HEPATIC EFFECT OF INHALED OZONE IN SPRAGUE-DAWLEY RAT

By
Whitney Slone Theis

SHANNON M. BAILEY, COMMITTEE CHAIR
MICHIELLE V. FANUCCHI
DALE A. DICKINSON

THESIS
Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Master of Science in Public Health

BIRMINGHAM, AL
2012
Copyright by
Whitney Slone Theis
2012
HEPATIC EFFECT OF INHALED OZONE IN SPRAGUE-DAWLEY RAT

WHITNEY SLONE THEIS

M.S.P.H. IN ENVIRONMENTAL TOXICOLOGY

ABSTRACT

Ozone (O₃) is a primary component of photochemical smog and represents a serious public health concern as more than 50% of the U.S. population lives in areas exceeding national ambient air quality standards for this toxic pollutant. Recent findings indicate that the effects of inhaled O₃ extend to extra-pulmonary tissues like the heart and liver. We hypothesize that inhaled O₃ exposure-related events in the lung will cause downstream alterations in the liver proteome. To test this, we exposed male Sprague-Dawley rats to 0.5 ppm ozone for 8 hr a day for 5 days. After exposures, livers were removed and proteomic studies were performed. Histopathology and serum liver enzyme measurements showed that 5 day O₃ exposure did not cause overt liver injury. Proteomic and mass spectrometry analysis successfully identified 10 proteins that were significantly altered, with 8 proteins increased and 2 decreased in abundance, as a consequence of O₃ exposure. Importantly, several stress-induced proteins were significantly changed in response to O₃; Grp78 and protein disulfide isomerase increased, whereas glutathione-S-transferase significantly decreased. No significant changes were detected when measuring the protein content of HO-1 and cytochrome P450s 2E1 and 2B as compared to control. Additionally, no significant lung injury was detected as measured by inflammatory cells and protein content in bronchoalveolar lavage fluid. In summary, these results suggest that an environmentally-relevant exposure to O₃ modifies the liver proteome by altering key stress proteins in the liver. We propose that O₃ and other
inhaled pollutants may represent important unrecognized risk factors for exacerbating liver diseases.

Key words: Ozone, liver, systemic effects, ER stress, liver disease
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT ........................................................................................................... iii</td>
</tr>
<tr>
<td>LIST OF TABLES .................................................................................................. vi</td>
</tr>
<tr>
<td>LIST OF FIGURES ............................................................................................... vii</td>
</tr>
<tr>
<td>1 INTRODUCTION ................................................................................................. 1</td>
</tr>
<tr>
<td>1. Formation of ozone ....................................................................................... 1</td>
</tr>
<tr>
<td>1. Ozone: A public health concern .................................................................. 6</td>
</tr>
<tr>
<td>1. Reaction of ozone with epithelial lining fluid ......................................... 7</td>
</tr>
<tr>
<td>1. Pulmonary effects of ozone ......................................................................... 12</td>
</tr>
<tr>
<td>1. Systemic effects of ozone ........................................................................... 12</td>
</tr>
<tr>
<td>1. Hepatic effects of ozone .............................................................................. 13</td>
</tr>
<tr>
<td>1. Effect of ozone on other organ systems .................................................... 16</td>
</tr>
<tr>
<td>1. Effects of ozone on pre-existing diseases ................................................... 17</td>
</tr>
<tr>
<td>1. Summary ......................................................................................................... 19</td>
</tr>
<tr>
<td>2 INHALED OZONE MODIFIES THE RAT LIVER PROTEOME ................................... 22</td>
</tr>
<tr>
<td>3 CONCLUSION ................................................................................................. 53</td>
</tr>
<tr>
<td>3. Inflammation and the unfolded protein response ....................................... 53</td>
</tr>
<tr>
<td>3. Effect of ozone exposure on mitochondrial respiration .............................. 55</td>
</tr>
<tr>
<td>3. Future directions ......................................................................................... 59</td>
</tr>
<tr>
<td>3. Summary ........................................................................................................ 62</td>
</tr>
<tr>
<td>GENERAL LIST OF REFERENCES ........................................................................ 63</td>
</tr>
<tr>
<td>APPENDIX: INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM (IACUC) ................................................................. 70</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Bronchoalveolar lavage cell differential analysis</td>
<td>45</td>
</tr>
<tr>
<td>2 Hepatic proteins significantly altered in abundance as a result of 0.5 ppm O₃ exposure: results from 2D-IEF SDS PAGE and mass spectrometry</td>
<td>46</td>
</tr>
<tr>
<td>3 Description of cellular pathways and function of identified proteins</td>
<td>47</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>INTRODUCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ozone formation</td>
</tr>
<tr>
<td>2</td>
<td>Composition of epithelial lining fluid and secondary products formed</td>
</tr>
<tr>
<td>3</td>
<td>Reaction of O₃ with unsaturated fatty lipids in epithelial lining fluid (ELF)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure</th>
<th>INHALED OZONE MODIFIES THE RAT LIVER PROTEOME</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Illustration of O₃ production for animal exposures</td>
</tr>
<tr>
<td>5</td>
<td>Master map of proteins differentially altered by inhaled ozone</td>
</tr>
<tr>
<td>6</td>
<td>Protein levels of HO-1 and the xenobiotic enzymes CYP2E1 and CYP2B in the liver of O₃ and FA rats</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Figure</th>
<th>CONCLUSION</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Effect of O₃ on liver mitochondrial respiration</td>
</tr>
</tbody>
</table>
INTRODUCTION

Formation of Ozone

Ozone (O$_3$) is a highly reactive triatomic molecule and a primary component of photochemical smog. A brief overview of O$_3$ formation will be given here in order for the reader to become familiar with the origin of O$_3$ in the atmosphere, and the basic chemical reactions that govern O$_3$ concentrations relevant to public health. O$_3$ is naturally found in the stratospheric and tropospheric layers of the atmosphere. Stratospheric O$_3$ is considered 'good' O$_3$ as it forms the O$_3$ layer and absorbs harmful UV light from the sun reaching the Earth's surface. Tropospheric, ground level, O$_3$ is also important because it can aid in production of the hydroxyl radical (OH·), which allows for the elimination of volatile organic compounds (VOCs) in the troposphere (Atkinson, 2000). However, it is important to note that disruption in VOC production vs. degradation results in excess formation of O$_3$ that is 'bad' and detrimental to human health. Factors affecting surface concentrations of O$_3$ include: (1) intrusion of O$_3$ from the stratosphere to the troposphere, which can then enter the planetary boundary layer (PBL) (Roelofs and Lelieveld, 1997); (2) formation of O$_3$ from two primary precursors - VOC and nitrogen oxides (NO$_x$); and (3) transportation of precursors by weather patterns (Logan, 1985). Figure 1 provides a schematic outline of the atmospheric chemical processes resulting in O$_3$ formation that are discussed in this section. The chemistry
described below is not an exhaustive explanation of the atmospheric reactions that occur and govern ambient $O_3$ concentrations. This is simply a general overview. Chemistry between $NO_x$ and VOCs is a complex set of non-linear reactions in the presence of sunlight and originates from industrial, diesel, and biogenic sources.

In the stratosphere, $O_3$ formation primarily occurs from molecular oxygen interacting with short wavelength UV light forming two ground state oxygen radicals.

$$ O_2 + hv \rightarrow 2 \ O (^3P) $$

Ground state oxygen atoms in turn react with molecular oxygen to form $O_3$.

$$ O (^3P) + O_2 + M \rightarrow O_3 + M \quad M=air $$

$O_3$ is reactive and can photolyze, producing $O_2$ and either a ground state oxygen atom or an electronically excited oxygen atom. The electronically excited oxygen atom quickly returns to a ground state.

$$ O_3 + hv \rightarrow O_2 + O (^1D) $$

$$ O (^1D) + M \rightarrow O (^3P) $$

The cycle completes itself as $O_3$ can then interact with the ground state oxygen atom to produce molecular oxygen.

$$ O (^3P) + O_3 \rightarrow 2O_2 $$

The above reactions are known as the Chapman reactions and balance the concentrations of $O_3$ in the stratosphere (Chapman, 1930). The $O_3$ produced in the stratosphere can then pass into the troposphere and contribute to ground level $O_3$ concentrations.

The production of VOCs and $NO_x$ by biogenic and anthropogenic sources are a major contributor to ambient $O_3$ concentrations. The most significant production of $O_3$ in
the polluted troposphere is driven by chemical reactions involving nitric oxide (NO) and nitrogen dioxide (NO₂). NO is the predominant NOₓ and will oxidize to NO₂ in the presence of O₃. NO₂ will subsequently photolyze to form NO and a ground state oxygen atom.

\[
\text{NO} + \text{O}_3 \rightarrow \text{NO}_2 + \text{O}_2
\]

\[
\text{NO}_2 + \text{hv} \rightarrow \text{NO} + \text{O} \left( ^3\text{P} \right)
\]

The ground state oxygen atom will interact with molecular oxygen in air to form O₃.

\[
\text{O} \left( ^3\text{P} \right) + \text{O}_2 + \text{M} \rightarrow \text{O}_3 + \text{M}
\]

O₃ can further be consumed as shown above to further produce NO₂. These reactions are in equilibrium, but the addition of VOCs drives a net production of O₃.

VOCs are characterized as any carbon containing gas phase compound, excluding carbon monoxide (CO) and carbon dioxide (CO₂), with anthropogenic or biogenic beginnings. These are a wide array of compounds, including, but not limited to alkanes, alkenes, and aromatic hydrocarbons, which also have a wide range of half lives. A brief understanding of the complex atmospheric chemistry that takes place proceeds from the oxidation of VOCs by the OH radical. OH radicals are primarily formed when excited oxygen atoms interact with water vapor.

\[
\text{O} \left( ^1\text{D} \right) + \text{H}_2\text{O} \rightarrow 2\text{OH}^-
\]
The easiest and simplest VOC degradation to visualize is methane (CH₄). Like the large majority of VOCs, CH₄ is oxidized by OH⁻ to produce an alkyl radical. The following series of reactions require NO and produce HO₂ and alkoxy radicals (Atkinson, 2000).

\[
\begin{align*}
\text{OH} + \text{CH}_4 & \rightarrow \text{H}_2\text{O} + \text{CH}_3^+ \\
\text{CH}_3^+ + \text{O}_2 & \rightarrow \text{CH}_3\text{O}_2^+ \\
\text{CH}_3\text{O}_2^+ + \text{NO} & \rightarrow \text{CH}_3\text{O}^+ + \text{NO}_2 \\
\text{CH}_3\text{O}^+ + \text{O}_2 & \rightarrow \text{HCHO} + \text{HO}_2 \\
\text{HO}_2 + \text{NO} & \rightarrow \text{OH} + \text{NO}_2
\end{align*}
\]

The production of HO₂ and alkoxy intermediates reacts with NO to form NO₂ and as previously shown will photolyze and produce O₃. NO₂ produced in the above VOC degradation will photolyze, as shown previously, and result in O₃ formation. The above representation of methane degradation shows that VOC degradation is dependent upon atmospheric NO concentrations and it produces NO₂ without the O₃ intermediate. This increases NO₂ in the atmosphere available for photolysis and continued O₃ formation.

Ambient O₃ concentrations are also affected by weather patterns and geographical location. In urban areas wind speed will alter vertical mixing and increase accumulation of VOCs and NOx. Areas near mountain ranges can also have this same effect. Additionally long range transport of O₃ precursors can increase ambient O₃ in non-urban areas where anthropogenic sources of precursors are relatively low in comparison to urban areas.
Figure 1. Scheme of NOx and VOC atmospheric chemistry resulting in O3 formation. O3 is formed in the Earth’s stratosphere and troposphere. O2 in the stratosphere absorbs UV light forming ground state O atoms which react with O2 to form O3. O3 also absorbs UV light and the cycle is completed with formation of molecular oxygen. O3 found in the stratosphere can move through the tropopause and into the troposphere to interact with water vapor and form OH. OH participate in VOC degradation and a series of reactions occur. HO2 and allylic intermediates and a net production of NO from NO2. NO2 is the major contributor to O3 formation in the troposphere. NO2 is photolyzed producing NO and O3. NO2 production through VOC degradation forms O4 without O3 as an intermediate resulting in a net increase in ground level O3.
Ozone: A Public Health Concern

Ozone inhalation represents an important public health concern. Under the Clean Air Act of 1970 the Environmental Protection Agency (EPA) set air quality standards for harmful air pollutants, including, but not limited to, sulfur dioxide (SO$_2$), particulate matter (PM), NO$_2$, and O$_3$. Standards are set with susceptible populations in mind. Those populations susceptible to O$_3$ exposure include children, elderly, and those with pre-existing pulmonary disease like bronchitis and obstructive pulmonary disease (COPD). According to the most recent National Ambient Air Quality Standards (NAAQS), O$_3$ is not to exceed 0.075 ppm over an 8-hour time average. Despite these standards approximately 50% of the population lives in areas that exceed NAAQS for O$_3$ (Environmental et al., 2008). It has been shown that O$_3$ inhalation leads to decreased lung function, increased airway epithelial cell damage and exacerbation of pre-existing lung diseases (EPA, 2006; Joad et al., 2000; Michalec et al., 2002). Considerable research has been devoted to showing the pulmonary effects from O$_3$ inhalation.

There are also O$_3$ associated occupational hazards. Occupational Safety and Health Administration (OSHA) has set the permissible exposure limit (PEL) for O$_3$ to 0.1 ppm over an 8 hour work-shift. A number of industries generate O$_3$ to aid in purification of drinking water, waste treatment, wet paper, etc. These industries include pulp mills, outdoor construction and copy machine companies, and steel mills. Thus there is potential for O$_3$ exposure not only in ambient air but also in the workplace.
Due to the high reactivity and modest solubility of \( \text{O}_3 \) its diffusion through the epithelial lining fluid (ELF) of the lung is very limited (Pryor, 1992). The ELF is a thin non-homogeneous aqueous layer that spans in thickness between 20 µm and 0.1 µm from the conducting airways to the lower alveolar regions covering the airway epithelium (Bastacky et al., 1995; Gil, 1985). It is estimated that \( \text{O}_3 \) can only react with lipid bilayers where the ELF is very thin in the alveolar region where macrophages could possibly stick out into the airway (Pryor et al., 1995). There are a number of small molecular weight antioxidants (ascorbate, uric acid, and glutathione), proteins, and lipids in the ELF (Uppu et al., 1995). It is estimated that the lipid-protein pool in the ELF is 90% and 10%, respectively (Uppu et al., 1995). The net flux of \( \text{O}_3 \) is determined by its high reactivity and modest solubility. This process termed ‘reactive absorption’ describes the process whereby \( \text{O}_3 \) chemically reacts with ELF constituents at the air-liquid interface of the lung further driving its uptake (Postlethwait et al., 1994). Thus, it is believed the ELF-derived secondary products are the key mediators responsible for initiating cellular responses from \( \text{O}_3 \) exposure in the lung (Kafoury et al., 1998 1995; Postlethwait et al., 1998; Pryor et al., 1995). Figure 2 represents ELF constituents, their interaction with \( \text{O}_3 \), and a simplification of the secondary products generated.
Figure 2: Composition of epithelial lining fluid and secondary products formed. The airways are covered in epithelial lining fluid (ELF) composed of antioxidants such as ascorbate and glutathione as well as proteins and lipids. Because $O_3$ is incapable of passing through the ELF to interact with epithelial cells, $O_3$ reacts with ELF constituents to form secondary products.
Lipids present in the ELF include both poly and mono-unsaturated fatty acids. Reaction of O$_3$ in an aqueous environment like the ELF with monounsaturated fatty acids yields predictable products including aldehydes, hydroxyhydroperoxides, and small amounts of the Criegee ozonide termed lipid ozonation products (LOP) (Figure 3) (Kafoury et al., 1998; Pryor et al., 1995). LOPs are more stable, have longer half-lives than O$_3$, and thus most likely contribute to some of the toxic effects from inhaled O$_3$ (Pryor et al., 1995). Experiments with O$_3$ and red blood cell membranes show that 88% of the O$_3$ reacts with unsaturated fatty acids, while the remaining 12% reacts with proteins (Uppu et al., 1995). However, these calculations don’t take into account reaction of O$_3$ with ELF antioxidants, which most likely serve as protection against initial O$_3$ exposure.

LOPs have been shown to activate phospholipases (PLC) A$_2$, C, and D contributing to O$_3$-induced lung inflammation and injury. Ozonation of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), an unsaturated fatty acid, produced predictable aldehyde and hydroxyhydroperoxide molecules of varying carbon chain lengths (Kafoury et al., 1998). These products were shown to differentially activate all three phospholipases and release inflammatory mediators in cultured airway epithelial cells (Kafoury et al., 1998). In the same study it was shown that PLA$_2$ activity could be significantly increased when exposed to aldehyde and hydroxyhydroperoxide products for 1 hr at low micromolar concentrations but not by the Criegee ozonide. In contrast, only hydroxyhydroperoxide products activated PLC. This is in accordance with other
studies that have shown that LOPs are biologically active and can stimulate inflammatory mediator release such as arachidonic acid (Devlin et al., 1994; Wright et al., 1994).

Additionally, autoxidation of unsaturated fatty acids produce reactive aldehyde products such as 4-hydroxynonenal, which are shown to be cytotoxic and lead to cellular dysfunction (Kirichenko et al., 1996). The cascades initiated by the ELF secondary products result in activation of the NFκB pathway and subsequent production of cytokines and inflammatory cell recruitment into the airways (Kleeberger et al., 2001; Last et al., 2005). O₃-induced production of cytokines, chemokines, and LOPs could initiate events observed in other organ systems. While this is purely speculative studies discussed later suggest that O₃ inhalation leads to an increase in inflammatory mediators in other organs and upregulate proteins involved in the inflammation process.
Figure 3: Reaction of $O_3$ with unsaturated fatty acids in epithelial lining fluid (ELF). $O_3$ directly attacks the double bonds in unsaturated fatty acids. In an aqueous environment products yielded are aldehydes and hydroxyhydroperoxides with small amounts of the Criegee ozonide produced. These are termed lipid ozonation products (LOP) and are hypothesized to exert the biochemical effects seen following $O_3$ exposure. An equilibrium exists between the aldehyde and hydroxyhydroperoxide with hydrogen peroxide produced ($H_2O_2$).
Pulmonary Effects of Ozone

The effect of O₃ inhalation on the respiratory tract has been extensively studied. A wide variety of studies have investigated the effect of O₃ with varying concentrations, durations, effect on development, the aging population, and effect on pre-existing respiratory conditions (EPA, 2006). Epidemiological, animal, and human exposure studies all show that O₃ inhalation alters lung function, inflammation, xenobiotic metabolism, lung immune response, and morphological changes in airway epithelium (Gohil et al., 2003; Watt et al., 1998; Wiester et al., 1996). Despite the multitude of research it is still not entirely clear the cause of O₃-induced lung injury. As explained previously, it is hypothesized that the secondary mediators play a role in some of the effects witnessed from O₃ inhalation but they may not be causal links. When examining exposure data it is most frequently divided into studies showing acute, higher concentrations for short periods of time, versus studies demonstrating effects from chronic exposures at lower O₃ concentrations for longer periods of time. These effects are often dose and time dependant (EPA, 2006).

Systemic Effects of Ozone

The impact of O₃ on organs beyond the respiratory system is becoming a more active area of research. A number of studies associate O₃ exposure to increased cardiovascular-related morbidity and mortality (Brook et al., 2004; Chuang et al., 2009; Chuang et al., 2007; Chuang et al., 2010; Park et al., 2005). Epidemiologic and clinical
studies show an association between high ambient O₃ days with increased hospital admissions (Brook et al., 2004). Additionally, high ambient O₃ days cause increased blood pressure, blood lipids, and decreased glucose tolerance in humans (Chuang et al., 2010). It is important to note that air pollution is a mixture of many constituents that include not only O₃ but also particulate matter (PM). PM is also an air pollutant that has been studied and associated with both pulmonary and non-pulmonary effects (Jerrett et al., 2009). Delineating the effects between O₃ and PM seen in epidemiological studies becomes difficult when you consider that air pollution isn't just one constituent. However, studies with research animals models exposed to O₃ alone do reveal cardiovascular effects. For example, studies by Ballinger, Postlethwait, and colleagues show that an exposure to 0.5 ppm O₃ induces vascular dysfunction, increases aortic mitochondrial DNA damage, and increases progression of atherosclerosis in murine models (Chuang et al., 2009). These studies are important because they have taken a more mechanistic approach to understand how O₃ inhalation has the potential to alter cardiovascular health. Additionally, mice genetically predisposed to obesity display increased sensitivity to O₃ (Shore et al., 2003). Genetically obese mice such as the ob/ob, db/db, and Cpefat models all display increased markers of inflammation after O₃ exposure as compared to the control obese counterparts (Shore et al., 2009).

**Hepatic Effects of Ozone**

In addition to effects on the heart, inhaled O₃ also leads to changes in other peripheral organ systems like the liver. Some of the earliest studies investigating hepatic
effects showed an altered clearance of the drug pentobarbital after exposure to O₃ (Gardner and Illing, 1974; Graham et al., 1981). Experiments initially found that female CD-1 mice exposed to 1 ppm O₃ exposure for 3 hr/day had increased pentobarbital induced sleeping time (Graham et al., 1981). The greatest increase in sleeping time as compared to a filtered air control was at the second and third days. Differences were lost with exposures lasting 4 or more days. When these results were compared between sex, mouse strain, and other rodent models such as rats and hamsters; females in every animal model were more sensitive with no differences observed in males (Graham et al., 1981). There is also a dose and time dependent effect. A single acute dose of O₃ will elicit an immediate increase in sleeping time after a 3 hr exposure; however, as the concentration of O₃ decreases more exposure days are required to observe a difference in pentobarbital induced sleeping time (Graham et al., 1985). For example, the peak difference in sleeping time for 0.5 ppm O₃ occurred after 3 days of exposure, whereas a 0.1 ppm O₃ exposure requires 15 days of exposure before an effect is witnessed. This suggested that O₃ exposure impacted the drug metabolizing systems in the liver responsible for clearance of pentobarbital.

The liver is a vital organ in the metabolism of both endogenous and exogenous compounds. A broad class of enzymes known as cytochrome P450s (CYP450) are responsible for drug metabolism as they enzymatically modify compounds so that they become more hydrophilic and are excreted (Hewitt et al., 2007). Altering these enzymes could result in significant changes in the half lives of drugs, thus altering drug-drug interactions, dosage, and toxicity. This could have extensive impact on populations
exposed to elevated ambient O$_3$ especially with an increasing number of patients taking multiple prescribed and over-the-counter medications. While it is apparent that O$_3$ can alter pentobarbital clearance (Graham et al., 1981), the mechanism responsible for these changes is not clear. When CYP450 protein was measured in female CD-1 mice there were no significant changes between O$_3$ and filtered air control animals. Neither was the activity of reactions representing $O_-$, and $N$-demethylation, but activity of hydroxylation measured by aniline hydroxylase was significantly increased in acute exposures (Graham et al., 1982).

Since this early work of Gardner and colleagues (Gardner and Illing, 1974; Graham et al., 1981) other groups have investigated this early phenomenon of inhaled O$_3$ on the liver. Alterations in nitric oxide (NO) production have been investigated and associated with changes in hepatocytes following O$_3$ exposure (Laskin et al., 1994). Primary hepatocytes isolated from female rats previously exposed to O$_3$ for 3 hr had significantly increased NO production both spontaneously and with incubation of inflammatory mediators like interferon-$\gamma$ (IFN-$\gamma$) and lipopolysaccharide (LPS). In addition, an increase in protein synthesis was observed in isolated hepatocytes. These results were dose-dependent between 0.5 ppm and 2 ppm with statistical significance reached at 1 ppm O$_3$. Changes in NO production were attributed to an increase in inducible NO synthase (iNOS). Changes in NO production may be important for liver metabolism as NO is a signaling molecule with multiple biological actions including reduction of mitochondrial respiration through inhibition of cytochrome c oxidase (CcOx) (Brown, 1995) and modification of proteins from production of reactive nitrogen.
species (Hill et al., 2010). Last and colleagues used a microarray approach to examine the effects of O$_3$ on the mouse liver transcriptome (Last et al., 2005). Exposing C57BL/6 mice to 1 ppm O$_3$ for 8 hr/day for 3 days, they showed that O$_3$ exposure caused a significant down-regulation of a number of genes responsible for xenobiotic, carbohydrate, and fatty acid metabolism with all but three of their reported significant changes decreasing in mRNA transcript. However, this study did not detect O$_3$-dependent changes in CYP450 isoforms 9, 10, and 13; enzymes involved in pentobarbital metabolism (Last et al., 2005). Speculation about how O$_3$ is eliciting these hepatic effects is still unexplained. It is known that O$_3$ exposure results in an increase in inflammatory markers produced by activated alveolar macrophages (Laskin et al., 1998). Thus, it was hypothesized in both of the most recent studies that an inflammatory response in the lung was an important factor initiating downstream hepatic events. However, in neither of the above studies were they able to show increased circulating amounts of inflammatory cytokines (Laskin et al., 1998; Last et al., 2005). ELISAs performed with antibodies for inflammatory cytokines such as IL-6 and TNF-$\alpha$ did not detect changes between filtered air and O$_3$ exposed groups (Last et al., 2005).

Effects of Ozone on Other Organ Systems

In addition to the cardiovascular and hepatic effects that have primarily been discussed, studies have also investigated other tissues and organ systems as well. The effects of O$_3$ on nervous tissue, thymus, spleen, reproduction and development, and ocular tissues have all been reported (EPA, 2006). Some behavioral and neurobehavioral
changes have been observed in animal models following O₃ (Colín-Barenque et al., 1999) however, in mouse models there is some disagreement on findings. In utero exposures did not produce significant changes in behavior at O₃ concentrations of 0.2, 0.4, and 0.6 ppm (Petruzzi et al., 1995), but in utero exposures in mice that continue through birth and weaning led to behavior changes such as hot plate avoidance and subtle changes in handedness at slightly higher concentrations of 0.3, 0.6, and 0.9 ppm O₃ (Petruzzi et al., 1999). Other studies show concentrations of 1 ppm for 12 hr/day during the entirety of gestation results in alterations in the anterior cerebellar lobe in rat pups (Rivas-Manzano and Paz, 1999). The effects surrounding the thymus and spleen involved T-cell mediated systemic immunity (Fujimaki et al., 1987). These effects have considerably been disregarded because observations were at very high O₃ exposures (≥1 ppm) and unable to be reproduced at lower concentrations. This same criticism is true of many of the studies conducted looking at effects of O₃ exposure to skin. Studies looking at dermal O₃ exposure suggest that O₃, concentrations between 1-5 ppm, alters the antioxidant defense systems of the subcutaneous layer and perhaps making the skin more susceptible to subsequent UV exposure (Cross et al., 1998; Valacchi et al., 2000).

Impact of Ozone on Pre-Existing Diseases

When O₃ exposure is combined with a pre-existing pathology in animal models effects are even more exaggerated. For example, mice with acetaminophen-induced hepatotoxicity are more sensitive to liver injury when O₃ exposure follows (Aibo et al., 2010). Mice were administered a dose of acetaminophen to intentionally induce
hepatotoxicity and 2 hr after administration mice were then exposed to O₃ for 6 hr at 0.25 or 0.5 ppm. Exposure to 0.5 ppm O₃ and acetaminophen increased plasma levels of IL-6 and keratinocyte-derived chemokine (KC) 1 hr post cessation of O₃ but these levels reduced back to baseline 24 hr after cessation of O₃. Other changes include significant increases in hepatocellular necrosis, liver enzymes, and the oxidative stress sensitive genes heme oxygenase-1 (HO-1), metallothionein-1 (MT-1), and catalytic subunit of glutamate-cysteine ligase (GCLC) when compared to acetaminophen alone. It is also interesting to note that in this study, down regulation of several genes responsible for DNA and cellular repair were observed 24 hr after cessation of O₃ suggesting an impaired ability for liver regeneration. The mechanism responsible for O₃-dependent exacerbation of acetaminophen toxicity is unknown, but it is clear that O₃ elicits a systemic response that enhances the toxic effects of this potent hepatotoxic drug.

As an extension of this concept, genetically obese animal models were used by Shore et al. (Johnston et al., 2008; Johnston et al., 2010; Shore et al., 2003) to determine whether obesity alters O₃-induced airway hyperreactivity, a common measure of asthma. Studies conducted by this group showed that both diet-induced and genetically obese animals were not only more sensitive to O₃-induced airway hyperresponsivness via methacholine challenge, but inflammatory markers were also increased in the lungs and in the plasma. One hallmark of obesity is chronic low-level systemic inflammation such as TNF-α and IL-6. While the goal of these studies was not to identify hepatic function alteration in obese animals after exposure to O₃ it does raise questions regarding the effect of an additional oxidant exposure such as O₃ on a pre-existing pathology like non-
alcoholic fatty liver disease (NAFLD). This is important as an increase of pro-
inflammatory markers in a pre-existing pathology that already displays an increase in
inflammation could increase disease severity and progression.

Other air pollutants have also shown to have systemic toxic effects. Exposure to
particulate matter (PM), an air pollutant also regulated by the NAAQS, was shown to alter inflammation and insulin resistance (Sun et al., 2009). More specifically, exposure to PM with a diameter < 2.5 µm (PM$_{2.5}$) in a diet-induced obesity animal model displays an increase in serum inflammatory markers, reduced p-Akt, which is involved in insulin signaling, increased blood glucose, and an increase in visceral adipocyte size (Sun et al., 2009). Additionally, p47 null animals have attenuated responses to PM$_{2.5}$ induced changes in a diet induced obesity model (Xu et al., 2010). The common theme among these studies is the potential for O$_3$ exposure to further exacerbate a pathology that produces a chronic inflammatory state.

Summary

O$_3$ is a highly reactive atmospheric pollutant. Increases in ground level O$_3$
formation are detrimental to human health especially susceptible populations such as children, elderly, and persons with pre-existing pulmonary conditions. Many areas of the United States do not meet the national air quality standards set for ambient O$_3$
concentrations. It is becoming clear that O$_3$ is not only a pulmonary toxicant, but is increasingly recognized as an environmental pollutant that leads to systemic perturbations
despite the fact that O₃ is incapable of passing into the vasculature. Some of the most convincing examples of this are the cardiovascular and hepatic events that occur following O₃ exposure. Epidemiological and experimental animal model studies show increased cardiovascular risk and vasculature oxidative stress due to O₃ exposure. Animal models show significant down regulation of mRNA transcripts involved in xenobiotic, fatty acid, and carbohydrate metabolism in the liver after O₃ exposure. Additionally, hepatocytes exhibit larger production of NO in isolated hepatocytes from O₃ exposed animals compared to filtered air controls.

The following section displays results from an O₃ exposure regimen aimed at using an O₃ concentration more relevant to human health exposure. As described previously, much of the systemic effects observed have been at very high acute concentrations, thus extrapolation to human consequence becomes difficult. Fortunately, recent work from Ballinger and colleagues showed that O₃ exposure at lower concentrations elicit cardiovascular effects increasing risk for atherosclerosis. Herein, this Master’s thesis project aims to investigate the effect of O₃ inhalation on the liver proteome using proteomic techniques coupled with mass spectrometry and measurement of xenobiotic metabolizing enzymes implicated in pentobarbital metabolism and liver disease progression. Based on previous research investigating pentobarbital induced sleeping time, we chose to measure the protein levels of CYP2B an enzyme known to participate in pentobarbital metabolism. We also measured the levels of CYP2E1 a prominent xenobiotic enzyme most known for acetaminophen metabolism as well as implicated in disease severity. We hypothesize that O₃ exposure will alter hepatic
proteins and xenobiotic metabolism enzymes. This thesis is intended to serve as a stepping stone for future experiments aimed at determining the detrimental effects of a key environmental pollutant on human health.
INHALED OZONE MODIFIES THE RAT LIVER PROTEOME

WHITNEY S. THEIS¹,³, KELLY K. ANDRINGA¹,³, TELISHA MILLENDER-SWAIN¹,²,³ DALE A. DICKINSON¹,³, EDWARD M. POSTLETHWAIT¹,³, AND SHANNON M. BAILEY¹,²,³

Departments of Environmental Health Sciences¹ and Pathology², and the Center for Free Radical Biology³, University of Alabama at Birmingham, Birmingham AL USA

In preparation for Toxicology Sciences
Format adapted for thesis
Introduction

Approximately 50% of the U.S. population resides in areas where ambient ozone (O$_3$) concentrations exceed the current 0.075 ppm 8-hr time average set by the National Ambient Air Quality Standards (NAAQS) (Environmental et al., 2008). O$_3$ is a primary component of photochemical smog and presents a serious public health concern when air quality is poor. Persons particularly sensitive to O$_3$ exposure include the elderly, young children, and those with pre-existing pulmonary diseases such as asthma and chronic obstructive pulmonary disease (COPD). It is established that exposure to high levels of O$_3$ leads to decreased lung function, increased pulmonary hyperreactivity, airway epithelial cell damage and remodeling, and increased epithelial permeability (Bhalla and Crocker, 1987; Dye et al., 1999; Lippmann, 1989).

In addition to adverse pulmonary effects, recent studies show that inhalation of O$_3$ also causes tissue injury and changes to metabolism in organs distal to the lung like the heart and liver. Recent epidemiologic studies show a strong association of air pollution exposure with increased cardiovascular-related morbidity and mortality resulting in increased hospital admissions related to cardiac events (Brook et al., 2004; Chuang et al., 2007; Chuang et al., 2010; Park et al., 2005). Additional studies show that successive days of high ambient O$_3$ exposure correlates with increased blood pressure, blood lipids, and decreased glucose tolerance in humans (Chuang et al., 2010). Ballinger and colleagues showed that exposure to 0.5 ppm O$_3$ induces vascular dysfunction, increases aortic mitochondrial DNA damage, and increases progression of atherosclerosis in mice that are genetically predisposed to cardiovascular disease (Chuang et al., 2009). This
study is important because not only does it provide direct evidence that inhaled O\textsubscript{3} damages the cardiovascular system, but also shows that O\textsubscript{3} has the potential to exacerbate pre-existing conditions like atherosclerosis.

Inhaled O\textsubscript{3} may also induce metabolic changes in other peripheral organ systems. For example, studies show that pentobarbital-induced sleeping times increase after O\textsubscript{3} exposure suggesting an alteration in the metabolism and clearance of the compound (Gardner, 1979; Graham et al., 1981). Since this early work, other groups have addressed the effect of inhaled O\textsubscript{3} on liver. Last and colleagues using a microarray approach examined the effects of O\textsubscript{3} (1 ppm) on the liver transcriptome and showed that O\textsubscript{3} inhalation led to significant decreases in mRNA levels of xenobiotic, carbohydrate, and fatty acid metabolism genes (Last et al., 2005). Other groups have shown that acute exposures of O\textsubscript{3} (1-2 ppm) for 3 hr increased nitric oxide production in isolated hepatocytes, as well as increased protein synthesis (Laskin et al., 1994). The goal of the current study was to determine whether inhalation of O\textsubscript{3} at a concentration lower than many of the previous studies alters the hepatic proteome and specific xenobiotic metabolizing enzymes in an animal model without pre-existing disease.

**Materials and Methods**

**O\textsubscript{3} Exposure Protocol**

Male Sprague-Dawley rats (250-275 g) were purchased from Harlan Laboratories (Barrier 217 VAF, Indianapolis, IN) and were provided food and water *ad libitum*. Animals were housed two per cage and maintained under a standard 12 hr light-dark
cycle. Twelve rats, 6 per group, were exposed to either filtered air (FA) or ozone (O₃) at 0.5 ppm for 8 hr/day for 5 days between 9:00 AM - 5:00 PM in the University of Alabama at Birmingham (UAB) Environmental Exposure Facility. Standard laboratory rat chow was removed from cages before the start of exposures each day to prevent rats from ingesting chow that may contain oxidized macronutrients (e.g., lipids) as a consequence of O₃. Prior to exposures, animals were acclimated to the chambers for at least 72 hr. O₃ was generated from 100% O₂ using a silent arc electrode model OZ1PCS-V/SW (Ozotech Inc, Yreka, CA) and mixed with FA and flowed into 0.8 m³ stainless steel chambers (~22°C, 50% relative humidity). O₃ concentrations were continuously monitored using a Thermo-Environmental Model UV Photometric analyzer (Thermo-Environmental, Franklin, MA). FA controls were housed in separate chambers with equal air exchange throughout the duration of the exposure regimen (Figure 4). All procedures were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Animals were anesthetized with a 50 mg/kg i.p. injection of sodium pentobarbital and euthanized via exsanguinations. Tissues were harvested within 1 hr of the cessation of the exposure.
Figure 4: Illustration of O₃ production for animal exposures. Oxygen is subjected to UV light to form O₃ and then mixed with chemical, biological, radiological air. The Air/O₃ mixture is then streamed through a mass flow controller at a specific concentration and streamed into stainless steel chambers where animals are housed throughout the experiment. O₃ can be monitored overtime to ensure correct concentrations.
Bronchoalveolar Lavage and Cell Differential Analysis.

A cannula was inserted into the trachea via a midline tracheotomy, the chest cavity opened via a midline thoracotomy and 9 mL of warmed (37°C) saline (pH 7.0; 310 mOsm) was gently instilled and withdrawn 3 times to yield bronchoalveolar lavage fluid (BALF) (Ballinger et al., 2005). One hundred and fifty microliters of BALF was centrifuged (StatSpin Cytofuge (StatSpin, Inc, Norwood, MA)) and set aside to dry on glass slides with 3 individual wells. Slides were stained with a modified Wright-Geimsa stain and 100 cells per well were counted for a total of 300 cells under a light microscope using characteristics unique to each cell type (Laviolette et al., 1988). Total counts were averaged for each cell type and percent of total cells were calculated. Remaining BALF was assessed for cell barrier permeability by measuring for protein using the Bradford assay (Lu et al., 2006).

Plasma Chemistries for Liver Enzymes and Histology

Blood was collected via the abdominal aorta using a heparin coated syringe and centrifuged at 4°C at 2,000xg for 10 min to obtain plasma. Alanine and aspartate aminotransferase (ALT and AST, respectively) activities were measured in plasma using a spectrophotometric assay per manufacturer’s directions (Pointe Scientific, Inc, Canton MI). Briefly, a decrease in NADH absorbance (due to NAD+ oxidation) was recorded under constant temperature conditions (37°C) over a time period of 10 min at 340 nm. The rate was measured and ALT and AST levels are reported in international units/liter (IU/L). Liver tissue was formalin fixed and paraffin embedded. Sections were mounted on slides and stained with hematoxylin and eosin (H&E). Slides were scored for injury, steatosis, and inflammation by a pathologist blinded to the experimental groups.
Two Dimension Isoelectric Focusing/SDS-PAGE (2D IEF/SDS-PAGE)

Livers were excised and homogenized in ice-cold 0.25 M sucrose, 5 mM Tris-HCl, and 1 mM EDTA, pH 7.4, containing protease inhibitors (phenylmethylsulfonyl fluoride (40 µg/mL), leupeptin (5 µg/mL), and pepstatin A (7 µg/mL) to obtain post-nuclear supernatant fractions of liver (Mantena et al., 2009). Protease inhibitors were included to prevent proteolytic degradation of samples for proteomic analyses. Post-nuclear supernatant fraction was prepared by centrifugation of liver homogenates at 568xg for 10 min at 4°C. Protein concentrations were determined using the Bradford protein assay and bovine serum albumin (BSA) as a standard (Bradford, 1976). Proteomic analyses were performed by methods described in (Andringa et al., 2010). Post-nuclear supernatant from liver homogenates (100 µg) was added to IEF gel strip rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (w/v) n-dodecyl-β-D-maltoside, 0.002% (w/v) bromophenol blue, ampholine electrophoresis reagent (Sigma, St. Louis, MO, range pH 3-10), 0.04 M DTT and 2 mM tributylphosphine. Following protein extraction, samples were applied to IEF gel strips (Invitrogen ZOOM Strips, pH 3-10, Carlsbad, CA) and rehydration of IEF strips was done overnight. For SDS-PAGE, IEF gel strips were placed horizontally on top of a 10% resolving gel with 4% stacking gel, and sealed into place using warm agarose (1%, w/v) and gels were run at 100 V for 1 ½ hr. After electrophoresis, gels were stained with Sypro Ruby™ (Invitrogen, Carlsbad, CA) for total protein. Protein stained gels were imaged using a Bio-Rad ChemiDox XRS imaging system (Bio-Rad Laboratories, Inc, Hercules, CA) (Andringa et al., 2010).
2D Gel Image Analyses

Gels were analyzed by methods described in (Andringa et al., 2010). Differences in protein density were performed using PDQuest Image Analysis software (Bio-Rad, Hercules, CA). Briefly, individual spots on 2D gels were identified using program software and visually matched for accuracy. A master gel (reference gel) was created to serve as a reference for FA and O₃ groups and the PDQuest software program uses an algorithm that automatically matches spots across all gels. Manual verification is used to correct for any incorrect spot matching to the reference gel or spots not initially detected by software. In order to correct for any differences in protein loading the density for all spots in each gel was normalized to total density in verified protein spots for that particular gel.

Protein Identification with Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI-TOF) Mass Spectrometry

Protein identification was performed as previously described (Andringa et al., 2010). Briefly proteins were excised from gels and processed using standard methods in the UAB mass spectrometry shared facility (http://www.uab.edu/proteomics). For MALDI-TOF analysis a focused laser beam was used to ionize peptides from a solid matrix into a gas phase. The resulting ions are injected into a tube, accelerated, and allowed to drift towards the detector. The time-of-flight is measured and is proportional to their molecular weight. Samples were de-stained with three successive 30 min washes in 50%, 50 mM NH₄HCO₃/50% acetonitrile solution. Samples were treated with 10 mM dithiothreitol in 50 mM NH₄HCO₃ for 60 min at 60°C to reduce cysteine residues, which
was followed by alkylation of free cysteines with 55 mM iodoacetamide in 50 mM NH$_4$HCO$_3$ for 60 min at room temperature. This was followed by 16 h incubation at 37°C with trypsin (12.5 ng/µL, Promega Gold Trypsin) to digest proteins. The resulting peptide solution was extracted by two successive 30 min washes in a 50/50 solution of 5% formic acid and acetonitrile, supernatants were, collected, and dried using a Savant SpeedVac. Lyophilized peptide samples were resuspended in 0.1% formic acid, desalted (C18 ZipTips, Millipore), and diluted 1:10 with a saturated solution of α-cyano-4-hydroxycinnamic acid matrix before application to MALDI-TOF target plates. Samples were analyzed with a Voyager De-Pro mass spectrometer in the positive mode and resulting spectra were analyzed using Voyager Explorer software. Peptide masses were submitted to the MASCOT database (see www.matrixscience.com) for protein identification.

**Western Blot**

Equal amounts of protein from post-nuclear supernatant fractions (40 or 50 µg) were loaded and separated on 12% SDS-PAGE gels then transferred for 1 hr to nitrocellulose membranes. Blots were blocked overnight in 5% (w/v) non-fat milk in Tris base saline solution with Tween-20 (TBS-T) while shaking at 5°C. Levels of heme oxygenase-1 (HO-1) protein were detected using a 1:5000 dilution of primary antibody (Stressgen, Ann Arbor, MI). Levels of cytochrome P450 2E1 (CYP2E1; Chemicon International) and 2B (CYP2B) were detected using 1:1000 primary antibody dilutions. After successive washes in TBS-T, blots were incubated in horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody (1:10,000) in 5% (w/v) non-fat milk in TBS-T
for 1 hr (Sigma, St. Louis, MO). Bands were visualized using luminol-based chemiluminescence reagents and quantification of band densities were achieved using Quantity One software (King et al., 2010). To ensure equal loading of lanes, blots were re-probed for GAPDH using 1:1000 primary antibody or a SDS polyacrylamide gel was run in tandem and stained with Sypro Ruby™.

Statistical analyses

Statistical significance was assessed using an unpaired Students’ t-test and significance was determined with a p-value < 0.05.

Results

Lung Cell Differential and BALF Protein

BALF from animals exposed to FA or O₃ was assessed for alveolar cell barrier permeability and inflammatory cell infiltration as described in Methods. BALF protein concentrations were measured using the Bradford assay. No significant change in BALF protein concentration were observed between FA and O₃ exposed groups; 63.0 ± 27.9 and 72.9 ± 46.2 µg/mL, respectively. Cell differential analysis revealed no significant changes in macrophage, lymphocyte, neutrophil, or basophil infiltration into the airway space after exposure to 5 days of O₃ compared to FA (Table 1).

Effect of O₃ exposure on plasma liver enzymes and histology

Plasma ALT and AST enzyme activities were measured as indicators of hepatocellular damage due to O₃ exposure. After 5 days of 0.5 ppm O₃ exposure AST enzyme activity levels for FA and O₃ groups were 47.2 ± 2.30 and 55.2 ± 13.2 IU/L,
respectively. Similarly, ALT enzymes activity levels for FA and O₃ groups were 48.9 ± 3.7 and 51.0 ± 8.9 IU/L, respectively. No significant changes were observed in liver enzyme activities compared to the FA group. Additionally H&E stained liver sections scored for changes to liver morphology, inflammation, and injury showed no differences between O₃ and FA groups (data not shown). These results demonstrate that inhaled O₃ at this concentration does not cause significant toxicity to liver.

**Effect of ozone exposure on liver proteome**

Representative 2D IEF-SDS-PAGE gel images from both filtered air and ozone treatment groups are shown in Figure 5. A global 2D proteomic approach was performed to determine whether O₃ inhalation could alter liver proteins. Following separation of proteins based on isoelectric point and molecular weight, proteins were analyzed for differences in abundance using PDQuest software tools as described in Methods. O₃ exposure resulted in a significant change in the expression of 23 proteins as compared to FA control. Of these 23 proteins, mass spectrometry successfully identified 10. A master map showing the location of the identified proteins and their assigned spot numbers is provided in Figure 5A. Unique protein identifications with statistical analysis and information gathered from mass spectrometry are provided in Table 2. Each spot shown in the master map matched the expected molecular weight and isoelectric point of each identified protein (Table 2). Total protein density is represented (Figure 5B) showing that changes in individual protein density were not due to global variation in gel protein loading. The 10 identified proteins are categorized into 4 broad groups; cytoskeletal, energy metabolism, drug metabolism, and protein folding/ER stress (Table 3).
**Protein folding/ER stress proteins.** Two proteins involved in ER stress were increased by O₃. Protein disulfide isomerase (PDI) increased by 32.3% and Heat shock protein 5, more commonly referred to as GRP78 or BIP, increased by 52.0% compared to protein levels measured in FA controls. PDI assists in the proper folding and disulfide bond formation of proteins within the ER (Zhang and Kaufman, 2008). GRP78, also located in the ER and a chaperone protein, plays an important role in the regulation of the unfolded protein response that is activated during ER stress (Gentile et al., 2011).

**Drug metabolism proteins.** Proteins that significantly changed in protein expression compared to FA control samples are; microsomal cytochrome b5, catechol-O-methyltransferase (COMT), and glutathione-s-transferase (GSTmu-1). Cytochrome b5 increased in expression by 42.6%. This protein is important in many P450 enzymatic reactions involved in fatty acid desaturation, metabolism of xenobiotics, and cholesterol.
Figure 8. Master map of liver proteins differentially altered by inhaled O₃. Rats were exposed to filtered air (FA) or O₃ (0.5 ppm) for 3 h/day for 5 days. After exposure, livers were removed and post-nuclear supernatant was analyzed for global protein expression using 2D gel proteomics and mass spectrometry techniques.

A. Shown above are proteins that were identified using mass spectrometry. Note that circled protein "spots" are identified and numbered in Table 2 with respective p-values.

B. Graphical representation of total protein density between FA and O₃ 2D gels showing equal protein loading across all gels. Data represent mean ± SD of n=6 animals per group.
synthesis/breakdown (Finn et al., 2011). COMT protein increased by 50% and is highly abundant in the liver. Its function is important in catalyzing the O-methylation of catechol containing molecules, which was first described in 1958 (Axelrod et al., 1958). COMT has been implicated in pathologies such as vascular disease and even Parkinson’s disease due to the high number of catecholamines present and the reactive semiquinones produced upon their metabolism (Zhu, 2002). Glutathione-s-transferase decreased in expression by 27%. GSTmu-1 is a phase II xenobiotic metabolism enzyme responsible for the conjugation of glutathione groups to endogenous and exogenous compounds for excretion (Hayes et al., 2005). This enzyme is considered protective due to its ability to conjugate reactive metabolites produced from production of reactive oxygen species (Hayes et al., 2005).

Energy metabolism and cytoskeletal proteins. Three proteins involved in energy metabolism, galactokinase, glycerol kinase, and \( \beta \)DH1 were altered by \( O_3 \). Both galactokinase and glycerol kinase increased in expression by 40.6 and 29.7%, respectively, whereas BDH significantly decreased in expression by 31.6% compared to FA controls. BDH1 is a mitochondrial protein that catalyzes the first step of ketone body metabolism, converting \( \beta \)-hydroxybutyrate to acetoacetate (Zhang et al., 1989). Cytoskeletal proteins that significantly increased were \( \beta \)-actin and \( \alpha \)-tubulin, which are responsible for cell cycle, division, and motility (Shepard and Tuma, 2010).
Effect of O₃ exposure on cytochrome P450 and HO-1 protein levels

Some of the first reports of hepatic perturbations resulting from O₃ exposure demonstrated prolonged pentobarbital-induced sleeping times in rodents, suggesting a decrease in xenobiotic metabolism (Graham et al., 1981; Graham et al., 1985). These results were demonstrated at O₃ concentrations in the high 1-3 ppm range. Proteins levels of cytochrome P450 2E1 and 2B were measured to determine if O₃ exposure altered specific drug metabolizing enzymes at environmentally relevant concentrations (0.5 ppm). CYP2E1 is a prominent inducible drug metabolizing enzyme implicated in pathology for its ability to metabolize and activate compounds such as ethanol, acetaminophen, and benzene (Lu and Cederbaum, 2008). CYP2B is one P450 isozyme known to metabolize pentobarbital (Rice et al., 1994). After 5 days of 0.5 ppm O₃ exposure, protein expression levels of CYP2E1 or CYP2B had not significantly changed as compared to FA controls (Figure 6A and B). Additionally, the stress inducible form of heme oxygenase (HO-1) was measured in this study. Analysis showed no significant difference in HO-1 protein levels between FA and O₃ groups (Figure 6C).
Figure 6. Protein levels of HO-1 and the cytochrome oxidase CYP2E1 and CYP2B in liver of O$_3$ and FA rats. Rats were exposed to filtered air (FA) or O$_3$ (0.5 ppm) for 3 hr/day for 5 days. After exposure, livers were removed and homogenized for western blot analysis for A. HO-1, B. CYP2E1, C. CYP2B, and D. GAPDH as a loading control. O$_3$ exposure did not significantly alter protein expression. Data represent mean ± SD for n=6 animals per group. HO-1, heme oxygenase-1; CYP2E1, cytochrome P450 2E1; CYP2B, cytochrome P450 2B; and GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Discussion

A new disease paradigm is emerging in the field of environmental health sciences suggesting that inhalation of environmental pollutants like O$_3$ extends beyond the pulmonary system; the initial site of exposure. Importantly, inhaled O$_3$ may be responsible for tissue injury of other organ systems like cardiovascular and hepatic systems. In support of this new concept, data show that both particulate matter (PM) and gaseous pollutants generated from industry and diesel exhaust leads to not only pulmonary injury, but also cardiovascular disease (Brook et al., 2004). Epidemiological studies also cite increased cardiovascular risk from high ambient O$_3$ days with significant changes in blood lipids, blood pressure, and factors important for glucose metabolism (Chuang et al., 2010). While human exposures to inhaled pollutants typically involve a heterogeneous mixture of PM and O$_3$, animal studies show O$_3$ alone results in cardiovascular damages and hepatic changes (Chuang et al., 2009; Laskin et al., 1994; Last et al., 2005).

The molecular events responsible for O$_3$ induced systemic effects are poorly defined. The reaction of O$_3$ within the lung is complex and results in the production of a diverse array of biologically active products. O$_3$ reacts with mono-unsaturated fatty acids to produce multiple aldehyde and hydroxyhydroperoxide species, as well as small amounts of the Criegee ozonide (Kafoury et al., 1998; Pryor et al., 1995). O$_3$ exposure in this study was done using an O$_3$ concentration of 0.5 ppm for 8 hr/day. This exposure regimen replicates a typical work day where ambient O$_3$ concentrations have increased beyond the current NAAQS standard of 0.075 ppm. While the 0.5 ppm concentration
used within this study is higher than this standard, it is important to note that much of the previous research uses even higher concentrations between 1-3 ppm O₃ (Laskin et al., 1994; Last et al., 2005). High doses of O₃ are used in experimental studies because rodents are less susceptible to O₃ than humans for a variety of reasons. It is generally accepted that because rats are obligate nose breathers and have a more complex nasopharyngeal architecture that facilitates removal of inhaled reactive gases; concentrations above those encountered during ambient exposures are needed during rodent studies. All of these factors will ultimately alter the dose of O₃ reaching the lung when compared to humans. Thus, the higher concentration used in this study is considered to compensate for these factors and have greater potential to be environmentally and biologically relevant (Cole and Freeman, 2009).

Following O₃ exposure, we did not observe any significant changes to overall protein levels or inflammatory cells within BALF extracted from lungs (Table 1). Increased protein in BALF would indicate damage to the airways and a loss of airway epithelial cell barrier integrity. It has been shown that repeated O₃ exposures result in an increase of neutrophils, lymphocytes, and macrophages. Neutrophil counts typically decrease back to baseline at 1 ppm O₃ with lymphocyte and macrophage numbers still elevated (Schelegle et al., 2003). We also did not detect any changes in plasma levels of ALT or AST enzyme activities, common measurements for hepatocellular damage. Taken together, these findings indicate that 5 days of 0.5 ppm O₃ exposure did not cause significant lung or liver injury. When we measured the protein levels of CYP2E1 and 2B and HO-1 we were not able to detect significant changes. As previously mentioned,
earlier work showed that rodents exposed to O$_3$ had altered drug clearance activities as reported by an increase in pentobarbital-induced sleeping times (Graham et al., 1981). We chose to measure CYP2E1 and 2B since it has been shown that CYP2E1 is an inducible enzyme important in metabolism and activation of a number of endogenous and exogenous environmental compounds, and CYP2B is induced by and metabolizes pentobarbital. Our data does not support earlier research showing alterations in pentobarbital-induced sleeping times (Graham et al., 1981) since we did not see a changes in CYP2B protein levels. However, it does support data showing no change in gene expression for these two CYP enzymes following inhaled O$_3$ exposure (Last et al., 2005). The absence of any change in HO-1 expression could be anticipated considering its up-regulation typically occurs very early in response to stress. HO-1 is the inducible form of the heme-oxygenase enzyme family. Only one time point was measure in this study, thus if O$_3$ exposure elicits an early hepatic stress response (e.g., HO-1 induction) any time before 5 days it may have been missed.

Despite the absence of overt lung and liver injury, and any significant changes in select xenobiotic enzymes measured in this study, O$_3$ exposure resulted in alterations to other proteins in the liver as evidenced by our proteomics analyses. Considering only a few studies have investigated the effects of inhaled O$_3$ on the liver (Graham et al., 1981; Laskin et al., 1994; Last et al., 2005) we took a global proteomic approach to determine whether O$_3$ exposure alters expression of liver proteins. Using 2D gel electrophoresis techniques and mass spectrometry we were able to successfully identify 10 proteins that were significantly altered in O$_3$ treatment groups.
These proteins were grouped into 4 different categories; cytoskeletal, drug metabolism, energy metabolism, and ER stress-related proteins with 8 proteins significantly increased and 2 decreased. One of the most intriguing changes observed from our mass spectrometry data is the increase of PDI and GRP78. Both proteins are located in the endoplasmic reticulum and are involved in the proper folding of proteins and maintenance of ER homeostasis. The ER is a complex membranous network acting as the site for proper folding and maturation of newly synthesized proteins. It is also sensitive to various signals and tightly regulated in order to maintain proper protein and lipid homeostasis. One such signal is the over accumulation of malfolded proteins, which activates the unfolded protein response (UPR). GRP78 binds all three proteins responsible for the activation of UPR (IRE1α, ATF6, and PERK) rendering the signaling pathway inactive (Zhang and Kaufman, 2008). It is postulated that GRP78 is released from the previous three proteins responsible for UPR activation and is sequestered in the ER lumen where it binds malfolded proteins and subsequently the UPR is activated (Rutkowski and Kaufman, 2004). An upregulation of GRP78 suggests that a mild form of ER stress occurred at our 5 day time point in order to bring the ER lumen back to homeostasis. Indeed, overexpression of GRP78 protects cells from known stressors such as high fat diet alleviating injury and promoting restoration of ER homeostasis (Kammoun et al., 2009; Ye et al., 2010). Additionally, studies investigating the effect of PM exposure show induction of ER stress pathways in the liver of animal models with pre-existing liver disease (Laing et al., 2010).
Despite the absence of an alteration in CYP2E1 and 2B we did observe changes in other drug metabolism enzymes. The glutathione S-transferase family are phase II drug metabolism enzymes and GSTm1 is one of 7 GST mu isoforms (Hayes et al., 2005). Substrates for cytosolic GSTs include, but are not limited to environmental chemicals, like acrolein and DDT, xenobiotics and endogenous reactive lipid species (Hayes and McLellan, 1999). Conjugation of GSH with many of these compounds produce less reactive products that are subsequently excreted thus ameliorating cellular oxidative stress. GST enzymes are typically increased in response to stress and considered a conserved adaptive response as it occurs throughout the animal kingdom (Leiers et al., 2003). In this study, GSTm1 protein was decreased by 27.5%. Animal models null for GSTmu1 display altered drug clearance (Fujimoto et al., 2006). In the context of this study decreased GSTmu1 protein could present an inability to compensate for increased stress or an altered ability to clear exogenous and endogenous compounds that GSTmu1 is responsible for in populations co-exposed to O₃. In fact, a proteomic analysis of alcohol-induced liver injury found that chronic ethanol consumption in a rodent model increased GSTmu1 but in an ethanol binge model GSTmu1 did not continue to elevate and instead decreased, perhaps indicating an inability to compensate for the injury (Aroor et al., 2012). We also saw a 50% increase in COMT protein levels. COMT, a phase II drug metabolism enzyme, will O-methylate catechol-containing compounds including dopamine and epinephrine but also exogenous catechol-containing dietary phytochemicals and metabolites of toxic aryl hydrocarbons (Zhu, 2002). Increases in COMT may also be protective because accumulation of endogenous catecholamines can spontaneously or enzymatically be converted to semiquinone/quionone intermediates that
are cytotoxic (Stokes et al., 1999; Zhu, 2002).

Other identified proteins in this study that have been associated in pathology are the two cytoskeleton proteins β-actin and α-tubulin. Adduction of these proteins is seen in alcohol-induced liver injury mainly by interaction with acetaldehyde and compromising the cytoskeletal network in hepatocytes (Shepard and Tuma, 2010). Increases in both transcript and protein were found to be associated in an animal model of pancreatic injury (Zhong and Omary, 2004). It was postulated that this may play a cytoprotective role by increasing the amount of cytoskeletal protein in order to replace damaged or modified β-actin and α-tubulin in the cell. Taken together, these results demonstrate that inhaled O₃ initiates reactions that alter protein expression patterns in an organ (liver) distal from initial site of exposure.

With the discovery that environmental O₃ can elicit effects that extend beyond the pulmonary system it is important to understand how these effects on multiple organ systems may impact health. Importantly, the systemic effects observed with O₃ alone may be even greater when coupled to pre-existing diseases and pathologies. Animals predisposed to heart disease display greater vascular injury when exposed to O₃ (Chuang et al., 2009). Rodents administered acetaminophen, a known hepatotoxicant, show increased liver injury following O₃ exposure (Aibo et al., 2010). Also, genetic predisposition to obesity in multiple animal models increases airway hypersensitivity and inflammatory cytokines as compared to unexposed obese controls (Shore et al., 2009; Shore et al., 2003). Thus, the current study highlights that additional susceptible
populations to O$_3$ may exist outside of those with pre-existing pulmonary disease and include individuals with pre-existing cardiovascular disease, liver disease, or manifestations of the cardiometabolic syndrome. Future studies will be aimed to investigate the O$_3$-dependent mechanisms causing changes in the liver proteome, as well as, elucidating how O$_3$ may exacerbate underlying conditions such as fatty liver disease.

Acknowledgments

This work was supported by NIH grants R01AA15172 and R01AA18841 to SMB and ES P01 111617 and HL R01 54696. The authors would also like to thank Dr. S. Barnes and Mr. L. Wilson of the UAB Mass Spectrometry Shared Facility. Mass spectrometers in the Shared Facility came from funds provided by NCRR grants S10RR11329, S10RR13795 plus UAB HSF General Endowment Fund. Operational funds came from UAB Comprehensive Cancer Center Core Grant (P30CA13148), Purdue-UAB Botanicals Center for Age-Related Disease (P50AT00477), UAB Center for Nutrient-Gene Interaction in Cancer Prevention (U54CA100949), UAB Skin Disease Research Center (P30AR050948), and UAB Polycystic Kidney Disease Center (P30DK74038).
### Table 1: Bronchoalveolar lavage cell differential analysis

<table>
<thead>
<tr>
<th></th>
<th>Filtered Air</th>
<th>Ozone</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume recovered (mL)</td>
<td>6.54 ± 0.52</td>
<td>6.23 ± 0.48</td>
<td>0.32</td>
</tr>
<tr>
<td>% yield</td>
<td>73 ± 7.00</td>
<td>69 ± 7.00</td>
<td>0.32</td>
</tr>
<tr>
<td>Cell count*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>99.2 ± 0.76</td>
<td>97.5 ± 1.95</td>
<td>0.1</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>1.30 ± 1.37</td>
<td>2.20 ± 0.68</td>
<td>0.75</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.33 ± 0.82</td>
<td>0.42 ± 1.90</td>
<td>0.23</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.00 ± 0.00</td>
<td>0.17 ± 0.40</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Lavage fluid was recovered as described in Methods. *Cell counts were generated using the average of 3 independent counts of 100 total cells/count and are represented as a percentage of total counted cells.
Table 2: Hepatic proteins significantly altered in abundance as a result of 0.5 ppm O$_3$ exposure: results from 2D-IEF SDS PAGE and mass spectrometry.

<table>
<thead>
<tr>
<th>Spot #</th>
<th>Protein Identification</th>
<th>Mass (kD)</th>
<th>Isoelectric point (pI)</th>
<th>% Change</th>
<th>p-value</th>
<th>MOWSE score</th>
<th>Peptides matched</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytochrome b5 type A (microsomal)</td>
<td>11.4</td>
<td>5.26</td>
<td>41.6</td>
<td>0.038</td>
<td>166</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Catechol-O-methyltransferase (COMT)</td>
<td>29.8</td>
<td>5.41</td>
<td>50.9</td>
<td>0.025</td>
<td>132</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Galactokinase 1 (Galk1)</td>
<td>42.8</td>
<td>5.24</td>
<td>29.7</td>
<td>0.036</td>
<td>286</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>Actin, beta like 2</td>
<td>42.2</td>
<td>5.3</td>
<td>20.6</td>
<td>0.014</td>
<td>277</td>
<td>13</td>
</tr>
<tr>
<td>5</td>
<td>Protein disulfide-isomerase</td>
<td>57.3</td>
<td>4.82</td>
<td>32.3</td>
<td>0.002</td>
<td>784</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td>Tubulin, alpha 1C</td>
<td>50.6</td>
<td>4.96</td>
<td>41.1</td>
<td>0.003</td>
<td>336</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>Heat shock protein 5 (Grp78/BiP)</td>
<td>72.5</td>
<td>5.07</td>
<td>52</td>
<td>0.002</td>
<td>467</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>Glycerol kinase (GLPK)</td>
<td>58.2</td>
<td>5.49</td>
<td>40.6</td>
<td>0.01</td>
<td>220</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>Glutathione S-transferase mu 1</td>
<td>25.8</td>
<td>8.27</td>
<td>-27.5</td>
<td>0.021</td>
<td>350</td>
<td>15</td>
</tr>
<tr>
<td>10</td>
<td>D-β-hydroxybutyrate dehydrogenase, type 1 (Bdh1)</td>
<td>38.7</td>
<td>8.93</td>
<td>-31.6</td>
<td>0.033</td>
<td>187</td>
<td>8</td>
</tr>
</tbody>
</table>

Proteins identified as significantly altered were matched on all Filtered Air (FA) and Ozone (O$_3$) 2D/IEF/SDS-PAGE gels. Spot # is the same number used to identify circled proteins in Figure 5A. Data represents p-values determined using a Student's t-test.
<table>
<thead>
<tr>
<th>Protein Identification</th>
<th>Accession #</th>
<th>Pathway</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b5</td>
<td>AAB67609</td>
<td>Drug metabolism</td>
<td>Cypb5 mediates the rate of P450 dependent mono-oxygenation reactions through the transfer of the second electron from NADPH.</td>
</tr>
<tr>
<td>Catechol-O-methyltransferase</td>
<td>NP_036663</td>
<td>Drug metabolism/L-dopa metabolism</td>
<td>COMT enzymatically O-methylates catechol containing compounds. Both over expression and loss of activity has been implicated in vascular diseases as well as neuronal disorders.</td>
</tr>
<tr>
<td>Galactokinase 1 (Galk1)</td>
<td>NP_001008283</td>
<td>Cell cycle, energy metabolism</td>
<td>First enzyme required for the conversion of galactose to glucose forming galactose-1-phosphate.</td>
</tr>
<tr>
<td>Actin, beta like 2</td>
<td>NP_001099879</td>
<td>Cytoskeletal</td>
<td>Important for cell motility, structure and integrity.</td>
</tr>
<tr>
<td>Protein disulfide-isomerase</td>
<td>PO4785</td>
<td>Protein Folding/ER stress</td>
<td>PDI is responsible for inter and intra molecular disulfide bond formation within the endoplasmic reticulum.</td>
</tr>
<tr>
<td>Tubulin, alpha 1C</td>
<td>NP_001011995</td>
<td>Cytoskeletal</td>
<td>Alpha tubulin is one of two components of tubulin which is assembled to form microtubules. These are important for cell movement and maintenance of shape.</td>
</tr>
<tr>
<td>Heat shock protein 5 (GRP78/BiP)</td>
<td>NP_037215</td>
<td>Protein Folding/ER stress</td>
<td>Located within the ER; GRP78/BiP binds and inactivates the proteins responsible for UPR activation. Also directly binds misfolded proteins to assist in proper folding or clearance.</td>
</tr>
<tr>
<td>Glycerol kinase (GLPK)</td>
<td>Q63060</td>
<td>Energy metabolism</td>
<td>Catalyzes formation of glycerol 3-phosphate. Overexpression in H4IIE cells resulted in increased fat storage and alters activity of PPAR-alpha and other transcription factors (Sriram et al., 2010)</td>
</tr>
<tr>
<td>Glutathione S-transferase mu 1</td>
<td>AAA41286</td>
<td>Phase II drug metabolism</td>
<td>Facilitates conjugation of reduced glutathione to electrophilic compounds for increased excretion.</td>
</tr>
<tr>
<td>D-β-hydroxybutyrate dehydrogenase</td>
<td>AAB59684</td>
<td>Energy/lipid metabolism</td>
<td>Oxidoreductase, mitochondrial. Involved in fatty acid catabolism. BDH1 facilitates formation of the ketone body 3-hydroxybutyrate.</td>
</tr>
</tbody>
</table>
References


CONCLUSION

Inflammation and the Unfolded Protein Response

Data suggest that O$_3$-related events stemming from the lung that affect systemic organ systems are associated with increased inflammatory mediators. It has been shown that O$_3$ exposure increases liver NO production and interferon gene transcripts in animal models (Laskin et al., 1994; Last et al., 2005). Inflammation is important because it is designed to help combat, limit, and eliminate stress and tissue injury caused by irritants and pathogens sensed by cells. Cells induce inflammatory processes and mediators in order to clear the pathogen and/or upregulate proteins that will clear modified and damaged proteins (Charo and Ransohoff, 2006). However, chronic inflammation is detrimental and many times will increase the initial encountered stress. Several pathologies are associated with chronic inflammation such as arthritis (Harama et al., 2009), Alzheimer’s disease (Salminen et al., 2009), cardiovascular disease (Hansson and Libby, 2006), and diabetes (Hotamisligil, 2006). Several research groups have shown that addition of O$_3$ exposure to pre-existing pathologies can exacerbate disease and increase tissue injury. For example, O$_3$ exposure increases liver and serum pro-inflammatory mediators in a model of acetaminophen induced hepatotoxicity (Aibo et al., 2010). In a model of atherosclerosis, O$_3$ exposure decreases antioxidant enzymes and increases mitochondrial DNA damage (Chuang et al., 2009). Genetically obese rodents
display greater increases of hyperreactivity in response to methacholine and pro-inflammatory mediators as compared to non-treated obese counterparts (Shore et al., 2003). All of these data lead to the hypothesis that O₃ inhalation induces an inflammatory response that further affects organ systems downstream of the respiratory system. Furthermore, those pathologies that include chronic inflammation as a contributing factor to the severity of disease may worsen with the additional exposure to an oxidant such as O₃.

The results from this thesis project show an increase in liver proteins associated with the endoplasmic reticulum and activation of the unfolded protein response (UPR), particularly protein disulfide isomerase and GRP78 following inhalation of O₃. The endoplasmic reticulum is a complex membranous bilayer network that is important for posttranslational modifications and proper folding of newly synthesized proteins. The ER is also sensitive to environmental cues and changes in homeostasis. The ER responds to changes and initiates signaling cascades that are communicated to the cytoplasm and nucleus. In the ER there are three transmembrane proteins that are sensitive to these homeostatic changes; inositol-requiring 1α (IRE1α), double stranded RNA dependent protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Gentile et al., 2011). These proteins have been identified as activators of the UPR (Schröder and Kaufman, 2005). Under normal conditions these proteins remain inactive through the association with GRP78 (Liu et al., 2003; Shen et al., 2002). However, when the cell encounters stress and accumulation of misfolded proteins occurs it is hypothesized that GRP78 has higher affinity for the misfolded proteins and thus allows for the
activation of the UPR (Zhang and Kaufman, 2008). Once disassociated from GRP78, PERK and IRE1α homodimerize and autophosphorylate. ATF6 translocates to the Golgi where it is cleaved and activated (Gentile et al., 2011).

It is thought that the UPR is an adaptive mechanism with the purpose of alleviating the stress and bringing the cell back to homeostasis. The ER accomplishes this by: 1) activating pathways that decrease overall protein synthesis so that there isn’t a demand for protein folding (Zhang and Kaufman, 2008) and 2) increasing the volume of the ER lumen to diffuse the encountered stress (Gentile et al., 2011). The activation of PERK results in the phosphorylation of translation initiation factor α (eIF2α). Phosphorylated eIF2α limits the production of new peptides by inhibiting transcription ultimately decreasing the ER burden (Harding et al., 2000). IRE1α when active leads to a translational frameshift in X-box-binding protein 1 (XBP1). An active XBP1 translocates to the nucleus to upregulate genes to assist in protein folding and degradation (Ron and Walter, 2007). Cleaved ATF6 in the Golgi then translocates to the nucleus to act in similar ways as XBP1 by upregulating UPR target genes. However, chronic activation of the system is associated with increased inflammatory mediators (Zhang and Kaufman, 2008), uncontrolled accumulation of ROS (Tu and Weissman, 2002), and cell death through apoptosis (Schröder and Kaufman, 2005).

**Effect of Ozone Exposure on Mitochondrial Respiration**

As mentioned in the previous section, chronic ER stress can initiate a number of processes that would increase cellular injury. Increases in pro-inflammatory mediators,
release of Ca\(^{2+}\) stores, and ROS production can adversely affect mitochondria. ER stress can result in the release of Ca\(^{2+}\), which would be taken up by mitochondria and concentrated in the matrix. Excessive Ca\(^{2+}\) uptake can alter mitochondrial membrane potential resulting in a disruption of the electron transport chain and ROS production (Deniaud et al., 2008). The ER is also a site of ROS production. Disulfide bond formation involves protein disulfide isomerase (PDI) which accepts an electron. Another ER protein, ER oxidoreductin 1 (ERO1) assists in the transfer of the electron accepted by PDI to molecular oxygen (Tu and Weissman, 2002). During ER stress this could result in an uncontrolled production of ROS and further fuel UPR, inflammation and mitochondrial damage. Because the ER and mitochondria are closely linked, a preliminary investigation was done to determine whether O\(_3\) inhalation caused any changes in mitochondrial function. To determine O\(_3\)-induced mitochondrial effects, oxygen consumption was measured using isolated mitochondria from rat liver.

Using a separate cohort of animals, male Sprague-Dawley rats were exposed to 0.8 ppm O\(_3\) for 1, 3, or 5 days for 8 hr/day. Using the methods described in Chapter 2 animals were sacrificed and livers were removed and homogenized. Additional centrifugation steps were taken to isolate mitochondria from post-nuclear supernatant (PNS). Briefly PNS was centrifuged for at 8900 rpm for 10 min to obtain the mitochondrial fraction. The pellet was repeatedly resuspended, homogenized using a smooth Teflon pestle, and centrifuged to increase mitochondrial yield. Following final centrifugation mitochondria were resuspended a final time and protein was measured using the Bradford assay with bovine serum albumin as the standard. Using techniques
described previously (King et al., 2010), mitochondrial respiration was measured using the Clark-type oxygen electrode. State 3 (ADP-dependent) and state 4 (ADP-independent) respiration was measured using glutamate/malate or succinate/rotenone as oxidizable substrates.

State 3 respiration decreased after 1 day of 0.8 ppm O\textsubscript{3} exposure with the largest decrease occurring on day 3. After 5 days of O\textsubscript{3} exposure mitochondrial respiration did not only increase back to baseline but was greater than filtered air control. This trend in state 3 respiration was observed with both substrates used (Figure 7A and B). State 4 respiration also decreased after 1 day of 0.8 ppm O\textsubscript{3} exposure. However, on day 3 respiration had increased back to FA control and was greater than the FA control on day 5 with both glutamate/malate and succinate (Figure 7C and D). Statistical analyses of these data could not be performed as there were only 2 animals exposed to O\textsubscript{3} on each time point. However, if these results are representative they may indicate that O\textsubscript{3} exposure is resulting in an oxidative stress and thus decreasing mitochondrial respiration between day 1 and 5. This phenomenon would have been missed in our initial study as exposures continued to the fifth day before harvesting tissues.
Figure 7: Effect of O₃ on isolated liver mitochondrial respiration. Isolated mitochondria from animals exposed to 0.8 ppm O₃ for 8 h/day for 1, 3, or 5 days. Oxygen consumption was measured using glutamate/malate and succinate as oxidizable substrates. ADP was added and state 3 was measured as a rate A&C. State 3 and 4 respiration measured using the substrates glutamate and malate. B&D: State 3 and 4 respiration measured using the substrate succinate. Data represent an N=2 for O₃ exposed animals for each time point and FA animals is an average of 3 animals.
Decreases in mitochondrial respiration suggest a defect in the ability of the mitochondrion to shuttle electrons through the electron transport chain and effectively make ATP. This can lead to an increase in reactive oxygen species in the form of superoxide (O$_2^-$) at complexes I and III and increase cellular stress and damage. Glutamate and malate donate electrons to complex I (NADH dehydrogenase) while complex II (succinate dehydrogenase) accepts electrons from the oxidation of succinate to fumarate. Decreases in state 3 respiration or ADP-dependant respiration would suggest a defect in electron transport capabilities of the complexes. State 4 respiration is oxygen consumption in the absence of ADP. Increases in state 4 respiration indicate mitochondria membranes are leaky to protons and that they are less tightly coupled and are thus less efficient at making ATP. Impairments in the electron transport chain as seen through state 3 and 4 respiration could indicate an inability to make enough ATP in the presence of liver injury from toxicants like O$_3$.

Future Directions

Considering that proteins involved in ER stress may interact with mitochondria, more experiments are needed in order to determine if these effects are dose and time dependent and if different cellular processes are changed when the exposure regimen changes. Future experiments need to consider this and couple O$_3$ inhalation with a pre-existing pathology. Studies investigating the inhalation of other environmental pollutants like PM$_{2.5}$ on insulin resistance and adipocyte alterations provide insight into modeling future O$_3$ induced systemic effects experiments. This becomes even more relevant when
one considers the percentage of the population suffering from insulin resistance and other conditions of the cardiometabolic syndrome. Many studies today center around the notion that most pathologies do not exist because of a single exposure but instead pathologies are due to the combination of multiple exposures, lifestyle, and genetic make-up of individuals. A classic example of this is non-alcoholic fatty liver disease (NAFLD). Moreover, the activation of UPR and ER stress as described above is implicated in the pathogenesis of NAFLD particularly the development of steatosis (Gentile et al., 2011). The onset of NAFLD in the presence of O₃ which increased proteins associated with the UPR may exaggerate the disease.

Liver disease is among the top causes of death in the U.S. and NAFLD is largely considered a leading cause of liver dysfunction and cirrhosis (Gentile et al., 2011). Obesity is strongly correlated with NAFLD and studies diagnosing NAFLD in selected populations have shown that the percentage of patients who are obese and present with NAFLD is significant (Angulo and Lindor, 2002; Silverman et al., 1990).

NAFLD is a spectrum of liver disorders that develops in the absence of any other documentable liver diseases or significant alcohol consumption (Festi et al., 2004). Thorough clinical examination is required to rule out fatty liver disease due to alcohol consumption. In order to rule out alcoholic liver disease, alcohol consumption must be less than 40 g/day for a male and less than 20 g/day for a female (Neuschwander-Tetri and Caldwell, 2003). The initial stage of NAFLD, steatosis, is caused by increased accumulation of triglycerides in hepatocytes; the most abundant cell type in the liver.
Steatosis is considered a reversible stage of the disease and can be tolerated by patients. However, it is proposed that steatosis sensitizes or primes the liver for further injury such that a certain percentage of the population with NAFLD (i.e., steatosis) can progress to non-alcoholic steatohepatitis (NASH) (Brunt and Tiniakos, 2010). NASH is the continued accumulation of triglycerides in hepatocytes but is combined with infiltration of inflammatory cells (lymphocytes, eosinophils, and neutrophils) in the liver. If left undiagnosed, NASH, can further progress to fibrosis, which is defined by increased production of extracellular matrix due to hepatic stellate cell activation, and subsequent development of cirrhosis, the end stage of the disease. Importantly, it is now widely accepted that NAFLD is a manifestation of the cardiometabolic syndrome.

Progression of steatosis to NASH is believed to require a second insult; which forms the basis of the “two or multi-hit hypothesis”. The 1st “hit” is the development of steatosis. Triglyceride accumulation can occur due to increased transport of fatty acids to the liver, increased de novo synthesis of fatty acids, and decreased beta oxidation of fatty acids; largely due to insulin resistance (Mantena et al., 2008). Factors such as, diet, diabetes, obesity, and/or development of insulin resistance contributes to hepatic steatosis. The 2nd insult progresses simple steatosis to NASH. These insults may include an increase in oxidative stress mediators (reactive oxygen, nitrogen, and lipid species – ROS/RNS/RLS), inflammation, or environmental stressors. Indeed, several recent studies show that environmental pollutants like diesel exhaust particles (Sun et al., 2009; Tan et al., 2009; Xu et al., 2010) and environmental tobacco smoke increase hepatic steatosis (Bailey et al., 2009).
Summary

Based on the increasing volume of data suggesting cardiovascular and hepatic events related to ozone exposure we chose to investigate the effect of inhaled O₃ on the liver. Since there are only a handful of studies that have examined hepatic effects from O₃ inhalation we employed a global proteomic approach. We also chose to examine xenobiotic enzymes because of early research observing increased action of the anesthetic pentobarbital. From these experiments we found two enzymes that were important in ER homeostasis; PDI and GRP78. While the effects noted in this study were small we believe that the changes observed could be exaggerated when combined with pre-existing pathology such as NAFLD. Thus, future experiments are needed to combine NAFLD with multiple exposure times and concentrations of O₃ in order to determine if the changes in this study become even more exaggerated and allow for observations that occur earlier or later in exposure duration. This could lead to information showing that there are subpopulations that were once not considered at risk or susceptible to increased ambient O₃ concentrations.
References


NOTICE OF RENEWAL

DATE: March 24, 2011

TO: EDWARD M POSTLETHWAIT, Ph.D.
    RPHB-530 0022
    FAX: (205) 975-6341

FROM: Judith A. Kapp, Ph.D., Chair
      Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: PPG: Mechanisms of Species-Dependent Environmental Lung Injury (Dr. Postlethwait); Project # 1: Biochemical Determinants of Local Dose Govern 03 Toxicity
         Sponsor: NIH
         Animal Project Number: 110408062

As of April 27, 2011, the animal use proposed in the above referenced application is renewed. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>A</td>
<td>75</td>
</tr>
<tr>
<td>Mice</td>
<td>B</td>
<td>200</td>
</tr>
<tr>
<td>Rats</td>
<td>B</td>
<td>50</td>
</tr>
</tbody>
</table>

Animal use must be renewed by April 26, 2012. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 110400062 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.
MEMORANDUM

DATE: March 24, 2011

TO: EDWARD M POSTLETHWAIT, Ph.D.
RPHB-530 0022
FAX: (205) 975-6341

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on March 24, 2011.

Title of Application: PPG: Mechanisms of Species-Dependent Environmental Lung Injury (Dr. Postlethwait), Project # 1: Biochemical Determinants of Local Dose Govern 03 Toxicity

Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).