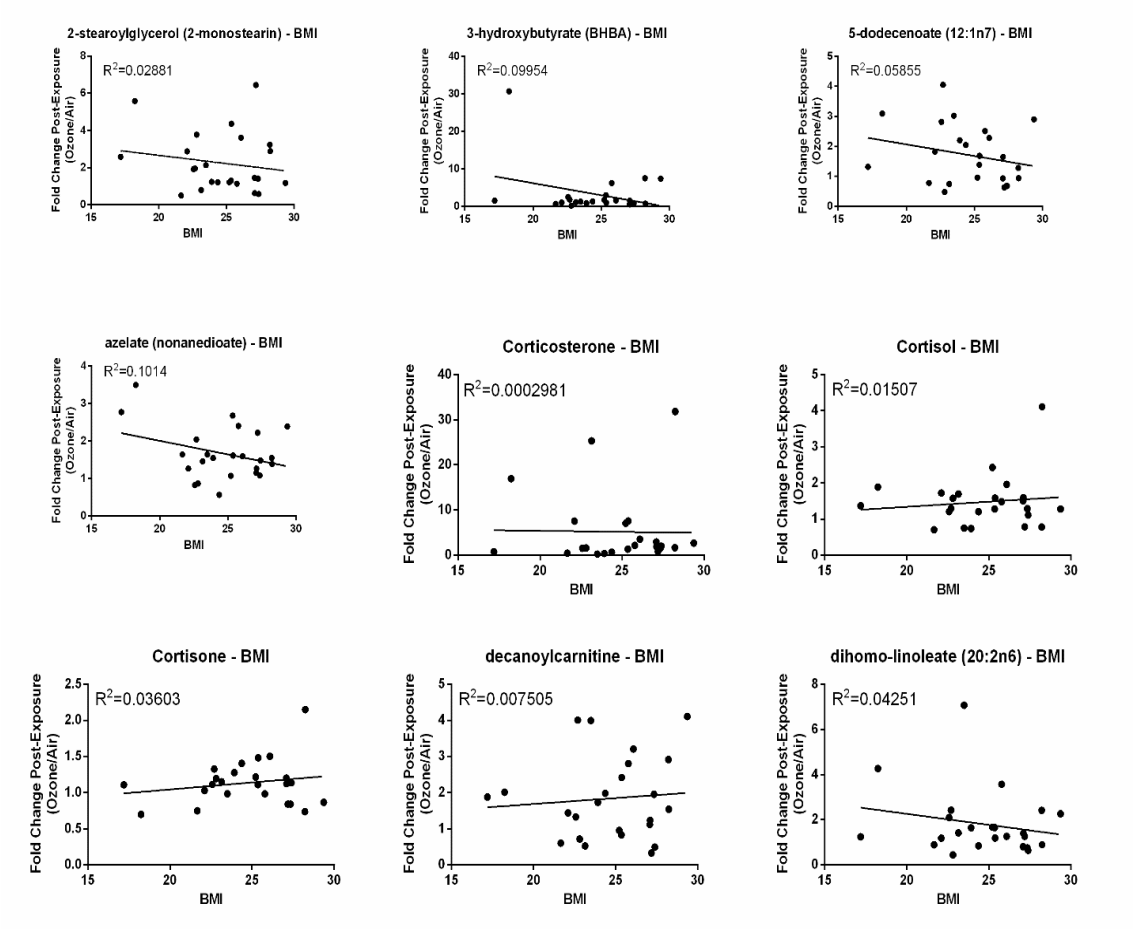

2-stearoylglycerol (2-monostearin) - BMI
R2=0.02881
3-hydroxybutyrate (BHBA) - BMI
R2=0.09954
5-dodecenoate (12:1n7) - BMI
R2=0.05855
azelate (nonanedioate) - BMI
R2=0.1014
Corticosterone - BMI
R2=0.0002981
Cortisol - BMI
R2=0.01507
Cortisone - BMI
R2=0.03603
decanoylcarnitine - BMI
R2=0.007505
dihomo-linoleate (20:2n6) - BMI
R2=0.04251
Fold Change Post-Exposure (Ozone/Air)
BMI

Figure E6C

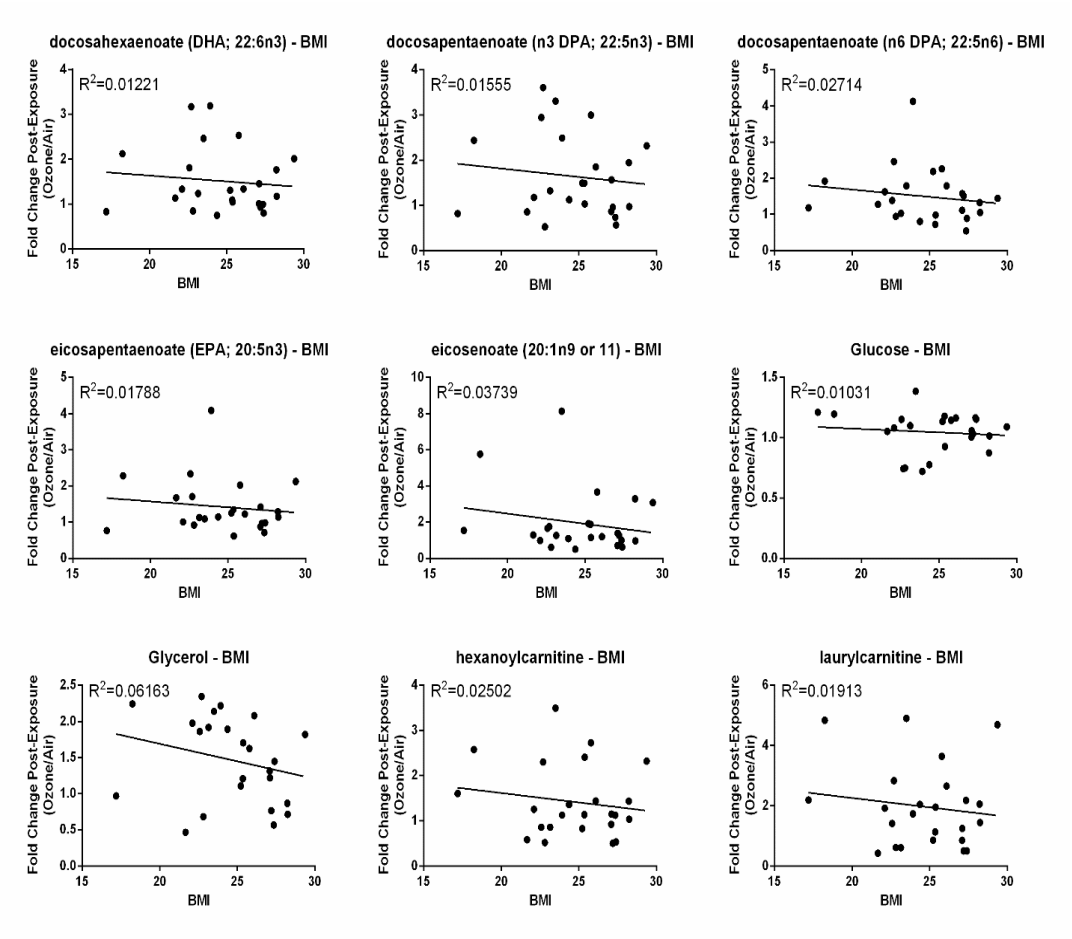

docosahexaenoate (DHA; 22:6n3) - BMI
R2=0.01221
docosapentaenoate (n3 DPA; 22:5n3) - BMI
R2=0.01555
docosapentaenoate (n6 DPA; 22:5n6) - BMI
R2=0.02714
eicosapentaenoate (EPA; 20:5n3) - BMI
R2=0.01788
eicosenoate (20:1n9 or 11) - BMI
R2=0.03739
Glucose - BMI
R2=0.01031
Glycerol - BMI
R2=0.06163
hexanoylcarnitine - BMI
R2=0.02502
laurylcarnitine - BMI
R2=0.01913
Fold Change Post-Exposure (Ozone/Air)
BMI

Figure E6D

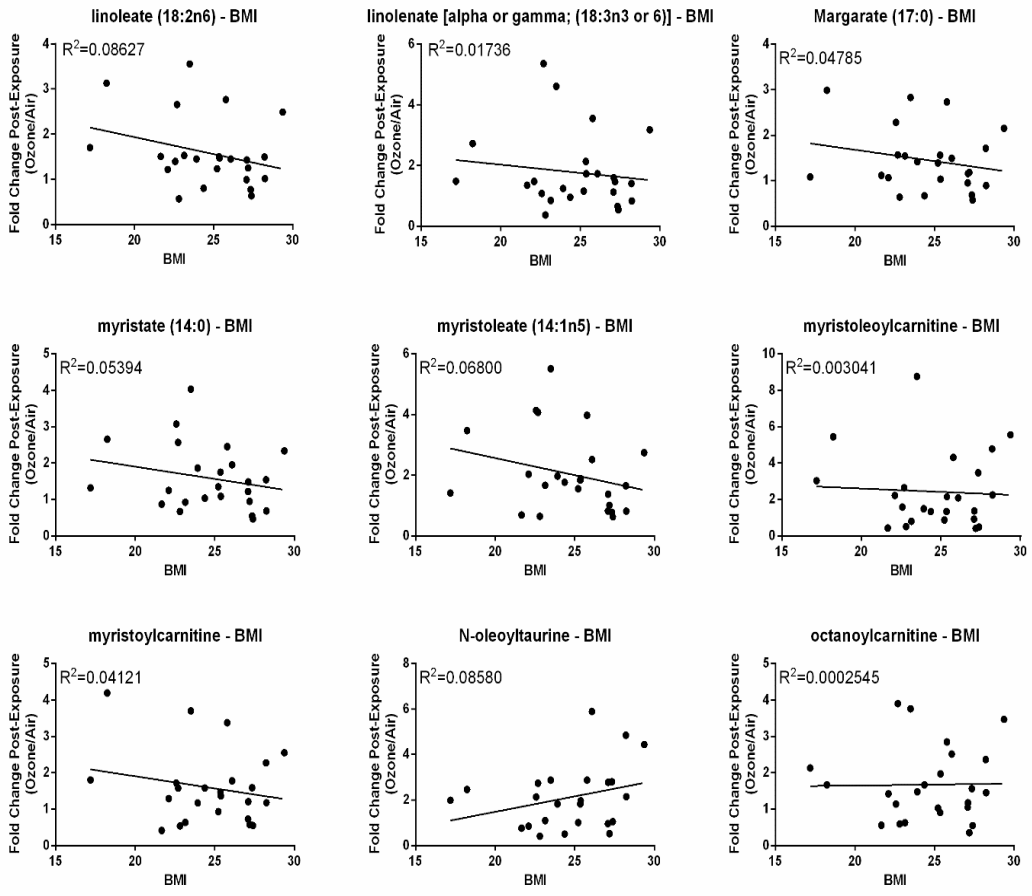
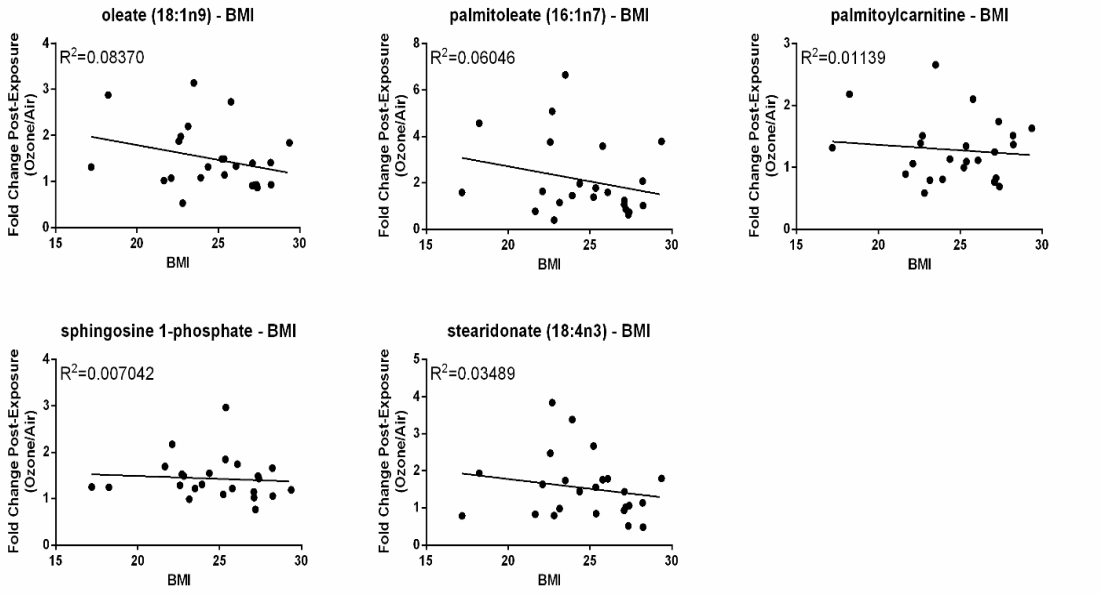

linoleate (18:2n6) - BMI
R²=0.08627
linolenate [alpha or gamma; (18:3n3 or 6)] - BMI
R²=0.01736
Margarate (17:0) - BMI
R²=0.04785
myristate (14:0) - BMI
R²=0.05394
myristoleate (14:1n5) - BMI
R²=0.06800
myristoleoylcarnitine - BMI
R²=0.003041
myristoylcarnitine - BMI
R²=0.04121
N-oleoyltaurine - BMI
R²=0.08580
octanoylcarnitine - BMI
R²=0.0002545
Fold Change Post-Exposure (Ozone/Air)
BMI

Figure E6E



*The data for various metabolites were derived from metabolomic assessment of serum samples collected post air or post ozone exposure. The ratio of ozone/air values that were found to be significantly increased (except for cortisone) were plotted against BMI to determine if ozone effects were influenced by increased BMI. This is a randomized clinical assessment where each subject arrived to the clinic for either air or ozone exposure during two separate clinical visits (each separated by at least two weeks). In this study, each subject was exposed to either 0.3 ppm ozone or clean air for 2hr in the morning time in a blinded manner (n=24; 20 males and 4 females). Serum samples collected immediately post exposure (within 1hr) were subjected to global metabolomic assessment (complete data provided in online supplement #2).

Figure E7A-E. Exploratory correlation plots showing the relationship between each subject's prior exercise habit (mild, 1; moderate, 2; or heavy, 3) and fold change in various lipid metabolites post ozone relative to air exposure.

Figure E7A

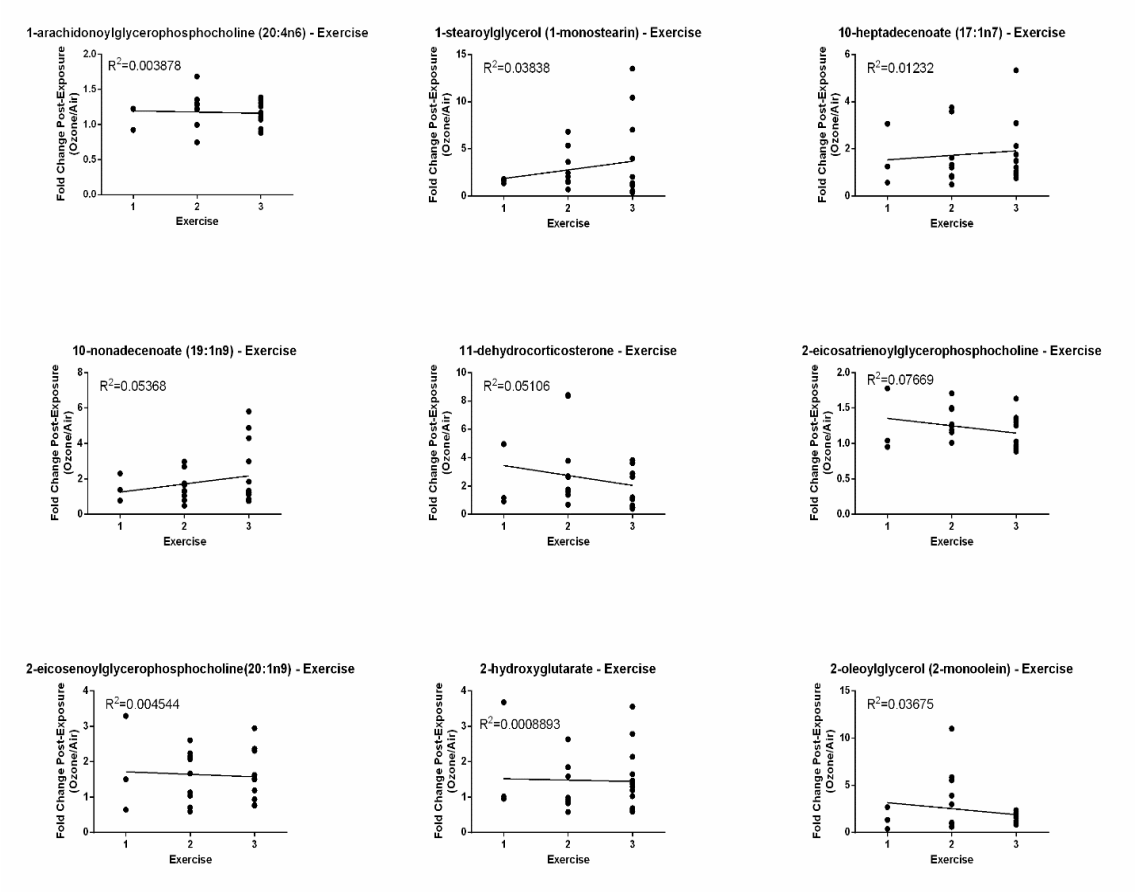


Figure E7B

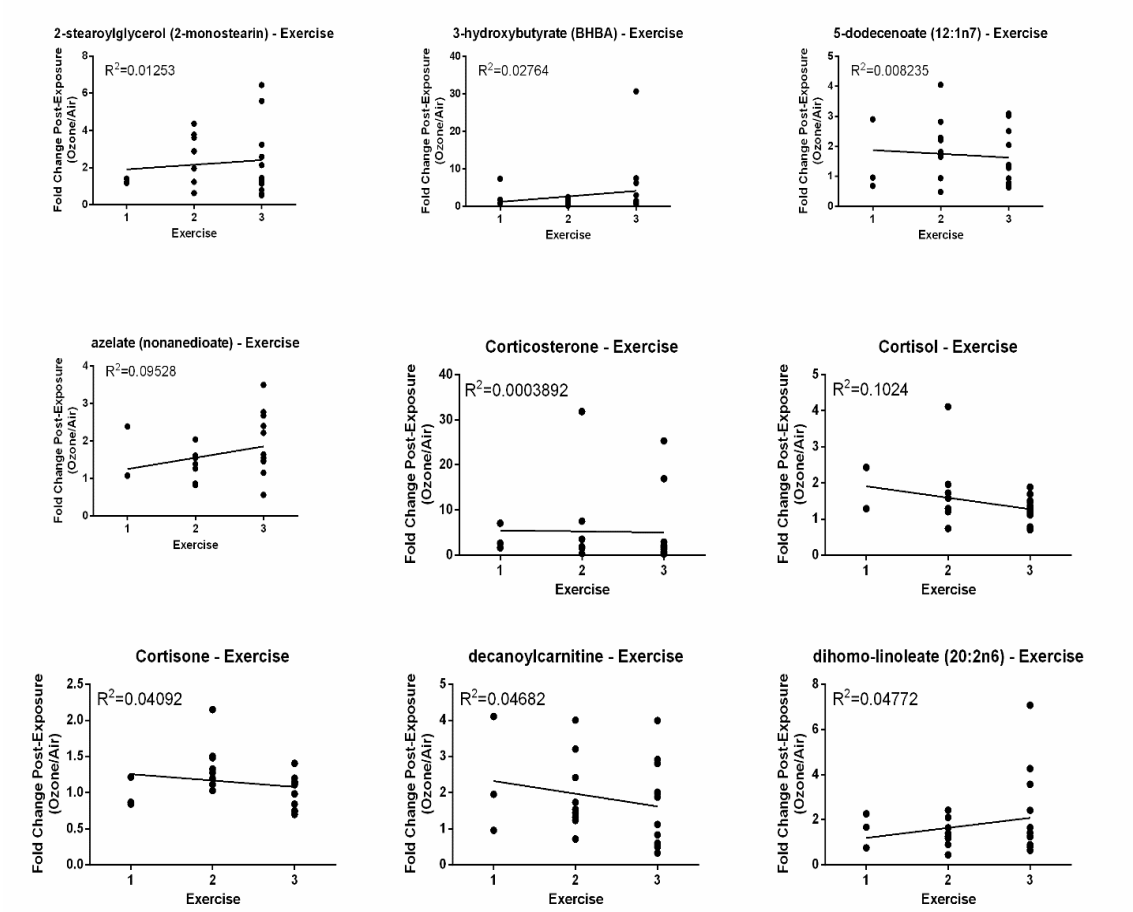

2-stearoylglycerol (2-monostearin) - Exercise
R2=0.01253
3-hydroxybutyrate (BHBA) - Exercise
R2=0.02764
5-dodecenoate (12:1n7) - Exercise
R2=0.008235
azelate (nonanedioate) - Exercise
R2=0.09528
Corticosterone - Exercise
R2=0.0003892
Cortisol - Exercise
R2=0.1024
Cortisone - Exercise
R2=0.04092
decanoylcarnitine - Exercise
R2=0.04682
dihomo-linoleate (20:2n6) - Exercise
R2=0.04772
Fold Change Post-Exposure (Ozone/Air)
Exercise

Figure E7C

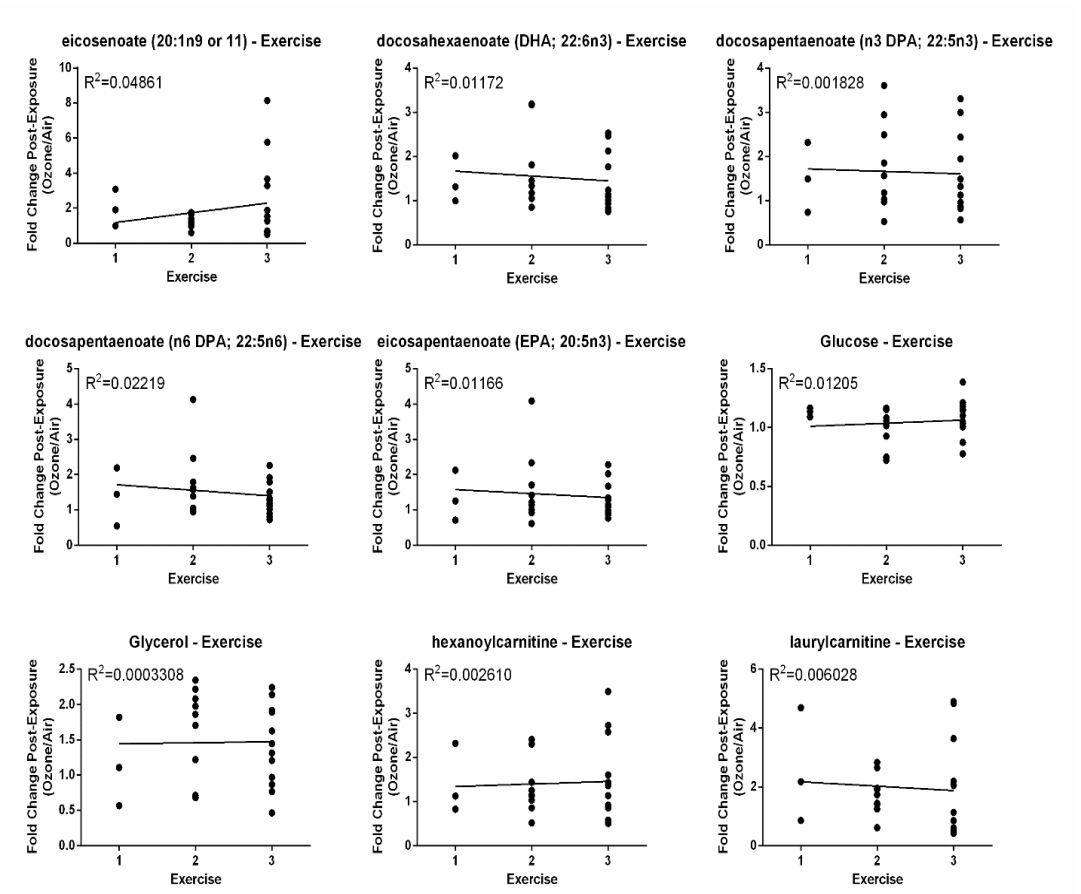

eicosenoate (20:1n9 or 11) - Exercise
R2=0.04861
docosahexaenoate (DHA; 22:6n3) - Exercise
R2=0.01172
docosapentaenoate (n3 DPA; 22:5n3) - Exercise
R2=0.001828
docosapentaenoate (n6 DPA; 22:5n6) - Exercise
R2=0.02219
eicosapentaenoate (EPA; 20:5n3) - Exercise
R2=0.01166
Glucose - Exercise
R2=0.01205
Glycerol - Exercise
R2=0.0003308
hexanoylcarnitine - Exercise
R2=0.002610
laurylcarnitine - Exercise
R2=0.006028
Fold Change Post-Exposure (Ozone/Air)
Exercise

Figure E7D

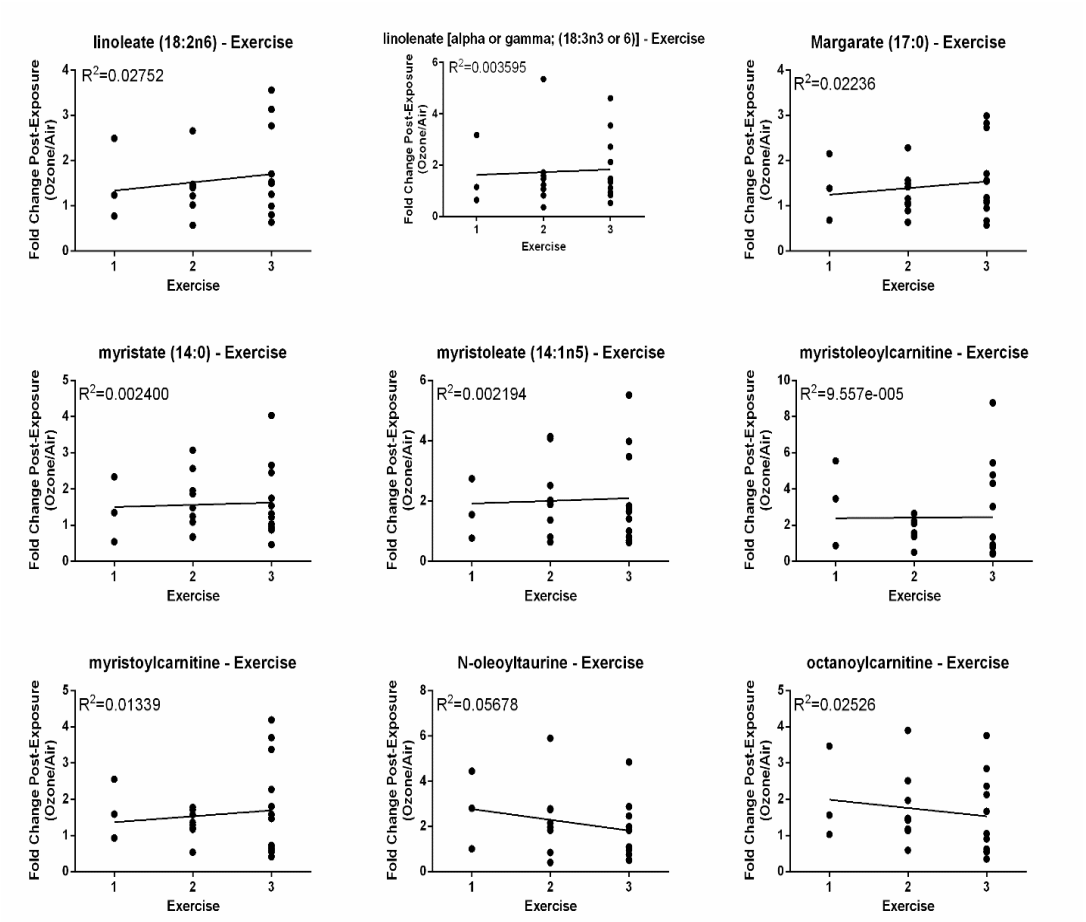
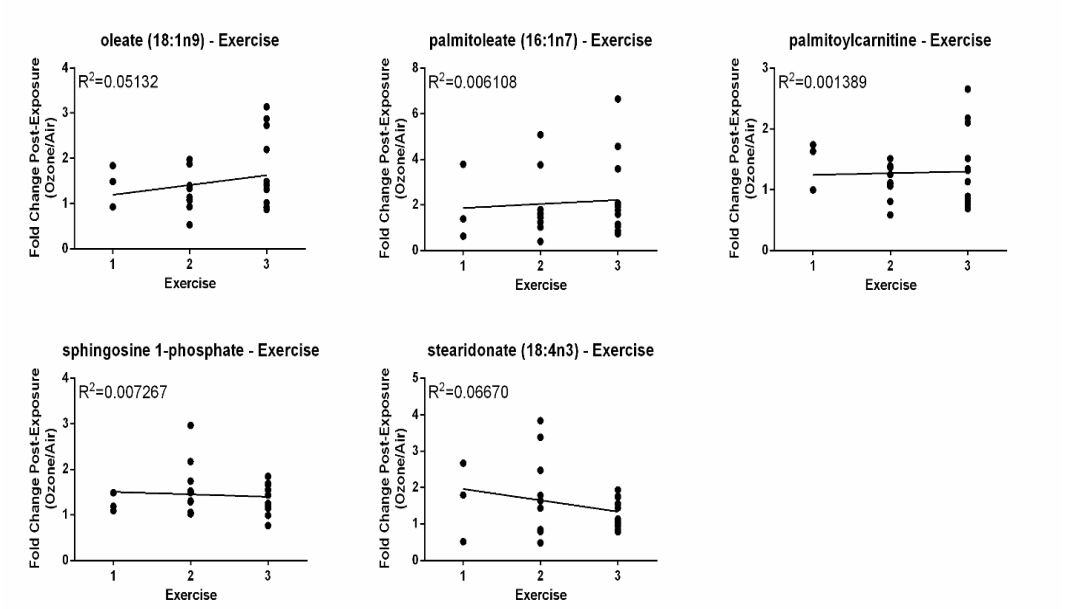

linoleate (18:2n6) - Exercise
R2=0.02752
linolenate [alpha or gamma; (18:3n3 or 6)] - Exercise
R2=0.003595
Margarate (17:0) - Exercise
R2=0.02236
myristate (14:0) - Exercise
R2=0.002400
myristoleate (14:1n5) - Exercise
R2=0.002194
myristoleoylcarnitine - Exercise
R2=9.557e-005
myristoylcarnitine - Exercise
R2=0.01339
N-oleoyltaurine - Exercise
R2=0.05678
octanoylcarnitine - Exercise
R2=0.02526
Fold Change Post-Exposure (Ozone/Air)
Exercise

Figure E7E



*The data for various metabolites were derived from metabolomic assessment of serum samples collected post air or post ozone exposure. The ratio of ozone/air values that were found to be significantly increased (except for cortisone) were plotted against each subjects prior exercise habit scaled based on questionnarre (mild, 1; moderate, 2; or heavy, 3) to determine if ozone effects were influenced by individuals prior exercise habits. Each subject arrived for either air or ozone exposure during two clinical visits, each were separated by at least two weeks. During each visit, subjects were exposed to either 0.3 ppm ozone or clean air for 2hr in the morning time in a blinded manner (n=24; 20 males and 4 females). Serum samples collected immediately post exposure (within 1hr) were subjected to global metabolomic assessment (complete data provided in online supplement #2). Note that these correlations are limited due to small number of subjects in each exercise group.

Figure E8A-E. Exploratory correlation plots showing the relationship between sex (1=female; 2=males) and fold ozone-induced change in various lipid metabolites post exposure.

Figure E8A

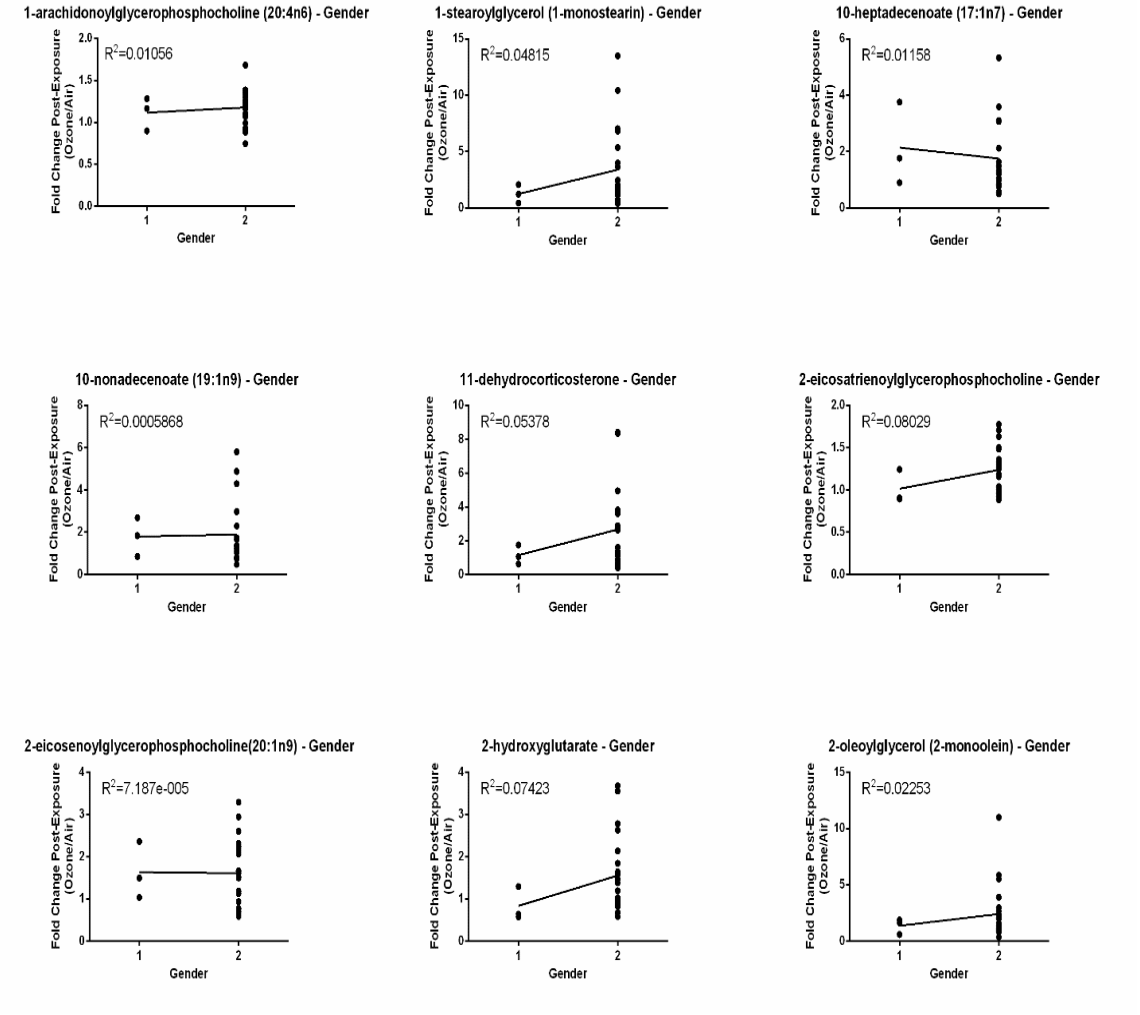


Figure E8B

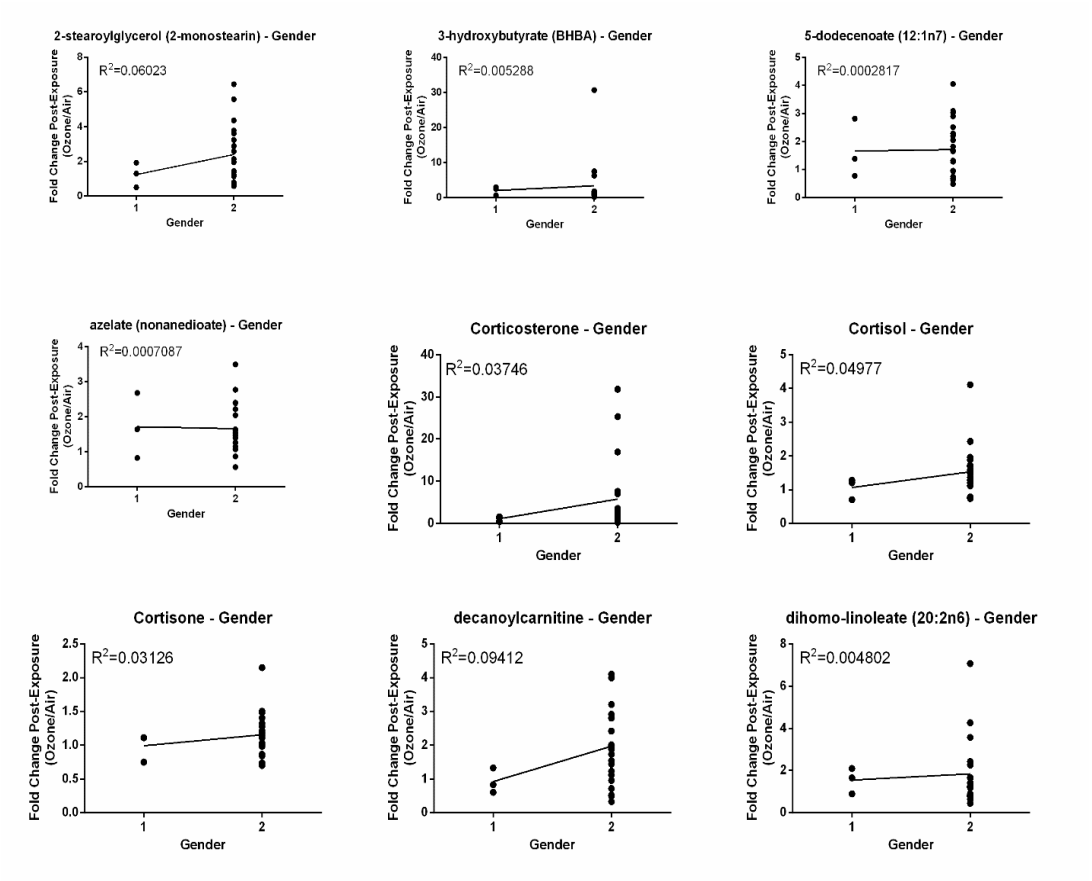

2-stearoylglycerol (2-monostearin) - Gender
R²=0.06023
3-hydroxybutyrate (BHBA) - Gender
R²=0.005288
5-dodecenoate (12:1n7) - Gender
R²=0.0002817
azelate (nonanedioate) - Gender
R²=0.0007087
Corticosterone - Gender
R²=0.03746
Cortisol - Gender
R²=0.04977
Cortisone - Gender
R²=0.03126
decanoylcarnitine - Gender
R²=0.09412
dihomo-linoleate (20:2n6) - Gender
R²=0.004802
Fold Change Post-Exposure (Ozone/Air)
Gender

Figure E8C

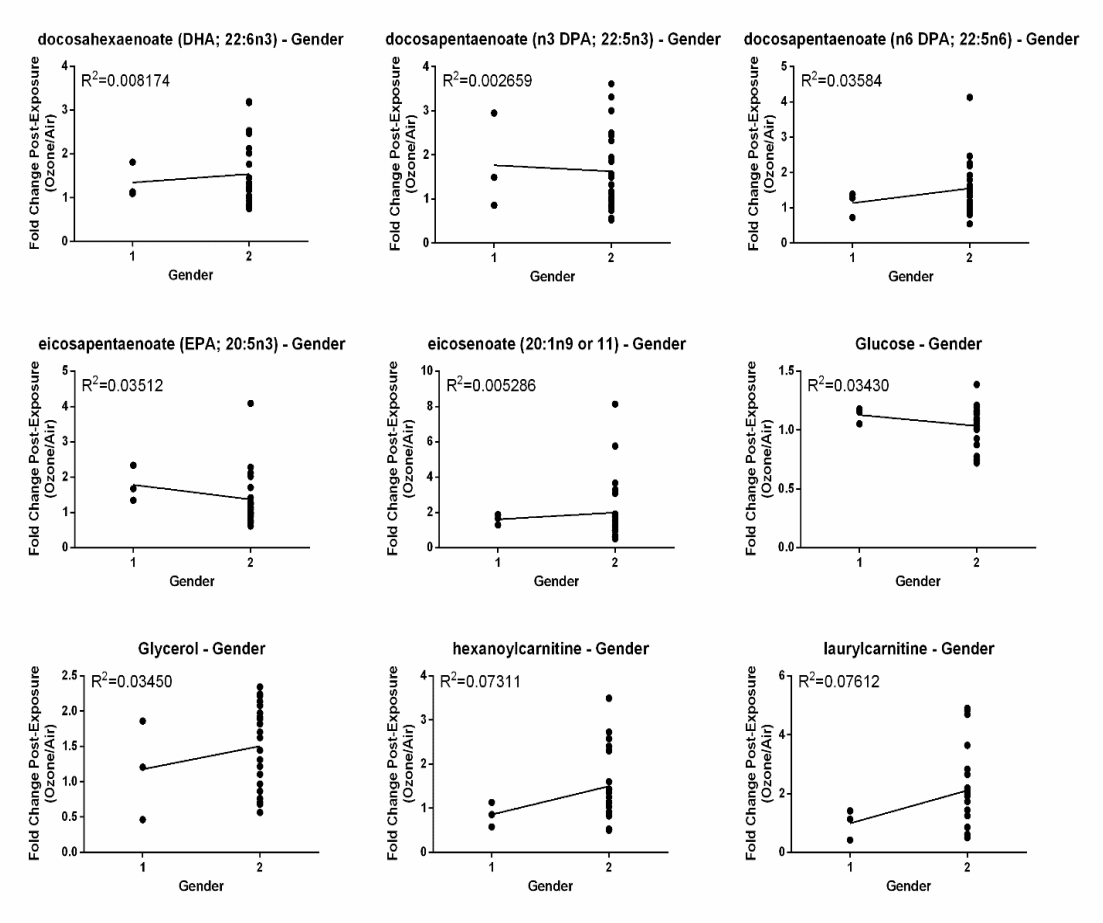

docosahexaenoate (DHA; 22:6n3) - Gender
R2=0.008174
docosapentaenoate (n3 DPA; 22:5n3) - Gender
R2=0.002659
docosapentaenoate (n6 DPA; 22:5n6) - Gender
R2=0.03584
eicosapentaenoate (EPA; 20:5n3) - Gender
R2=0.03512
eicosenoate (20:1n9 or 11) - Gender
R2=0.005286
Glucose - Gender
R2=0.03430
Glycerol - Gender
R2=0.03450
hexanoylcarnitine - Gender
R2=0.07311
laurylcarnitine - Gender
R2=0.07612
Fold Change Post-Exposure (Ozone/Air)
Gender

Figure E8D

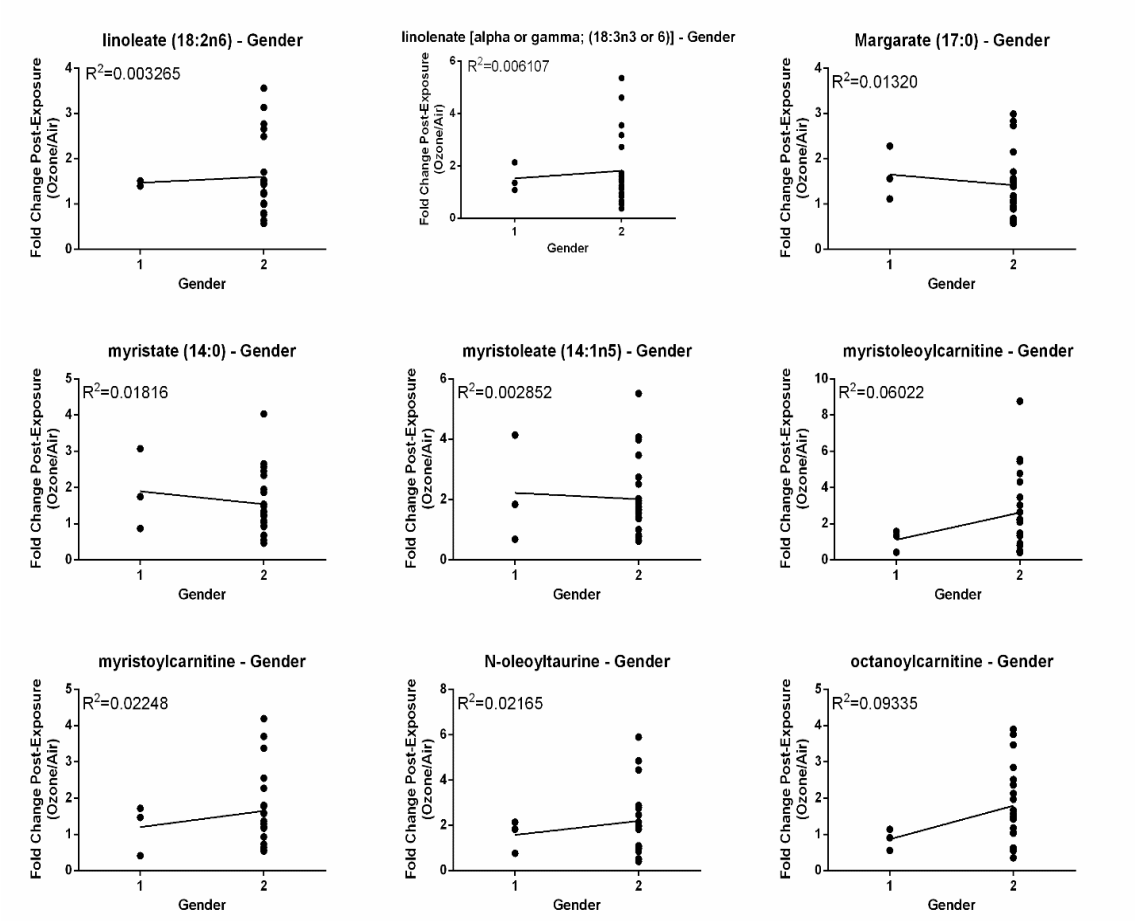
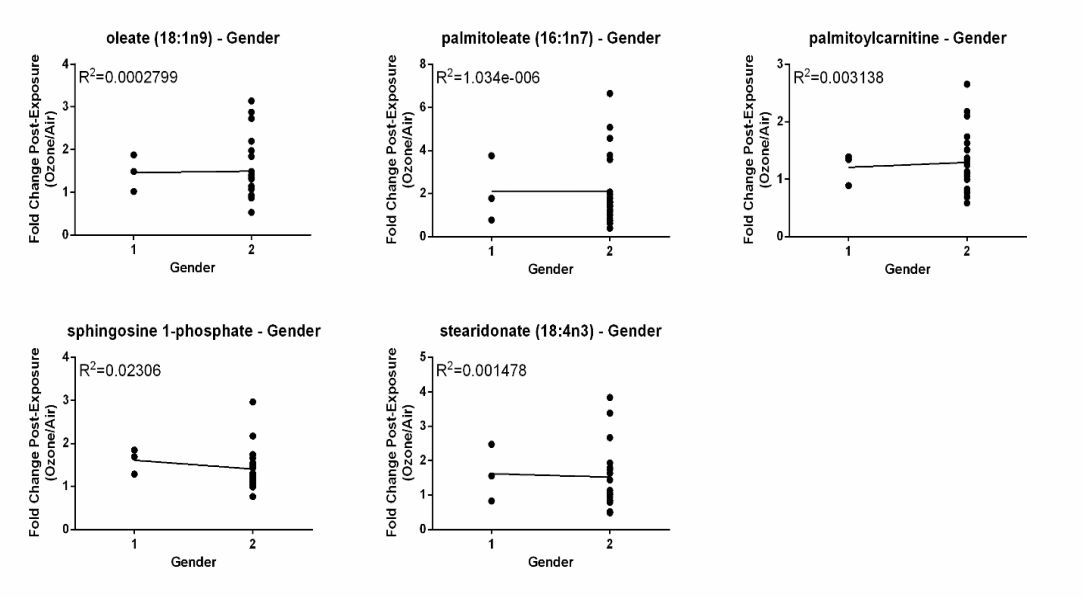

linoleate (18:2n6) - Gender
R2=0.003265
linolenate [alpha or gamma; (18:3n3 or 6)] - Gender
R2=0.006107
Margarate (17:0) - Gender
R2=0.01320
myristate (14:0) - Gender
R2=0.01816
myristoleate (14:1n5) - Gender
R2=0.002852
myristoleoylcarnitine - Gender
R2=0.06022
myristoylcarnitine - Gender
R2=0.02248
N-oleoyltaurine - Gender
R2=0.02165
octanoylcarnitine - Gender
R2=0.09335
Fold Change Post-Exposure (Ozone/Air)
Gender

Figure E8E



* The data for various metabolites were derived from metabolomic assessment of serum samples collected post air or post ozone exposure. The ratio of ozone/air values that were found to be significantly increased (except for cortisone) were plotted against sex (female, 1; males; 2) to determine if ozone effects were influenced by individual's sex. In the study, each subject arrived for either air or ozone exposure during two clinical visits, each separated by at least two weeks. During each visit, subjects were exposed to either 0.3 ppm ozone or clean air for 2hr in the morning time in a blinded manner (n=24; 20 males and 4 females). Serum samples were collected immediately post exposure (within 1hr) were subjected to global metabolomic assessment (data provided in online supplement #2). Note that these correlations are limited since there were only 4 females subjects included in this study whereas 20 were males.

Figure E9A-B. Exploratory correlation plots showing the relationship between body mass index (BMI) and levels of clinical markers in subjects post air or post ozone exposure.

Figure E9A

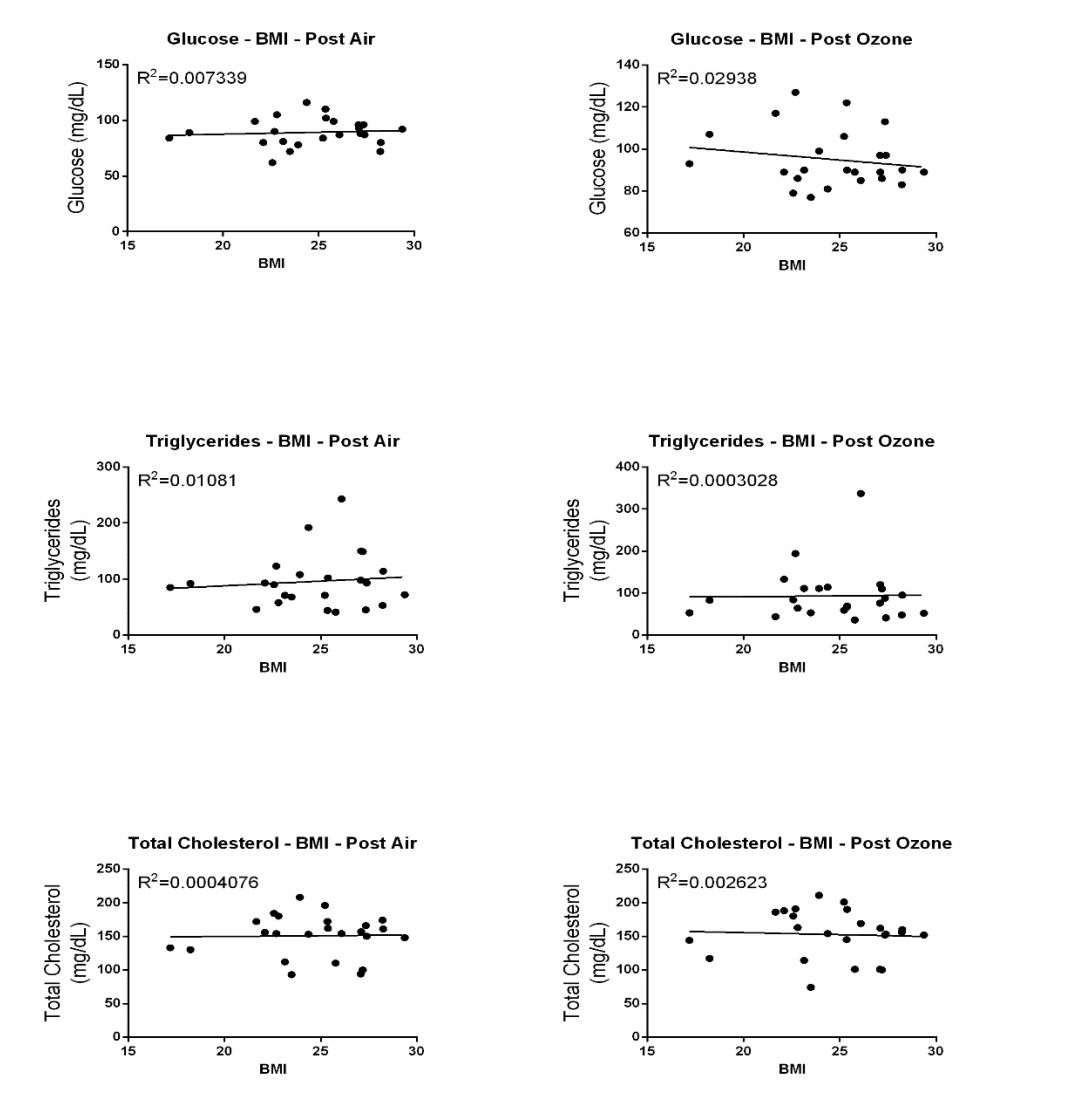
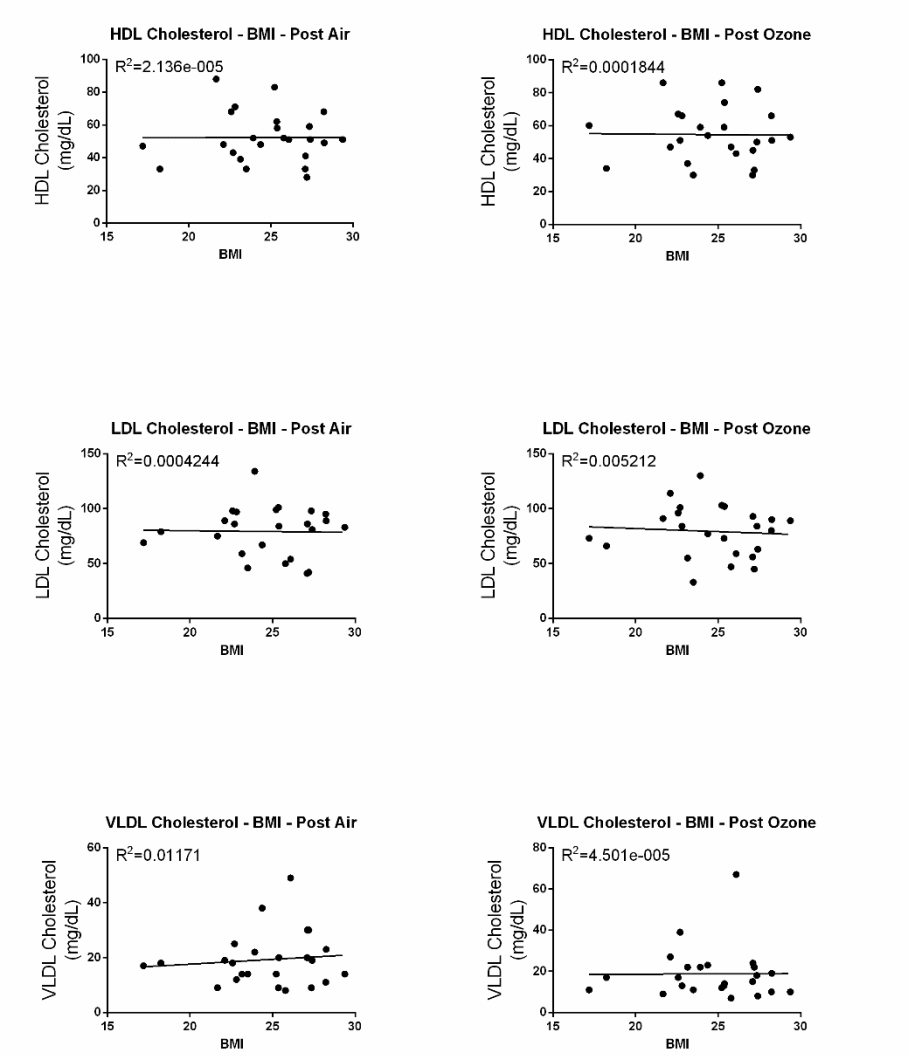


Figure E9B



*The data available to us for clinical markers of glucose and lipids collected post air exposure (left panel) or post ozone exposure (right panel) are plotted against BMI for all 24 subjects. In this study each subject arrived for either air or ozone exposure during two clinical visits, each separated by at least two weeks. During each visit, the subjects were exposed to either 0.3 ppm ozone or clean air during intermittent exercise for 2hr in the morning time in a blinded manner. Serum samples were collected immediately post exposure (within 1hr) and analyzed for lipids by LabCorp Inc (Durham, NC; complete data provided in online supplement #2). The plots indicate correlation between BMI values and the levels of lipid metabolites after air or ozone exposure (n=24; 20 males and 4 females).

Figure E10A-B. Exploratory correlation plots showing the relationship between each subject's prior exercise habit (mild, 1; moderate, 2; or heavy, 3) and levels of clinical markers in subjects post air or post ozone exposure.

Figure E10A

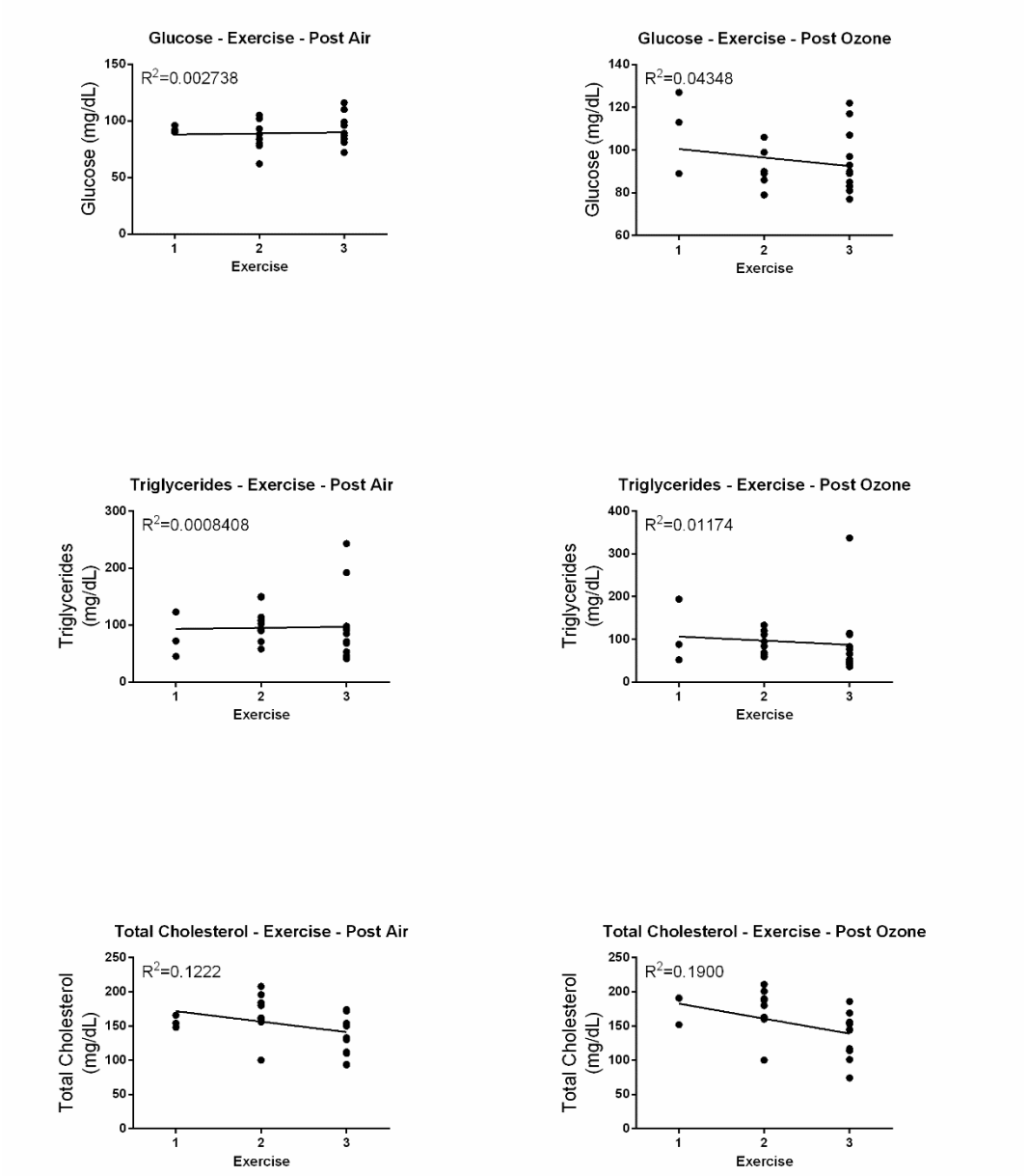
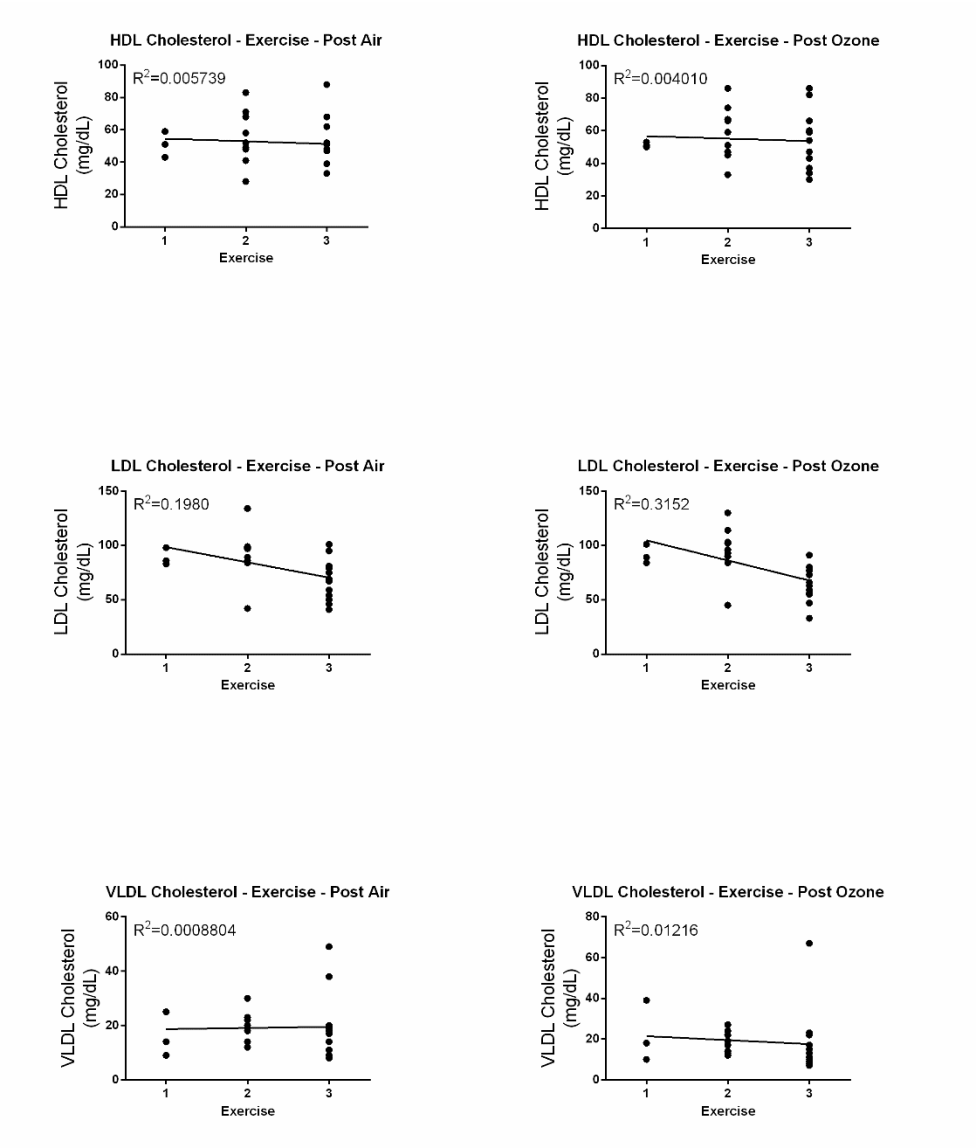


Figure E10B



* The data available to us for clinical markers of glucose and lipids collected post air exposure (left panel) or post ozone exposure (right panel) are plotted against each subject's prior exercise habit (mild, 1; moderate, 2; or heavy, 3) for all 24 subjects. In this study, each subject arrived for either air or ozone exposure during two clinical visits, each separated by at least two weeks. During each visit, subjects were exposed to either 0.3 ppm ozone or clean air during intermittent exercise for 2hr in the morning time in a blinded manner. Serum samples were collected immediately post exposure (within 1hr) and analyzed for clinical chemistry by LabCorp Inc (Durham, NC; complete data provided in online supplement #2). Note that the exploratory correlations includes limited number of subjects for each exercise level.

Figure E11A-B. Exploratory correlation plots showing the relationship between sex and the levels of clinical markers in subjects post air or post ozone exposure.

Figure E11A

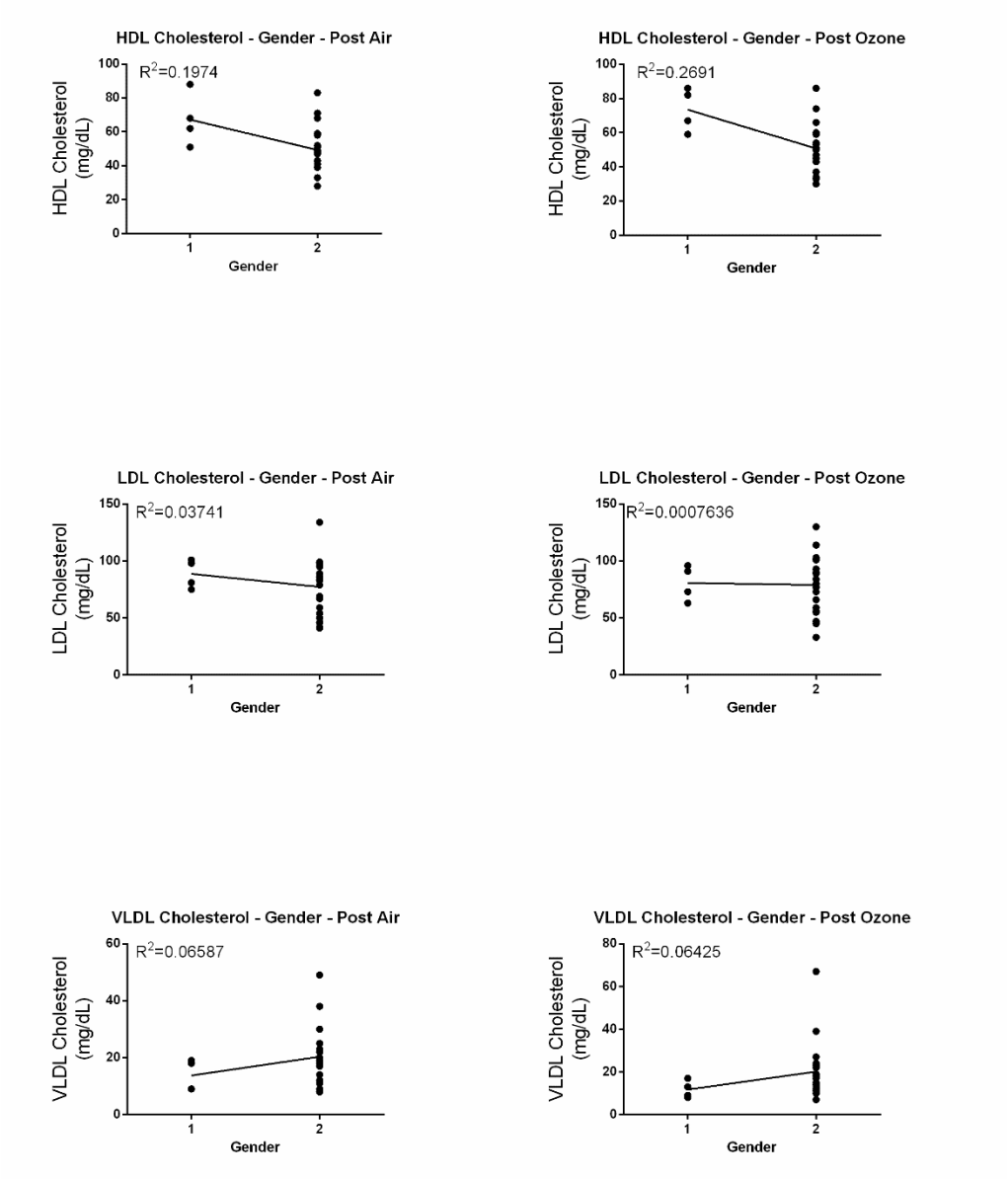
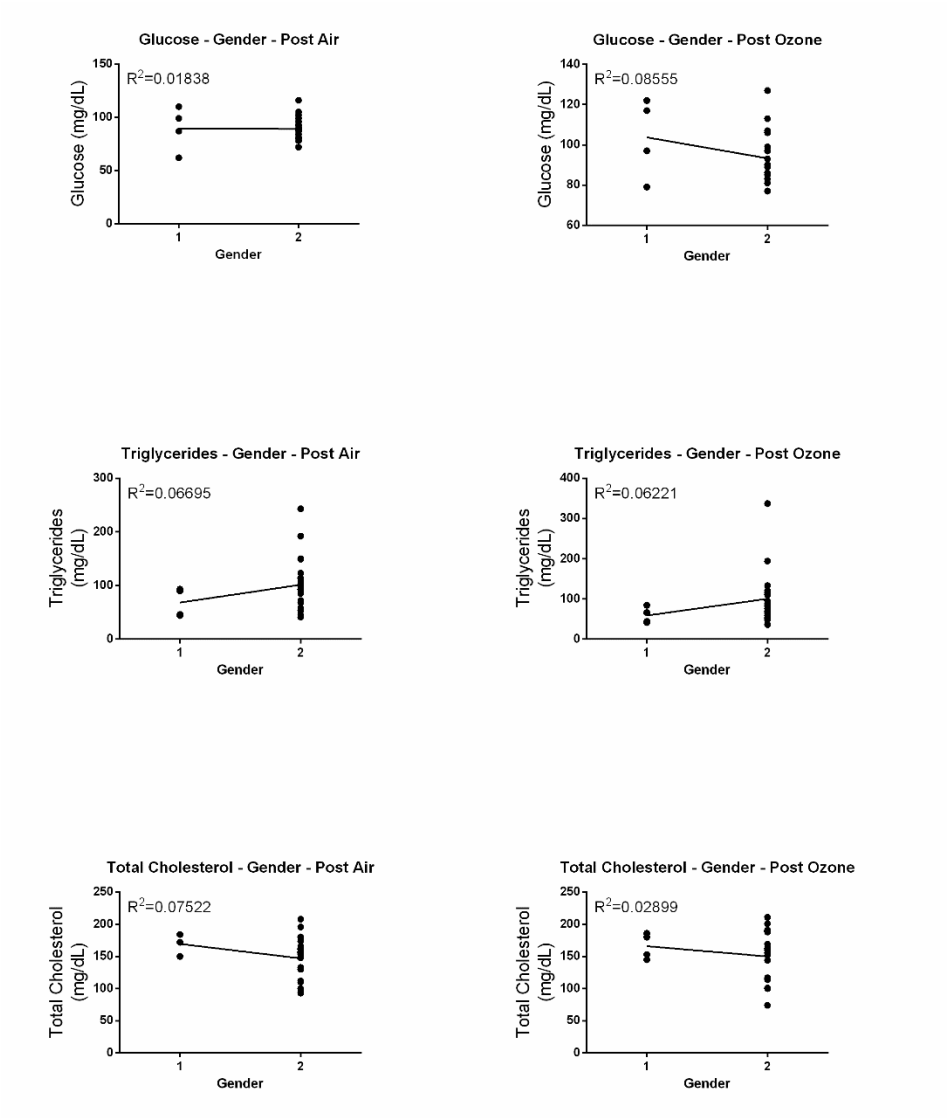


Figure 11B



*The data available to us for clinical markers of glucose and lipids collected post air exposure (left panel) or post ozone exposure (right panel) are plotted against sex (1, females; 2, males). In this study, each subject arrived for either air or ozone exposure during two clinical visits, each separated by at least two weeks. During each visit, the subjects were exposed to either 0.3 ppm ozone or clean air during intermittent exercise for 2hr in the morning time in a blinded manner. Serum samples were collected immediately post exposure (within 1hr) and analyzed for glucose and lipids by LabCorp Inc (Durham, NC; complete data provided in online supplement #2). Note that there were only 4 females in the study and therefore, the data are highly limited.

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