# Using Smog Chambers to Estimate the Toxic Effects of Reactive Atmospheric Mixtures

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# ABSTRACT

Melanie Doyle: Using Smog Chambers to Estimate the Toxic Effects of Reactive Atmospheric Mixtures
(Under the direction of Harvey Jeffries)

We live in a dynamic environment with atmospheric pollutants constantly transforming, interacting with one another, and generating secondary pollutants. Many of these secondary pollutants have not been identified and, because they are often more oxygenated, many are more toxic than their parent compounds. Continuous emissions from biogenic and anthropogenic sources into this reactive environment create problematic conditions for evaluating the respiratory toxicity of exposure to individual components of urban atmospheres. While previous investigations have studied individual atmospheric components of air pollution, the evaluation of "one atmosphere" effects has been limited by experimental complexities. In this work, new techniques were developed to create an air-liquid interface exposure system coupled with a controllable atmospheric reactor, or "smog chamber", and these were used to examine various reactive atmospheric mixtures using a laboratory setting that still mimicked the outdoor environment. This smog chamber – *in vitro* exposure system combines common techniques used in classic toxicology with an outdoor environmental chamber system that was developed to investigate chemical reaction mechanisms.

This dissertation is divided into three main parts that demonstrate new methods to study reactive atmospheric pollutants utilizing the smog chamber – *in vitro* exposure system. In the first part, 1,3-butadiene and isoprene were used to evaluate the differences in respiratory toxicity between unreacted parent pollutants and their complete mixture of products generated during photochemical transformations. The second part applied similar techniques to differentiate the roles that specific photochemical products play in the induction of toxicity mediators; in particular, the role of ozone effects compared to the other known, first generation products. In addition to determining the effects induced by product mixtures generated during photochemical transformations, understanding which products induce the greatest overall effect is particularly helpful when regulating ambient pollutants. In the final part, a preconditioning method was developed to ascertain if repeated ozone exposures modify the respiratory effects induced by subsequent challenges to atmospheric mixtures. Combined, these results characterize and estimate the toxic potential of a realistic, complex, reacting, polluted atmosphere.

*To my parents Darrell and Carolyn, my sister Dara, and my boyfriend Tim. Thank you for all your love, ever-lasting support and continuous encouragement. This was a very hard few years… a tough journey to which this path ends here. New roads lie ahead. No words could express my gratitude.*
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# TABLE OF CONTENTS

# List of Tables

# List of Figures

# List of Abbreviations

| | |
|---|---|
| Acet | acetaldehyde |
| ACR | acrolein |
| ANOVA | analysis of variance |
| BD | butadiene |
| BSA | bovine serum albumin |
| BSO | buthionine sulphoximine |
| C | Celsius |
| $CCl_4$ | carbon tetrachloride |
| CO | carbon monoxide |
| CO2 | carbon dioxide |
| ECD | electron capture detector |
| EDT | Eastern Daylight Time |
| ELISA | Enzyme Linked Immuno Absorbant Assay |
| EPA | Environmental Protection Agency |
| F12K | Hams F-12 serum |
| FBS | fetal bovine serum |
| FEP | fluorinated ethylene propylene |
| FGF2 | fibroblast growth factor 2 |
| FID | flame ionization detector |
| Form | formaldehyde |
| GC | gas chromatography |
| GM-CSF | granulocyte-monocyte-colony stimulating factor |
| GSH | glutathione |
| GSH-ET | glutathione reduced ethyl ester |
| HAP | hazardous air pollutant |
| HC | hydrocarbon |
| HPLC | high pressure liquid chromatography |
| IL-1 | human interleukin 1 |
| IL-10 | human interleukin 10 |
| IL-6 | human interleukin 6 |
| IL-8 | human interleukin 8 |
| IR | infrared |
| ISO | isoprene |
| ISO MON | isoprene monoxide |
| LDH | lactate dehydrogenase |
| LDT | local daylight time |
| MACR | methacrolein |
| MCP-1 | monocyte chemotactic protein 1 |
| METH | methanol |

| | |
|---|---|
| mRNA | messenger ribonucleic acid |
| MS | mass spectrometry |
| MVK | methyl vinyl ketone |
| NO | nitrogen monoxide |
| $NO_2$ | nitrogen dioxide |
| NOx | nitrous oxide |
| NTP | National Toxicology Program |
| $O_3$ | ozone |
| OH | hydroxyl radical |
| OSHA | Occupational Safety and Hazards Administration |
| PAN | peroxyacetyl nitrate |
| PFBHA | O-(1,2,3,4,5-pentafluorobenzyl) hydroxylamine hydrochloride |
| ppb | parts per billion |
| ppbV | parts per billion volume |
| ppm | parts per million |
| ppmC | parts per million carbon |
| PS | Penicillin Streptomycin |
| RT-PCR | reverse transcription polymerase chain reaction |
| SCTS | smog chamber toxicology system |
| TNF-α | tumor necrosis factor-alpha |
| TOL | toluene |
| UNC-CH | University of North Carolina- Chapel Hill |
| UV | ultraviolet |
| VEGF | vascular endothelial growth factor |
| VOC | volatile organic carbon |
| WHO | World Health Organization |

# 1. Study Overview

Air pollution is a significant health concern in the United States with a primary focus being the effects due to the reactive urban environment. The overall goal of this project was to combine, develop, and demonstrate new experimental techniques that can advance and prioritize the toxicologic study of pollutants found in the atmosphere. The fundamental idea is that compounds identified as hazardous air pollutants (HAPs) are not just emitted from various sources but can also be created and destroyed in the atmosphere. Thus, HAPs undergo various chemical reactions in the atmosphere that modify their chemical composition, properties, and toxicity. Here, environmental irradiative chambers were used to study toxicities of both chemicals emitted into the ambient air as well as their photochemical transformation products using real-time *in vitro* lung cell exposures. The primary toxicological endpoints evaluated were cytotoxicity and inflammatory gene expression.

To further analyze the effects of specific components of the inflammatory response, this approach was used to create composite or blended realistic atmospheric mixtures that could discriminate effects caused by the different photochemical transformation products, that is, compare the effects of only ozone, or ozone and acrolein, or ozone, acrolein, and methacrolein, or these and formaldehyde. To examine the mechanism of response, glutathione was blocked as a way to determine if there was a change in oxidative stress effects. The purpose of broadening the scope of the evaluation beyond the effects of ozone was to demonstrate the importance of studying not only the

ozone generated during pollutant episodes and its adverse effects, but also the other possibly more toxic components that are created simultaneously. Finally, this analysis evaluates possible effects, synergistic or antagonistic, induced from exposure to mixtures of atmospheric pollutants with known composition.

Photochemical air pollution is a heterogeneous mixture of different pollutants that individuals are continuously exposed to throughout there life. Previous experiences can influence subsequent responses individuals have to later exposures. To study these health effects subsequent exposures must be considered. In addition, individuals may exhibit either a sensitization or desensitization dependent upon their previous exposure history, including the concentration, duration and time in their life in which they were exposed. A method of preconditioning or multiple exposure analysis using human respiratory epithelial cells, with a repeated exposure protocol, was developed to model exposures of HAPs mixtures or different levels of single pollutants such as ozone, that are commonly present in urban smog, thus examining the response from repetitive, innate pollutant exposures.

# 2. Background of the Problem

## *2.1. Rationale*

The Greeks and Romans recognized that adverse health effects could be caused by many different types of air pollution. In recent times, regulations and restrictions have alleviated many of the worst problems. Nevertheless, in the United States, "In spite of these controls, air pollution remains a current problem" (Lumb 2005). Inhalation is the major route by which individuals are exposed to air pollutants. Once these compounds have entered the respiratory tract, they may be absorbed and interact with lung cells or the surrounding tissues (Castell et al. 2005). Classic methods to study air pollution, particularly the adverse effects of hazardous air pollutants (HAPs), include the use of epidemiology, human clinical studies, and animal and cellular toxicology studies. Each of these strategies has strengths and weaknesses that impact the effectiveness to advance knowledge. HAPs (also known as air toxics) are those pollutants that cause or may cause cancer or other serious health effects. Most HAPs originate from anthropogenic sources; however some are uncontrollable or have biogenic sources. Many HAPs are organics, or are classified as volatile organic compounds (VOCs).

While the best model to study the effects of air pollution is a holistic setting with both healthy and susceptible individuals, "*in vitro* tests have become an essential part of an integrated toxicity testing strategy" (Pauluhn 2005). These provide "a degree of experimental control" that has the potential for providing detailed information about

complex mixtures that is not possible with epidemiology or clinical studies, including determining the toxicokinetics and interactions causing the effects (Pauluhn 2005). Although *in vitro* results are sometimes harder to translate into human health risks, "their advantage is that many of the specific interactions occurring in complex mixtures can be identified at a technically less demanding and more step-wise level when compared to the costly and technically demanding *in vivo* inhalation studies" (Pauluhn 2005). Along with such advantages, however, there are limitations when using *in vitro* models. Most importantly is that the cells are "removed from their normal environment" therefore taking away interactions with neighboring cells and blood supply that may play important roles in the protection or exacerbation of toxicity (Devlin et al. 2005).

The majority of current studies are aimed at understanding potential health effects of urban air pollution by examining the adverse effects of the individual chemicals emitted, and are focused mostly on primary pollutants or simple mixtures of these. This limits the investigation of a most important class of air toxics: those that are generated in the atmosphere via photochemistry, also called secondary air pollutants. A common and important distinction for these products is that their chemical identities are often unknown. But, whatever they actually are, it is also likely that they are more toxic than their parent compound. In addition, individuals exposed to ambient HAPs are rarely exposed to only one pollutant at a time; therefore, the overall health effects of complex mixtures of hazardous air pollutants needs to be investigated in further detail. There is little understanding of the risk to human health via co-exposure to multiple pollutants, relative to that presented by single pollutant studies (Harkema and Wagner 2005). A major problem with current methods is that single atmospheric pollutants are studied

individually to evaluate their toxicity, rather than in mixtures comprised of common ambient pollutants or in a mixture representative of common urban environments. Further the common exposure methods (e.g., sampling into solution that is then 'instilled' into animals) frequently change pollutant characteristics that in turn could also change the potential exposure and risk outcome. Although previous research studying individual compounds has been useful in highlighting important hazardous compounds, more complex investigations are warranted.

Common atmospheric pollutants, including the HAPs, are constantly transforming in both indoor and outdoor environments. The toxic potential of these reactive compounds after further interacting with hydroxyl radicals and ozone (created by photochemical processes) is mostly not known. What is clear, however, is that these chemical processes are likely to further oxygenate the organic compound making them more likely to interact with cells.

Classic approaches used in animal and cellular toxicology also have not been able to analyze the effects of the unknown transformation products that may be important when assessing the risk of HAPs. The smog chamber toxicology system is an advanced approach that combines *in vitro* exposure techniques followed by assessment of suitable endpoints, with an outdoor environmental chamber system that creates a reproducible and yet complex atmospheric environment. The system dynamically produces chemical mixtures that generate full transformation products, including both known and unknown reaction products, that allow the toxicity of reactive, short-lived mixtures to be determined. This system is most beneficial with mixtures having atmospheric chemistries that might not be fully characterized. It can analyze effects induced by

pollutant mixtures, while also providing the opportunity to examine each individual component for single, additive, and synergistic effects. Once it is determined that such mixtures exhibit modified toxicity, efforts can then be allocated to investigate the full suite of products.

## 2.2. Sources of Air Pollution

Photochemical air pollution remains a major problem in many areas in the United States, despite improvements in urban air quality (Brasseur et al. 1999). The problem of air pollution began many centuries ago, but became more evident after the Industrial Revolution through the increased usage of coal in the $18^{th}$ and $19^{th}$ centuries. The compounds released into the atmosphere creating air pollution are continuously emitted from both anthropogenic and biogenic sources. These sources include both indoor and outdoor releases; however, many outdoor pollutants exist in much greater concentrations.

There are many biogenic or natural sources of air pollution that have mass emissions that are much greater than their anthropogenic, or man-made counterparts. Biogenic sources account for significant amount of VOCs emitted and include, but are not limited to releases from trees, plants, grass, volcanoes, biological decay, oceans, dust mites, and mold spores. Isoprene is the most common biogenic pollutant emitted by all deciduous species and is therefore always present in the outdoor environment.

Anthropogenic sources include three main types of sources: area sources, mobile sources, and point sources. Areas sources are described as many small pollution sources that emit less than 10 tons per year of a criteria pollutant or HAPs. Examples of areas

sources include: dry cleaners, gas stations, auto body shops, residential buildings (fire places mainly), and commercial buildings (primarily heating and cooling units). Mobile sources consist of both on-road vehicles (cars, SUVs, trucks, and buses) and off-road equipment (ships, airplanes, agricultural and constructional equipment). Considering urban areas alone, more than 50% of the hydrocarbon and nitrogen oxide pollutants come from motor vehicles. Point sources are characterized by major industrial facilities that emit more than 10 tons per year of any criteria pollutants or HAPs, or 25 tons per year of a mixture of air toxics. Examples of point sources include chemical plants, steel mills, oil refineries, power plants, industrial paper mills, copper smelters and hazardous waste incinerators. Overall, in most urban areas, point sources are less important when examining the releases of VOC's into the atmosphere; they account for less than 15% of total VOC. A few cities like Los Angles, CA and Houston, TX have large petrochemical facilities that produce billions of pounds per year of very reactive and sometimes toxics VOCs. Point sources in these locations can dominate the composition of the urban air, at least over significant fractions of the urban area.

## *2.3. Atmospheric Chemistry*

Chemistry and meteorology together play crucial roles in the fate and transport of emitted environmental pollutants by creating transient conditions that influence lifetime and transformation yields. Meteorological parameters including temperature, relative humidity, precipitation, wind speed and direction led to day-to-day variability associated with the transport and exposure of pollutants in the atmosphere. The health impact of environmental pollutants are not solely determined by the concentrations that result from

their emissions, but also by their atmospheric fates; therefore, understanding these transformation reactions is essential to developing appropriate health risk assessments.

Environmental pollutants constantly react in the dynamic atmosphere through chemical reactions including photolysis, reaction with photochemically created hydroxyl radicals, with nitrate radicals, and with ozone, which is also created photochemically during the cyclic oxidation of the oxides of nitrogen (NO and $NO_2$). As early as the 1950's, public health scientists became concerned with photochemical air pollution (e.g., Los Angeles in the 1940's), and the fundamental chemistry driving these reactions was established in the late 1960's (Finlayson-Pitts and Pitts 2000). These pollutants are not just emitted into the atmosphere, but are created and destroyed in the atmosphere; therefore in the presence of sunlight and nitrogen oxides, HAPs are converted into other pollutants by a complex series of chemical reactions. These other pollutants may or may not be HAPs. Many HAPs begin their life as hydrocarbons and are known to "fuel the photochemistry of the troposphere with their rich supply of hydrogen atoms" (Brasseur et al. 1999). Hydrocarbon VOCs are grouped into methane and non-methane VOCs, with methane being an important component of VOCs due to its environmental impact on global warming.

Non-methane VOCs or hydrocarbons, readily react with hydroxyl radicals; "a major source of the hydroxyl radical (OH) in both clean and polluted areas is created through the photodissociation of ozone by actinic UV radiation in sunlight to produce electronically excited oxygen atoms" (Finlayson-Pitts and Pitts 2000). The OH radical is a strong atmospheric oxidizer that generally is the most rapid atmospheric transformation process. Overall OH chemistry is considered to control the lifetime of most organic

species (Brasseur et al. 1999). They initiate chain reactions by attacking HAPs causing the oxidation of organics thus regenerating additional radicals that further propagate the reaction (Jacobson 2002). Products that are formed during these reactions include aldehydes, ketones, or both (Atkinson 1994). Oxygenated gas molecules of HAPs can absorb radiation of UV~290-400 nm, increasing their internal energy (electronic, vibrational and rotational states) and potentially breaking chemical bonds thus contributing to the radical pool, thus helping to oxidize other VOCs (Finlayson-Pitts and Pitts 2000).

## *2.4. Respiratory Pathophysiology*

### 2.4.1. Characteristics of the Respiratory Tract

The respiratory tract consists of the three major regions: the upper airways, the respiratory airways and the lungs. The nasal passages, the para-nasal sinuses, and throat form the upper airways. The larynx, trachea, bronchi, and bronchioles form the respiratory airways. The respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli are considered to be the lung region. The respiratory tract is covered in an epithelium with different location-dependent cell types. Each type plays a different role in the function and overall structure of the respiratory tract. This epithelium is exposed to pollutants during inhalation. The extent of damage and which region of the respiratory tract the pollutant attacks is dependent on the pollutant physical and chemical characteristics. In general, most gaseous HAPs reach into the lung region of the respiratory tract.

The lungs, "situated at the air/blood interface, are a prominent target organ for numerous types of chemically induced damage as a result of exposure to xenobiotics or other substances after inhalation" (Castell et al. 2005). A major target for non-soluble gaseous HAPs is the alveolar region of the lungs. This area is lined entirely with epithelial cells thus making it extremely vulnerable to attack by gaseous exposures. "Airway epithelial cells are positioned at the interface with the external environmental and are the first exposed to inhaled irritants, allergens and air pollutants" (Bosson et al. 2003). They consist of Type I and Type II cells that are not only a barrier but are in charge of "the production of surfactant proteins to reduce alveolar surface tension, the release of cytokines and growth factors to regulate inflammation and cell growth, and the release of matrix proteins, proteinases and proteinase inhibitors needed to regulate turnover of alveolar structure" (Hayashi 2005). Together, Type I and Type II alveolar epithelial cells are the most susceptible lung cells to inhalation exposures (Castell et al. 2005). Type II cells are involved in pulmonary defense mechanisms by secreting protein mediators that contribute to pulmonary inflammation and true to their function play a crucial role replacing damaged Type I cells (Brewis et al. 1995).

### 2.4.2. Chemical and Physical Response to Pollutants

Studying exposures to air pollutants not only gives information about the interaction of the pollutant with the cells lining the respiratory tract, it also provides vital knowledge on the level of pollutant required to induce an inflammatory effect (Henderson 2005).

Injured or environmentally stressed airway epithelial cells are capable of synthesizing and secreting a variety of proteins to induce intracellular signaling

orchestrating the process of inflammation, repair, apoptosis and possible remodeling. The most common response that occurs in the lung from inhalation of atmospheric pollutants or HAPs is the induction of inflammation at the inhaled site. Inflammation, in this context, can be defined as a "localized and protective response, serving to eliminate or isolate injurious agents and facilitate the repair and regeneration of damaged tissue" (Huether and McCance 2004). Although inflammation plays a protective role in the response from outside exposures, it can also exacerbate preexisting health diseases or conditions, such as asthma, thus causing a significant increase in symptoms and acute or chronic lung disease. Inflammation is also widely associated in the pathogenesis of a range of lung diseases, including chronic bronchitis, emphysema, adult respiratory distress syndrome, neonatal distress syndrome, extrinsic allergic alveolitis, and cystic fibrosis (Brewis et al. 1995). The primary mechanism leading to exacerbation of respiratory disease or conditions is the recruitment of cells associated with inflammation, which is a process regulated by lipid and protein mediators.

Cytokines are proteins that interact and bind to specific receptors on membranes and activate signal transduction mechanisms involved with inter- and intra-cellular communication, regulating cell differentiation, proliferation and cell secretory activities. They modulate the initiation and amplification of inflammation by the recruitment and activation of responding leukocytes, the activation of systemic responses, and induction of local repair processes critical to the resolution of inflammation (Niederman et al. 2001). During the initial inflammatory response, upregulation of specific cytokines, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF- α) occurs. Both IL-1 and TNF-alpha are considered proximal mediators and play key roles in propagating further

biochemical reactions produced primarily by macrophages. Epithelial cells then respond to these stimuli, thus increasing the production of other cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), granule-monocyte-colony stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), monocyte chemotactic protein-1 (MCP-1), and fibroblast growth factor-2 (FGF2), which later activates chemotaxis of monocytes, neutrophils and basophils (Theze 1999). These cytokines can also be directly released in response to an external stimulus. As inflammatory responses develop, anti-inflammatory mediators (e.g. IL-4, IL-10, IL-11, IL-13, and TGF-beta) are produced limiting the intensity and duration of the inflammation. Overall, these cytokines are readily used as biomarkers of response (Henderson 2005; Scherer 2005). There are two major advantages of using cytokine markers to study environmental exposures: 1) cytokines can be used as early indicators of biochemical changes leading to later morphological changes in the propagation of disease; and 2) due to their quantitative properties, their dose-response can be measured (Henderson 2005).

One of the primary focuses in this study is on IL-8. IL-8 is one chemokine member of the C-X-C family that is produced by several cell types, including epithelial cells (Mastonarde et al. 1995; Standiford et al. 1990). It plays a major role in the biological system attracting and activating neutrophils to injured sites. It has been suggested that serum concentrations of IL-8 could predict the prognosis of patients with acute lung injury (Pittet et al. 1997). IL-8 is very unique compared to the other chemokine cytokine family members, due to its low molecular weight, amino acid composition, and its folded structure (Wuyts et al. 1998). Thus it is fairly resistant to many environmental factors, such as pH, temperature, denaturing chemicals and

enzymatic reactions (Wuyts et al. 1998). Once inflammation sets in, IL-8 molecules are not easily reduced inside the body, and therefore IL-8's concentration can be used as a good indicator of cellular stress.

Several studies have shown that increased chemokine mRNA or the presence of chemokine protein under exposure conditions concurrently happens during the presentation of pulmonary inflammatory symptoms. This is true in recent studies examining the effects of exposure to common atmospheric pollutants (Ban et al. 2006, Bourbia et al. 2005, Carpagnano et al. 2005, Henderson 2005; Saber et al. 2006, Tesfaigzi et al. 2005, and Wegmann et al. 2005).

Along with inflammation, studies also quantify the release of lactate dehydrogenate (LDH) to measure cellular injury induced by pollutants exposures (Dong et al. 2005; Henderson 2005; Kaneko et al. 2005; Muller et al. 2005; Pauluhn 2005; Seagrave et al. 2005; and Sureshkumar et al. 2005). LDH is released when the integrity of the cellular membrane is damaged, thus making it an adequate marker of cell viability (Huether and McCance 2004).

## *2.5. Importance of Ozone*

Studies have estimated that as many as 13 million healthy individuals in the US alone are routinely exposed to ozone levels that are in excess of the National Ambient Air Quality Standards (NAAQS) (Harkema et al. 1997). Because large populations reside in areas with high levels of ozone for extended periods of time, it is important to determine whether progression of ozone induced airway inflammation occurs with repeated

exposures (Christian et al. 1998). The pathological interaction between ozone and other co-pollutants are both complex and is currently unpredictable; consequently studying ambient exposures is very difficult. Research has shown that prolonged exposure to ozone levels that are even below the 1-hr NAAQS can cause respiratory symptoms including reduced lung function, difficulty breathing and increase the reactivity in the airways in exercising healthy men (McDonnell et al. 1991). Because of this common condition of daily ozone exposure in much of urban U.S., in this study, repeated exposures of human respiratory epithelial cells to ozone was used to condition or pre-treat them before the cells were later challenged; this is believed to be a suitable technique to study the effects of pre-exposure conditions on subsequent cell response.

### 2.5.1. Effects of Single Ozone Exposures

The adverse respiratory effects due to single ozone exposures have been extensively studied and document that ozone disrupts normal lung function by inflammation and increasing epithelial permeability (U.S. EPA 2006). There have been multiple versions of the EPA Ozone Criteria documents that summarize and interpert these studies. The most recent document was published in February 2006 and was titled "Air Quality Criteria for Ozone and other Related Photochemical Oxidants"; this report has references to the ozone health effect knowledge database and sums the entire relevant work on ozone. Instead of crediting individual studies, in subsequent sections of this thesis, I will cite the Criteria document.

Ozone's chemical and molecular characteristics allow the interaction with a wide variety of different cellular components making it a destructive force during exposures. In brief, ozone's mechanism of toxicity occurs mainly through lipid and protein

ozonation, which leads to lipase activation and production of phopholipases, arachidonic acid, leukotrienes, and prostanoids effecting both the structure and function of the respiratory tract. Concurrently these compounds initiate the production of inflammatory cytokines that participate in the recruitment of inflammatory cells. Inflammatory cells play an important role by activating a second cycle of inflammatory responses that significantly alters epithelial permeability leading to overall increased response. This inflammatory cascade generates a mix of biological reaction products, which are then likely to further oxidize lipids and proteins found on the cell membrane or within the cell. Studies have shown that approximately 280 genes are expressed post-ozone exposure (80% suppressed and 20% induced) and play individual roles in the possible toxic response (U.S. EPA 2006).

Much of ozone damage within the cell or on the cell membrane occurs when ozone reacts directly with the target components. The formation of oxidized proteins, aldehydes, free radicals, ozonides and peroxides through intra- and extra-cellular reactions act as signal transduction molecules involved in inflammation. Interestingly, some of the formation products listed are also generated naturally in the atmosphere making their indirect exposure due to ozone reactions only one possible exposure method; thus, the possibility of direct exposures to these same body-induced compounds is also very probable. Of the many reaction products formed, aldehyde concentrations have been used as markers of response. Studies have found that the absolute absorption into the cell from direct aldehyde exposures in unknown; thus making these measurements difficult and uncertain.

In summary, environmentally relevant levels of ozone cause many types of adverse health effects including lung inflammation, decreases in host defenses against infectious disease, alterations in lung function and exacerbation of previous respiratory conditions. Some of these effects are reversible and well documented in the literature with attenuation occurring within a short period of time; however, the irreversible elements and how these will affect later challenges is also an interest in public health research.

### 2.5.2. Effects of Repeated Ozone Exposures

Epidemiology studies, human controlled studies, and animal toxicology studies have found different responses when examining the effects of repeated exposures to atmospheric pollutants, in particular to ozone. Although much of these indicate some form of tolerance or adaptation occurring from the repeated exposures, few still argue that an increased sensitization or hyper-responsiveness may transpire. Discussed below is a collective review of studies that are most relative to the repeated ozone exposure protocol and the markers of response used in this study.

#### 2.5.2.1. Epidemiology

Observational epidemiology studies provide relevant evidence as to whether ambient environmental factors, such as air pollution, can adversely affect public health. Studies have shown that single and repeated exposures to air pollutants can alter the induction of respiratory inflammation caused by later pollutant challenges. These alterations can be through either an increased or decreased sensitization to the response indicator.

Recently, few studies have focused on the response of repeated pollutant exposures without extensively investigating the effects of co-pollutants. Most recently, observations have supported a sensitization. For example, Chan and colleagues (2005) found an increase in inflammatory mediators indicated by the greatest effect observed on the 3rd day of ozone exposure, rather than the 1st ozone day. Subsequently, Kinney and Lippmann (2000) measured a greater response through respiratory flow indicators at the end of a five-week exposure period when compared to the first week measurements. In addition, Romieu et al. 1998 and Frischer et al. 1997 found significant increases in respiratory irritation and inflammatory markers over an extended ozone exposure period.

In contrast, a handful of studies in the mid-late 1990's examining the effects induced by repeated ozone exposures demonstrated an adaptive response or tolerance when examining inflammatory responses. This adaptation or tolerance is more widely found in the literature and accepted amongst health researchers. Frischer et al. 1993 and Kopp et al. 1999 documented a decrease in inflammatory markers after the considered "repeated ozone" exposure period. Interestingly, although Kinney et al. 1996 indicated acute inflammatory responses diminished creating a tolerance effect, LDH continued to increase therefore they suggested that cell death may be ongoing.

#### 2.5.2.2. Animal Toxicology

A large portion of studies, *in vivo* or *in vitro*, examine the effects of repeated exposures by evaluating higher concentrations of ozone (or other atmospheric pollutants) than those proposed in this study (Cho et al. 1999, 2000; Sun and Chung 1997; Van Bree et al. 2002; Wagner et al. 2002, 2003). Overall, these recent studies, using concentrations greater than naturally occurring ambient concentrations (i.e., greater than 500 ppb), found

slight changes when examining airway inflammation through airway responsiveness, respiratory function, and quantification of inflammatory cell infiltration from repeated ozone exposures.

Based on repeated ozone studies examining the effects of tolerance or sensitization at lower concentration levels, those closer to environmental ozone conditions, the scientific community still was unable to reach a consensus. A few of these studies that were similar to the exposure protocol tested for this project will be discussed below. Although a majority of the studies produced results suggesting an inhibited response to repeated ozone exposures, some studies still indicate both sensitization and tolerance when evaluating animal and human studies.

Tepper et al. 1991 repeatedly exposed rats to 60-250 ppb ozone, 9 hr/day, up to 78 weeks and found a small, but significant decrease in respiratory function in those exposed at 78 weeks. Although outdated, studies with comparable protocols repeatedly exposed rats or mice to 200 ppb ozone and found significant changes in lung function; however unlike Tepper et al. both enhancement and suppression of responses were found (Bartlett et al. 1974; Costa et al. 1983; Raub et al. 1983). At higher concentrations, Rohr et al. 2002 repeatedly exposed mice to 3.4 ppm ozone before challenging them to isoprene or isoprene oxidation products with concentrations ranging from 47-465 ppm. In this study they found no significant enhancement or suppression in respiratory function as well as irritation with or without repeated exposures. In 2003, Rohr et al. altered their repeated exposure study protocol to an isoprene and ozone mixture comprised of concentrations relative to those that cause known human pulmonary irritation rather than ozone alone; however the authors still could not find significant evidence that the mixtures of ozone or

products caused "direct lung epithelial cell damage" using these techniques. Although some changes in respiratory function were found in many of these studies, unfortunately they did not evaluate direct markers of cytotoxicity or inflammation similar to those later proposed in this study.

#### 2.5.2.3. Controlled Human Studies

In 1998, Christian et al. found that repeatedly exposing individuals to 200 ppb ozone for 4 days increased the induction of inflammatory markers such as IL-6, IL-8 and GM-CSF, and LDH response after exercising moderately for 30 minutes; however, similar to previous studies this response is later attenuated. Similarly, Jorres et al. 2000 exposed healthy individuals to 200 ppb ozone for 1-4 days and analyzed for an increase in inflammatory marker production. They also found a significant increase in IL-6 and IL-8 protein. Although these studies did not use an *in vitro* model, the protocols and markers of response used are similar to those proposed in this study.

#### 2.5.2.4. Collective Review of Repeated Ozone Exposures

Examining the effects of repeated exposures of ozone and other atmospheric pollutants is difficult due to the unknown mechanism causing the response. As shown above it is not clearly accepted or understood if sensitization or tolerance occurs. Tolerance or an inhibition of response is well recognized throughout; however some researchers still feel sensitization through repeated exposures is still a probable response mechanism. Understanding the development and progression of antioxidants to protect against damage induced by oxygenated species, and how these are altered during repeated exposures is part of the incomplete response mechanism. However, we do know that previous research examining single ozone exposures agree that ozone alone causes

neutrophil responses, induces gene expression of inflammatory mediators, epithelial cytotoxicity, changes in airway resistance, respiratory rate, and epithelial permeability (Hotchkiss et al. 1989; Jaspers et al. 1997; Krishna et al. 1997; Lippmann 1989; Mehlman and Borek 1987; NTP 1994; Samet et al. 1992; WHO 1978 Li et al. 1997).

Overall, these studies suggest that ozone may or may not be capable of modifying respiratory inflammation after repeated exposures at 200 ppb, a level at which is known to cause acute inflammatory effects (Holz et al. 2002). The combined research illustrated that typically ozone effects occurred with some delay, suggesting that other factors such as subsequent exposures might have caused inductions of some of the inflammatory markers or cytokine expression. This may be a confounding factor associated with previous studies that examined the effects of multiple exposures and found no increased response. Still, the overall conclusion must be that the mechanism or mechanisms by which ozone, single or multiple exposures, enhances inflammatory responses in the airways of humans are unclear due to the multiple pathways that are known (Wagner et al. 2002). These inconclusive results further warrant additional controlled repeated ozone exposure studies testing ambient-like ozone concentrations.

## *2.6. Study Strategy*

The UNC environmental chambers have been used for over 30 years to investigate and develop chemical mechanisms of atmospheric pollutants. These chambers operate with natural environmental conditions (real sunlight and ambient temperatures and humidity) and simulate the chemistry of environmental mixtures of HAPs and other organic compounds found in urban atmospheres, thus generating ozone

and a full range of photochemical products. As transformation products are discovered, prioritized and regulated, natural simulation chemistry is studied to predict potential changes in environmental risk assessments. Chamber studies are considered to be "the most direct experimental means of examining the relationship between emissions and air quality by simulating atmospheric conditions" (Finlayson-Pitts and Pitts 2000). Despite several limitations (possible chamber contamination and uncontrollable surface chemical reactions), chambers studies are "useful in studying the chemistry of photochemical air pollution under controlled chamber conditions in which emissions and meteorology are not complicating factors" (Finlayson-Pitts and Pitts 2000).

The smog chamber toxicology system was designed to investigate the toxicity of chemicals before and after photochemical reactions and to investigate interactions of the urban atmosphere using real-time exposures to human lung cells, thereby avoiding concerns about compositional modifications to the exposure mixture caused by collecting gases in liquid or other collection techniques. This system combined with a cultured human cell line is a good method to evaluate the toxicity of natural, complex gaseous pollutants and mixtures, such as HAPs.

### 2.6.1. Synthetic Urban Smog Experiments

Initial experiments were conducted mimicking urban smog using the outdoor environmental chambers and the in vitro exposure system (Sexton et al. 2004). This studied compared the production of IL-8 mRNA from photochemical transformations of the UNC synthetic urban mixture. The UNC synthetic urban mixture consists of 54 representative measured VOC's indicative of the average outdoor chemical composition of 40+ U.S. cities (Jeffries 1995). A549 cells, an immortalized human respiratory

epithelial cell-line, were exposed to reacted and unreacted urban mixtures for 5 hours and then analyzed post-exposure. Preliminary results indicated that both secondary reaction products generated from the photochemical reacted system, and the unreacted synthetic urban mixture had an effect on inflammatory mediator production in respiratory epithelial cells. Importantly, the photochemical products generated two to three times more IL-8 mRNA compared to the unreacted synthetic urban mixture. These results suggest further studies to evaluate the effects photochemical may play on the toxicity of other atmospheric pollutants or mixtures.

### 2.6.2. Optimization of Experimental Protocol

In this study one early objective was to create an experimental protocol that generate a chamber mixture with maximum first generation products produced from photochemical transformations of isoprene/NOx and 1,3-butadiene/NOx. A second objective was to create chamber mixtures that produced minimal amounts of ozone present during the cell exposure period, thus minimizing the ozone effects. The goal was to expose cultured human respiratory epithelial cells to chamber mixtures after sundown, also alleviating chamber constituent decay during the 5-hour exposure period. To begin, a complete review was performed of previous experiments conducted using the outdoor environmental chamber and isoprene/NOx or butadiene/NOx mixtures. This information was needed to identify possible initial conditions including hydrocarbon and nitric oxide concentrations, to evaluate the rate of photochemical degradation occurring in the chamber, and to examine the growth and decay of photochemical products generated.

Based on previous chamber experiments, 100 ppb nitric oxides and 500 ppb isoprene were chosen for the first experiment. This mixture was allowed to react from

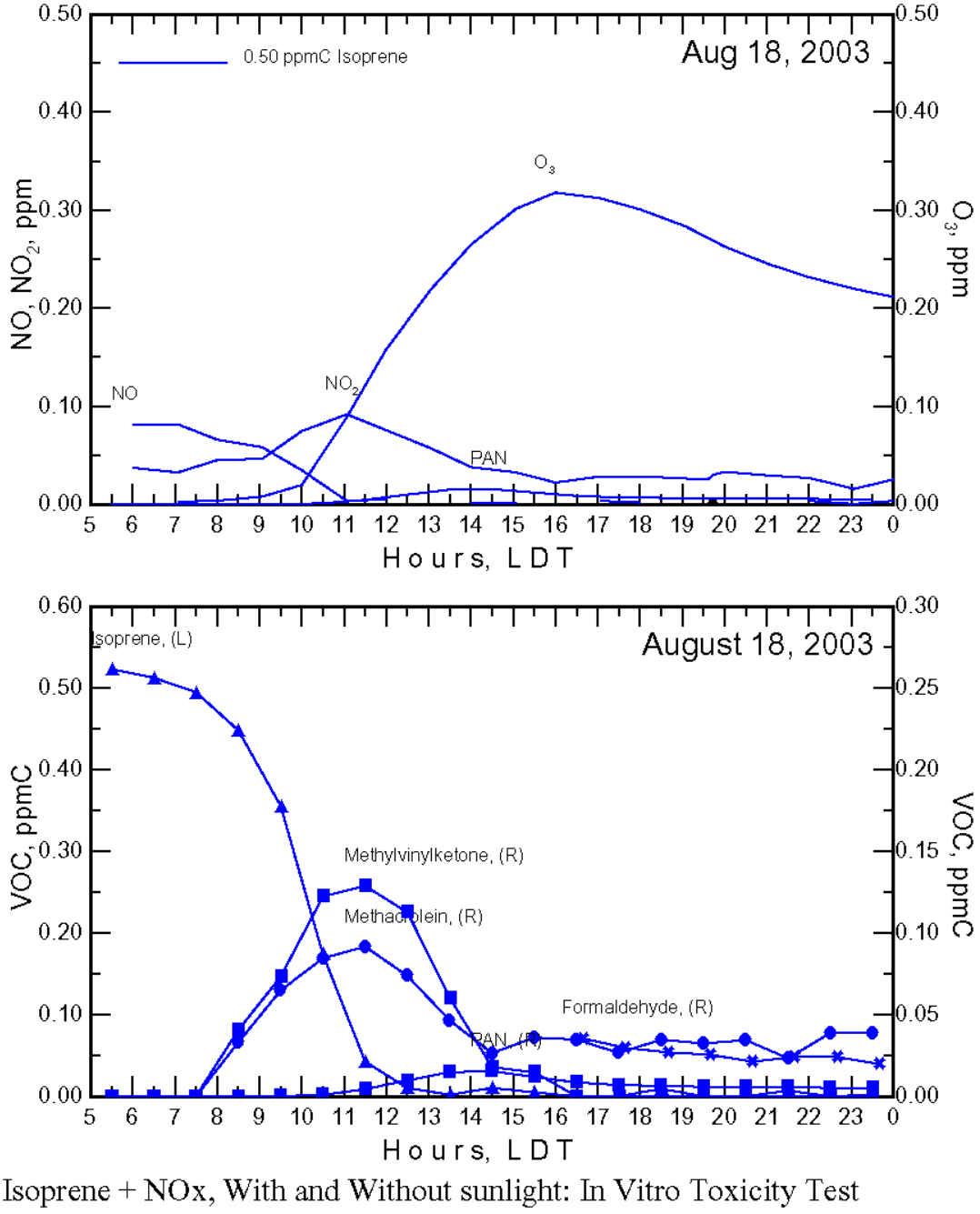
sunrise to sunset, approximately 13 hours. Figure 2-1 displays the time-concentration plot for the given experiment. During this experiment >300ppb ozone was generated and the initial products (methacrolein and methyl vinyl ketone) continued to react to form secondary and tertiary products during the 5-hour exposure period.

Therefore changes to the protocol were needed to alter the product concentrations found in the chamber during the 5-hour cell exposure period (approximately 1800-0100 LDT), as well as the total amount of ozone generated within the system. Modifications included: decreasing the initial hydrocarbon concentration, increasing the amount of nitric oxides, and adjusting the time the hydrocarbon-nitric oxides mixture was allowed to react with sunlight while inside the chamber. The latter adjustment was performed by changing the initial injection time from before sunrise to approximately 5 hours before sunset. This allowed the hydrocarbon mixtures only 5 hours to react producing mostly the first generation products. The photochemical reaction time was chosen based on modeling studies comparing the strength of the sun at a given time in the year and the particular hydrocarbon's photochemical reaction rate.

Figure 2-2 displays the time-series concentration plot showing the photochemical degradation of isoprene using the modified experimental conditions. Here 200 ppb isoprene and 50 ppb nitric oxides were injected into the chamber at 1430 LDT. The mixture reacted until sundown generating methacrolein, methyl vinyl ketone, formaldehyde, ozone and PAN. This figure differs from the previous one because this system generated at maximum only 200 ppb ozone, and during what would be the 5-hour cell exposure period, each of the initial products of interest could be found at near-

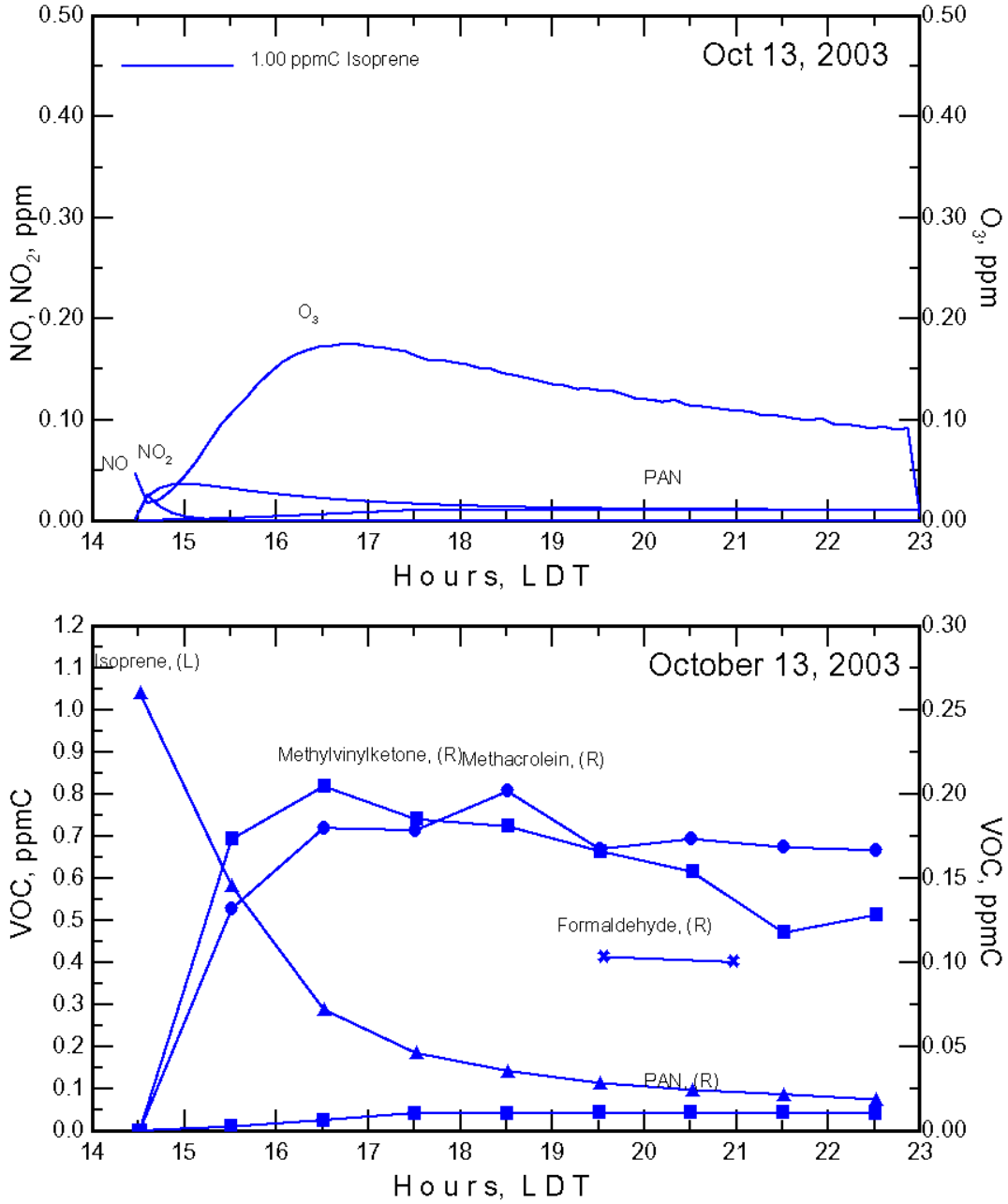
maximum levels thus suggesting minimal photochemical degradation generating secondary products.

The starting conditions are slightly modified per experiment depending on the hydrocarbon of interest and the time of year. Similar experiments were performed to optimize the complete experimental conditions used for 1,3-butadiene exposures. Below in Figure 2-3, are the complete time series concentration plots for isoprene (blue) and 1,3-butadiene (red) experiments generating maximum initial or first generation transformation products. The region between the dashed lines represents the 5-hour cell-exposure period for the given experimental days.




Isoprene + NOx, With and Without sunlight: In Vitro Toxicity Test

**Figure 2-1: Time Series Concentration Plots for 500 ppb ISO and 100 ppb NOx. These are the initial conditions for the first cell exposure using Isoprene.**




1,3-Butadiene/NOx vs Isoprene/NOx: In Vitro Toxicity Test

**Figure 2-2: Time series concentration plots for 200 ppb ISO and 50 ppb NOx.**

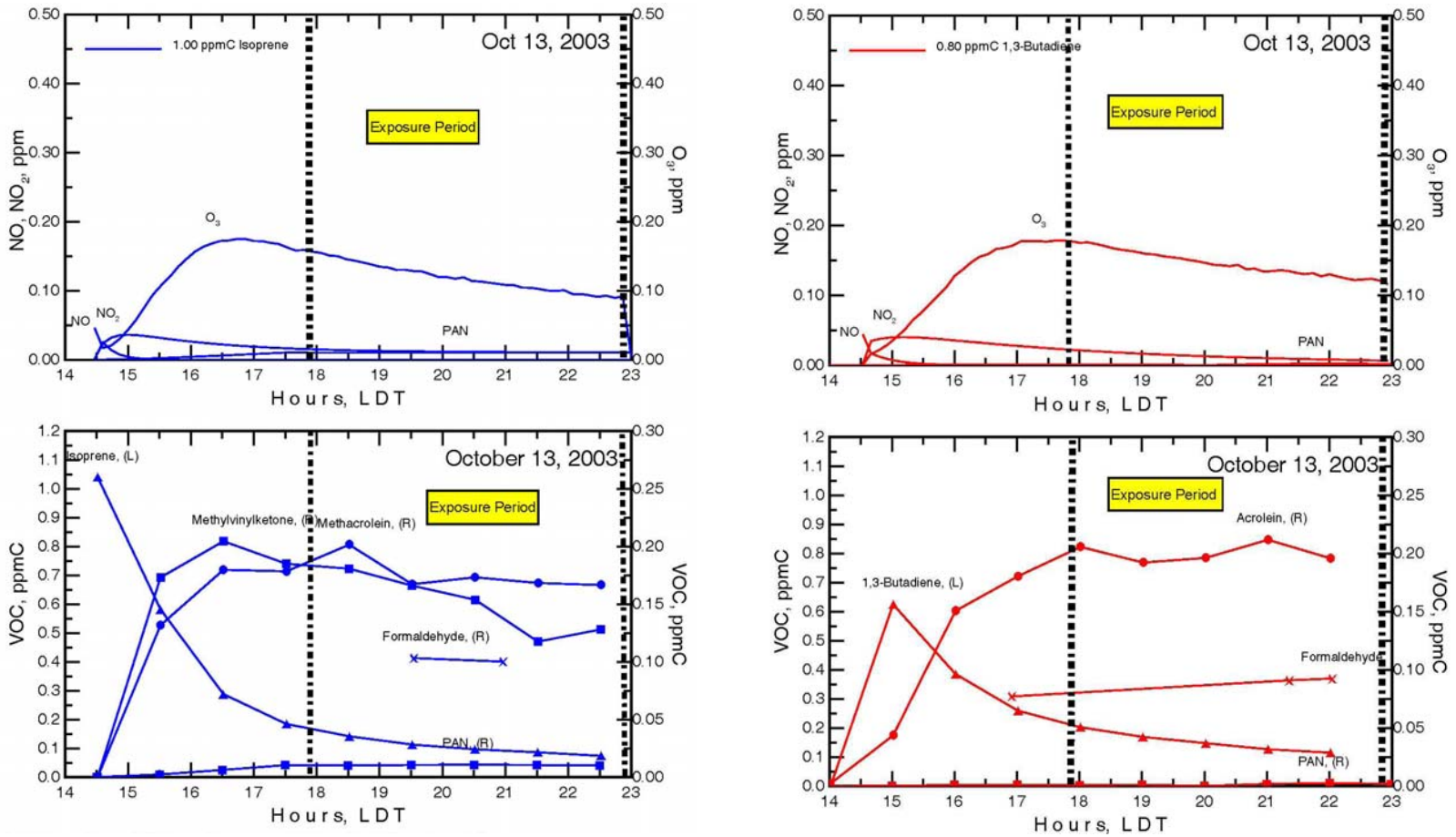



**Figure 2-3: Time series concentration plots, red and blue chamber data comparing 200 ppb hydrocarbon and 50 ppb nitric oxides after undergoing photochemical transformations for 5 hours.**

## 2.7. Direction and Implications

Implementing the Smog Chamber Toxicology System (SCTS) approach using isoprene (ISO), 1,3-butadiene (BD), and methanol (METH) can be used to examine if the photochemically generated transformation products of these toxicants induce greater adverse effects, cytotoxicity and inflammatory gene expression, than the original, unreacted HAP. One concern when working with pollutant mixtures and testing their toxicity on human respiratory epithelial cells is the question of which toxic agent is causing the toxicity. Along with comparing the effects of photochemistry, a second objective of this study was to examine the different roles that specific photochemical degradation products may play in the induction of cytotoxicity and inflammatory gene expression, and to discriminate these effects from the full photochemical product mixture. This set of experiments demonstrates the ability of the SCTS to better characterize and estimate the toxic potential of atmospheric pollutants in their natural mixed, complex states. The final objective was to evaluate if the response is altered, either through adaptation, tolerance or sensitization by the process of preconditioning human respiratory epithelial cells. This type of approach is believed to better mimic the natural response found in individuals living in polluted areas.

This work demonstrated an improved method that characterizes and estimates the toxic potential of atmospheric pollutants; the method allows scientists to study atmospheres with both rural and urban chemical characteristics, all within a laboratory setting. Taken together, the concepts behind the Smog Chamber Toxicology System when combined with a preconditioning protocol can enable health effects researchers to

mimic human responses to their natural environments using respiratory epithelial cells to model the adverse effects of atmospheric pollutants.

## 3. Hypothesis

Exposure to a photochemically transformed air pollutant mixture always has more respiratory health effects than exposure to the VOC precursors, or the ozone generated during the reactions, when these effects are measured by gene expression *in vitro*. Further, "preconditioning" *in vitro* samples by prior ozone exposures always shows an increased response compared to "new", unconditioned cells exposed only to the challenge mixture.

# 4. Approach

The approach in this part of the study was to 1) select three model VOC species that differ in their reaction products and complexities, and 2) evaluate these three systems under similar exposure conditions comparing the cellular responses to unreacted and reacted systems. The model species chosen are: 1,3-butadiene, isoprene, and methanol. More details on the reasons for choosing these compounds as models will be described below. The exact cellar responses that were determined will also be described in more detail below. The procedures to obtain the relevant chamber conditions and the exposure protocol will also be described below.

## 5. Objectives

Necessary steps to address the hypothesis include:

1. To evaluate the differences in respiratory cytotoxicity and inflammatory cytokine induction between unreacted hazardous atmospheric pollutants and the complete mixture of products generated during photochemical transformations.

2. To examine the roles of specific, known photochemical transformation products in inducing cytotoxicity and inflammatory cytokine expression. The transformation products evaluated in their natural mixed, complex states were acrolein, formaldehyde and ozone for 1,3-butadiene; methacrolein, methyl vinyl ketone, and ozone for isoprene; formaldehyde and ozone for methanol.

3. To determine if preconditioning human respiratory epithelial cells with ozone modifies the induction of cytotoxicity and inflammatory response caused by subsequent exposure to mixtures of the products generated during photochemical transformations of hazardous atmospheric pollutants.

# 6. Choice of Hazardous Air Pollutants Investigated

The following three chemicals were chosen for this work: 1,3-butadiene, isoprene, and methanol. These industrial pollutants are commonly emitted both naturally and anthropogenically into the environment. Butadiene and isoprene were chosen because they are structurally analogous compounds with similar industrial processes, sources, mechanistic chemistry, and toxicological properties. Methanol was chosen as a comparison pollutant since its also released from industry, however its chemistry being far less complex than both butadiene and isoprene, enables the evaluation of methanol's' full product toxicity without gaps in the chemical mechanistic history.

## *6.1. 1,3-Butadiene*

The compound 1,3-butadiene (BD) is not a new topic of concern when studying human health and the atmosphere. The Clean Air Act Amendments of 1990 added BD to EPA's hazardous air pollutants list, it ranks 36th in the top 50 most produced chemicals within the United States (OSHA 2002) and is one of the top 33 in the Toxic Release Inventory (US EPA 2001). With fluctuating emissions from both biogenic and anthropogenic sources (Hughes et al. 2001; Sorsa et al. 1996; Thorton-Manning et al. 1997) reaching as high as 3000 tons per year within the US (IARC 1992),

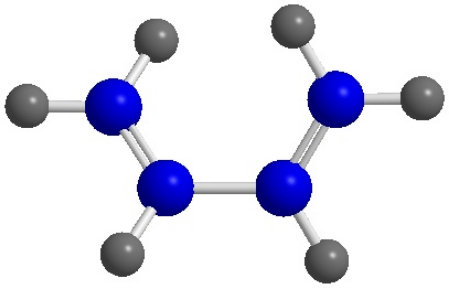


**Figure 6-1: 1,3-butadiene diagram**

the fate and transport of BD in the atmosphere plays an important role in the overall health concern after examining releases into the environment.

Once released into the atmosphere, BD reacts through partially known chemical mechanisms, including reactions with hydroxyl and other radicals as well as with ozone, which are all created during photochemical processes. During the daytime, only taking into consideration BD reacting with hydroxyl radicals (with OH concentrations ~$2x10^6$ molecule/$cm^3$), BD has a lifetime in the atmosphere of only 1-2 hours (Baker et al. 2005). This is significant for studying the adverse effects of the generated transformation products. These atmospheric reactions result in the formation of chemically identified products, detected but unidentified products, and (via mass balance) undetected and unidentified (Atkinson and Arey 2003; Baker et al. 2005; Claeys et al. 2004; Feltham et al. 2000; Jenkin et al. 1998). Previously, lack of knowledge of the full degradation pathways for BD made studying its full toxic potential difficult.

Known BD photochemical degradation products include acrolein (ACR), formaldehyde (FORM), organic nitrates, butadiene monoxide, CO, $CO_2$, ozone, PAN, 1,2-epoxy-3-butene, glycoladehyde, glycidaldehyde, 3-hydroxy-propanaldehyde, malonaldehyde (Atkinson 1990; Atkinson et al. 1994; Baker et al. 2005; Liu et al. 1999; Wayne et al. 1991; Yu and Jeffries 1997; Yu et al. 1995).

Although many studies have shown the adverse effects of BD and its known photochemical transformation products, little could be found using realistic ambient concentrations, non-carcinogenic end-points, or short term exposures. BD and all of its known initial or first generation photochemical products (primarily ACR, FORM,

acetaldehyde, furan, ozone) are known respiratory irritants (Gomes and Meek 2002; Krishna et al. 1997; Lippmann 1989; Liteplo et al. 2002; Mehlman and Borek 1987; NTP 1994; WHO 1978, 1989, 1992, 1995) , but nothing could be found on what respiratory effects these irritants have when an individual is simultaneously exposed to them all, for example in the neighborhood downwind of a polymer facility after a release of BD emissions from their process stacks.

## *6.2. Isoprene*

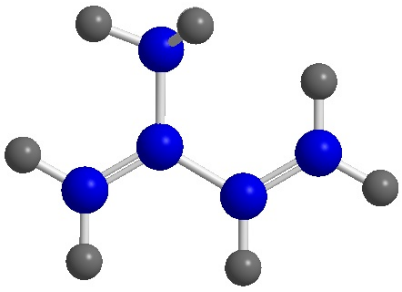


**Figure 6-2: Isoprene diagram**

Isoprene (ISO) is the most predominant hydrocarbon emitted by a number of deciduous species (IARC 1994) and therefore it is found naturally in many environments. It is also emitted from rubber, thermoplastic and polymer industries; it can be found in cigarette smoke, and it is expired in trace amounts during respiration in both humans (Johsson and Larsson 1969; Conkle et al. 1975; Krotoszynski et al. 1977; NTP 2002) and in animals (Peter et al. 1987). Worldwide ISO global emissions from vegetation range from 175-503 tons per year (Guenther et al. 1993) compared to the much larger estimated BD emissions in the United States alone. Similar to BD, outdoor concentrations of ISO range from 1-21 ppb, but are generally less than 10 ppb (IARC 1994; PHS 2002; Reimann et al. 2000).

Once ISO photochemically reacts in the atmosphere, its reactions result in the formation of methacrolein (MACR), methyl vinyl ketone (MVK), FORM, 3-methylfuran,

acetaldehyde, carbon monoxide, PAN, ozone, glycolaldehyde, hydroxyacetone, glyoxal, methylglyoxal, biacetyl, Criegee biradicadials and some unspecified multifunctional carbonyls (Atkinson and Arey 2003; Carter 1996; Palen et al. 1992; Sauer et al 1999; Tuazon and Atkinson 1990; Yu et al 1995).

Much work has been done to study the effects of these pollutants individually in both animal and human models, but very little can be found on their effects once combined in a natural urban setting (complex mixture effect). Little work has been done to study ISO mixtures similar to those comparable to isoprene's degradation products (Rohr et al. 2002). Although the toxicity of the mixture is still unclear, each product alone has been examined for, and is known to exhibit an increase in inflammatory responses (Alarie et al. 1998; Cunningham et al. 2000; Larsen and Nielson 2000; Liteplo et al. 2002; Rohr et al. 2002; U.S.EPA 2003; WHO 1989, 1995; Wilkins et al. 2001).

## *6.3. Methanol*

Methanol (METH), also known as methyl alcohol, wood spirit, carbinal, or wood alcohol (Reese and Kimbrough 1993), is a solvent not only in industrial facilities (Kumai et al 1983) but also in agricultural, laboratory, and consumer settings (Harris et al. 2004; Kawai et al. 1991; Saito and Ikeda 1988). Since the early 1980's, METH has been used as a fossil fuel alternative replacing petrol in automotive fuel (Dorman et al. 1994). Studies suggested that the added utilization of METH as an alternative fuel will increase low level exposures to METH vapors for the general public and in occupational settings (Osterloch et al. 1996). Such uses reinforce

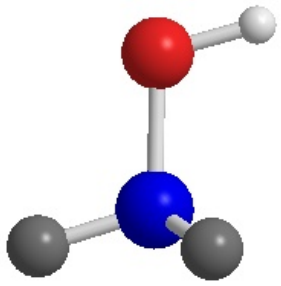


**Figure 6-3: Methanol diagram**

the importance of studying the adverse effects of low concentration exposures of METH and its transformation products.

The adverse effects seen from METH exposure have been reported since the early part of the century (Kawai et al 1991; Tyson 1912). METH causes species-specific toxicity due mainly to differences its enzymatic metabolism to formaldehyde (FORM) and formic acid within the body (Dorman et al 1994; Lee et al 1994; Tephly et al 1979; Tephly and McMartin 1984). Occupational exposures reported persistent headaches and other symptoms such as visual disturbances (dizziness, blurred vision, dimness of vision, hyperemia of the optic never head, inflammation of ganglion cells of the retina, atrophy of the optic nerve, and blurring of the optic disk edges) and nausea (Frederick et al 1984; Kawai et al 1991; Kingsley and Hirsch 1954; Reese and Kimbrough 1993). Although no human evidence has been found, animal studies determined METH to cause developmental toxicity through both inhalation and oral routes (Becker 1983; Harris 2004; Kruse 1992, Oyama 2002). There have been little to no inhalation studies performed at realistic environmental levels, thus emphasizing the importance of this study (Nelson et al 1985, Harris 2004; Lee et al. 1994). Although the mechanisms of toxicity at sublethal doses are not understood (Lee et al. 1994), we do know it is rapidly absorbed by route of inhalation (Osterloh et al. 1996) and is estimated that 58% of an inhaled dose is retained in the lungs after expiration (Sedivec et al 1981). EPA estimates that even in the worst traffic scenario, METH concentrations in the air do not exceed 200 ppm, (Osterloh et al. 1996), but our largest concern is not in the METH, but what damage can be done by the products formed once the METH reacts in the air after being released into atmosphere.

METH photochemically reacts to generate FORM, CO, ozone (McCauley et al. 1989; Niki et al. 1982). At concentrations similar with this proposed study, METH itself is primarily innate in comparison to its products FORM (Oyama et al. 2002) and ozone. Although toxicity studies are inconclusive, FORM by itself is a noxious chemical, highly unpleasant and a well recognized health hazard (Akbar-Khanzadeh et al. 1994; American Conference of Governmental Industrial Hygienists (ACGIH) 1992; Council on Scientific Affairs (CSA) 1989). Studying FORM alone, some researchers have found no changes in pulmonary function or irritations (Main and Hogan 1983; Levine et al 1984; Uba et al 1989) while others have reported significant responses, airway, throat, eye, nose, lung and skin irritations (Akbar-Khanzadeh et al. 1994; Kilburn et al 1989; Malaka and Kodama 1990; Schoenburg and Mitchell 1975), but still when examining the effects of photochemically reacting METH these effects of FORM need to be combined and evaluated with the known ozone response previously discussed.

Many of the products formed from photochemical reactions of ISO, BD and METH, i.e. ozone and FORM, are not merely formed through reactions with these compounds, but are generated during other photochemical transformation processes of most HAPs and are readily available in the natural environmental. Thus further emphasizing studying the complete photochemical degradation pathways for each compound, as well as the products formed during the process.

# 7. Methods and Materials

## *7.1. Chamber facilities*

Environmental irradiation chambers (also called smog chambers) that use sunlight can be used to study systems of natural transformation chemistry of pollutants (Jeffries et al. 1976; 2000; Sexton et al. 2004). The UNC Ambient Air Research Facility or site is off-campus, approximately 32 kilometers from the University of North Carolina at Chapel Hill (http://www.oneatmosphere.unc.edu/facilities.html). It is in the adjacent Chatham County and is approximately 10 kilometers from the small town of Pittsboro in an isolated rural area. Chatham County is one of the most rural, least industrialized counties in North Carolina and is heavily wooded. These conditions contribute to low background concentrations of NOx and non-methane hydrocarbons at this site are usually less than 5 ppb and less than 80 ppbC. More importantly, the air exhibits very low reactivity in the chamber.


Dual 150,000-liter UNC outdoor smog chambers made of sheets of Fluorinated Ethylene Propylene (FEP) Type A 0.13 mm Teflon film were used as photochemical reactors during the experiments. The film's transmission in the UV and visible regions of the solar spectrum is excellent, and it has only a few absorption bands in the IR, a property necessary to reduce the "greenhouse effect" inside the chamber. It has very low permeability for most chemical species and can be heat-sealed to form large durable panels. For this application its worst property is it s ability to hold a static charge for long periods of time and thus collect dust on its surface. The chambers are located in Chatham County, North Carolina. More descriptive information about the chambers has been published before (Jeffries et al. 1976; 2000 and on the Web). These chambers are ideal to study chemical systems that are part of real photochemical phenomena. The use of FEP Teflon film permits the transmission of the ultraviolet and visible sunlight that results in near-natural photochemistry inside the chamber.

To prevent condensation inside the chamber after sundown, we used a chamber dehumidification system before the photochemical experiment to lower the dew point below the expected nighttime minimum temperature. Condensation would cause product loss due to adsorption of oxygenated compounds into the moisture on the walls. In each experiment, a very small amount of carbon tetrachloride ($CCl_4$), used as an inert tracer, was injected and then monitored. The $CCl_4$ concentration can be used to calculate the dilution within each chamber. Over time each of the experiments were performed in slightly different experimental conditions (sunlight, humidity and photochemical reaction time). To provide a fixed set of controls, an additional set of cells were exposed to clean breathing air during each of the chamber runs; these controls were subject to the same transport and handling, and were exposed in the same incubator as the test cells.

## *7.2. Cell Cultures and In vitro Exposures*

A549 cells, a human lung epithelial cell line that has retained several alveolar type II-cell characteristics were used throughout this study. A549 cells were grown on membranous support (Costar-Clear Transwell™ inserts; Costar, Cambridge MA) as described in Jaspers, et al. (1997) in complete medium (F12K, 10% fetal bovine serum, antibiotics; all from Invitrogen, Carlsbad, CA). Upon confluency, the media was exchanged for serum-free media (F12K, 1.5µg/ml bovine serum albumin, antibiotics; all from Invitrogen) several hours before exposure. Just before transport to the smog chamber site, media located in the apical chamber was aspirated, while media in the basolateral compartment remained. This facilitates direct exposure of lung epithelial

cells to gaseous pollutants without significant interface of media, while the cells are maintained with nutrients from the basolateral side.

Primary human bronchial cells were obtained from healthy nonsmoking adult volunteers by cytologic brushing at bronchoscopy. The protocols for the acquisition of the primary human bronchial epithelial cells were reviewed by the University of North Carolina Institutional Review Board. Primary human bronchial epithelial cells were expanded to passage 2 in bronchial epithelial growth medium (BEGM, Cambrex Bioscience Walkersville, Inc., Walkersville, MD ) and then plated on collagen-coated filter supports with a 0.4 μM pore size (Trans-CLR; Costar, Cambridge, MA) and cultured in a 1:1 mixture of bronchial epithelial cell basic medium (BEBM) and DMEM-H with SingleQuot supplements (Cambrex), bovine pituitary extracts (13mg/ml), bovine serum albumin (BSA, 1.5 μg/ml), and nystatin (20 units). Upon confluency, all-trans retinoic acid was added to the medium and air liquid interface (ALI) culture conditions (removal of the apical medium) were created to promote differentiation. Mucociliary differentiation was achieved after 18-21 days post-ALI.

## *7.3. Smog Chamber – In vitro Exposure System*

The schematic shown in Figure 2 illustrates how the smog chambers were coupled to the in vitro exposure system. Inside tissue culture incubators we placed 8-liter, modular, cell-exposure chambers (Billups-Rothenberg, MIC-101™, Del Mar, CA) that hold the tissue culture plates. Humidification of the exposure chamber was achieved by placing a dish of sterile water inside the chamber. The 8-liter cell-exposure chambers are designed to have an inlet and an outlet connection for flowing gas through the exposure

chamber. Sample lines, directly coupled to the smog chambers through two externally circulated sample manifolds, were used to provide chamber gases to the cells during exposure (Sexton et al. 2004). Three cell-exposure chambers were used throughout these studies, two were supplied with the gas mixtures from the smog chambers and one was supplied with medical-grade clean air. For each experiment, one set of A549 cells was exposed to clean air to control for potential variations induced by tissue culture or transport of the cells. In addition, the clean air control cell-exposure chamber was used to hold the cells during pre- and post-exposure periods. The cell-exposure chambers were ventilated with either humidified medical-grade air from a cylinder or chamber air, which were mixed with 5% $CO_2$. The addition of $CO_2$ to chamber air was achieved using small pumps on the exhaust side and mass flow controllers (AALBORG, Orangeburg, N.Y.). Humidification of the control cell exposure chamber was achieved by passing medical-grade air from the gas cylinder through two midget impingers in series (Ace Glass, Vineland, N.J.), containing 15 milliliters of HPLC-grade water (Fisher Scientific). The impingers were heated at 28°C that resulted in a 50% relative humidity designed to match that in the smog chambers.

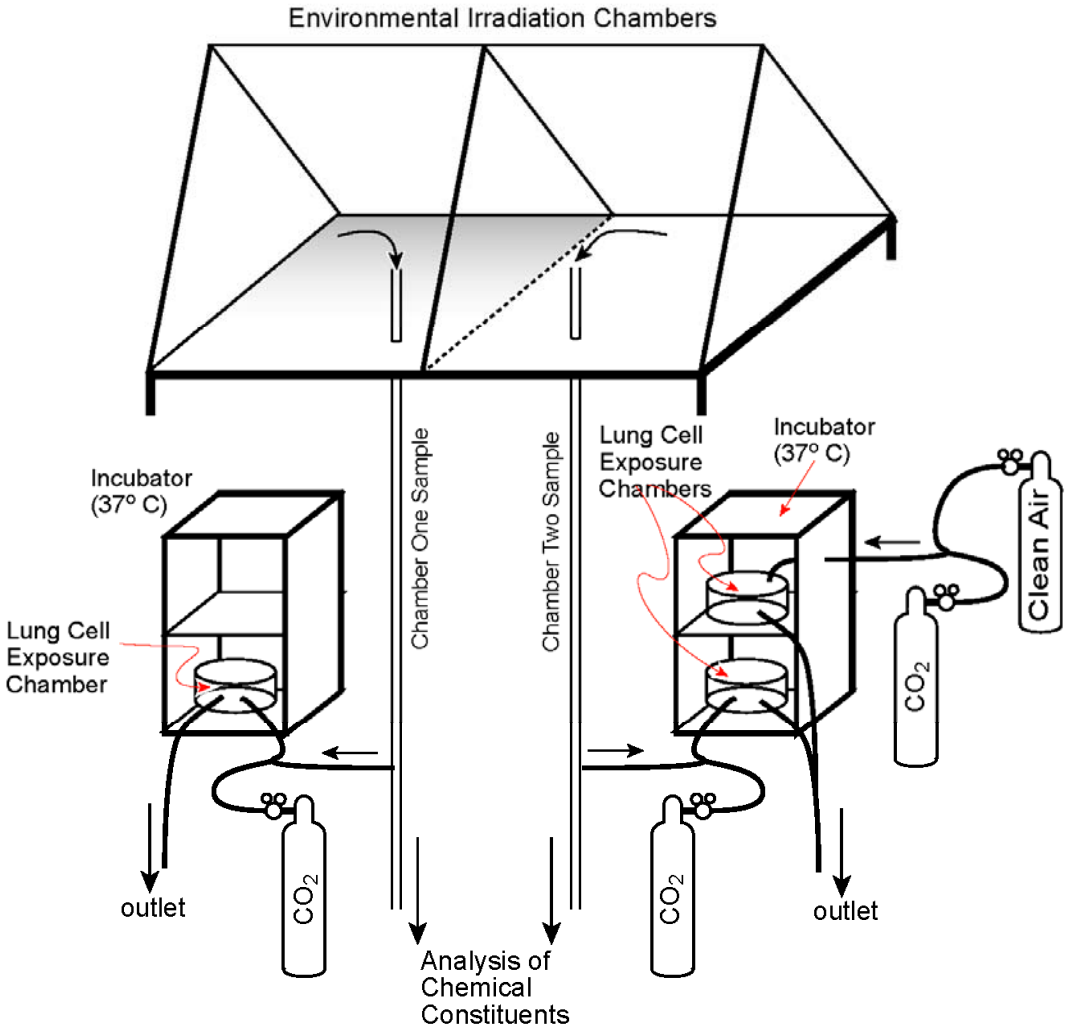



**Figure 7-1: Smog-Chamber-*In Vitro*-Exposure System**

## 7.4. Chemical Analysis

Five gas chromatographic (GC) methods were used to monitor volatile organic compounds within the chambers. One GC (Carle, Inc., Chandler Engineering, Tulsa, OK) was used to measure total hydrocarbon (THC), which was used for assuring low background concentrations while also measuring the total initial VOC injections. Samples were taken continually throughout the experiment, once an hour from each chamber and analyzed with two GCs (Carle, Inc.) using packed isothermal columns coupled to flame ionization detectors (FID). A Varian 3700 GC with electron capture detector (ECD), used to measure $CCl_4$ (our dilution tracer), PAN, and other N- or O-containing compounds, was also used continually every thirty minutes throughout the duration of the experiment. A Varian 3400 capillary GC-FID was used with a Varian Saturn 2000 ion trap mass spectrometer to analyze air samples taken before, at the beginning, and during each exposure to help analyze for both known and unknown products created during the photochemical reactions. FORM was measured continuously, using the automated Dasgupta-diffusion-tube sampler to obtain aqueous formaldehyde, which is then mixed with buffered 2,4-pentanedione and measured with fluorescence (Dasgupta 1988). Ozone was measured using an EPA standard reference method based on photometry with a Thermo Environmental Instruments Inc., Model 49 monitor. Nitrogen oxides were measured using an EPA standard reference method based on chemiluminescence with a Monitor Labs Incorporated Model 98-41 monitor.

## 7.5. Experimental Protocol

During each experiment, the dual smog chambers were used to examine two unique composite mixtures of photochemical transformation products. Pre-experiment procedures included drying, calibrations, and sample set-up. Approximately 15 hours before sunrise, the outside vents on each of the chambers were closed. Next, the chambers were dehumidified to below the estimated low temperature for that day using a nitrogen dehumidification system. While the chambers were dehumidifying, instrument calibrations were performed to calculate a current ozone calibration factor, and to confirm the retention times of common hydrocarbons on the GC's. GC and GC/MS samples were taken from both chambers prior to any injections to test for possible contaminants. Approximately 5 hours before sunrise, the pollutant that is being tested that day was injected into the chamber along with 50 ppb NOx. This mixture was allowed to sit in the chamber photochemically reacting until sundown.

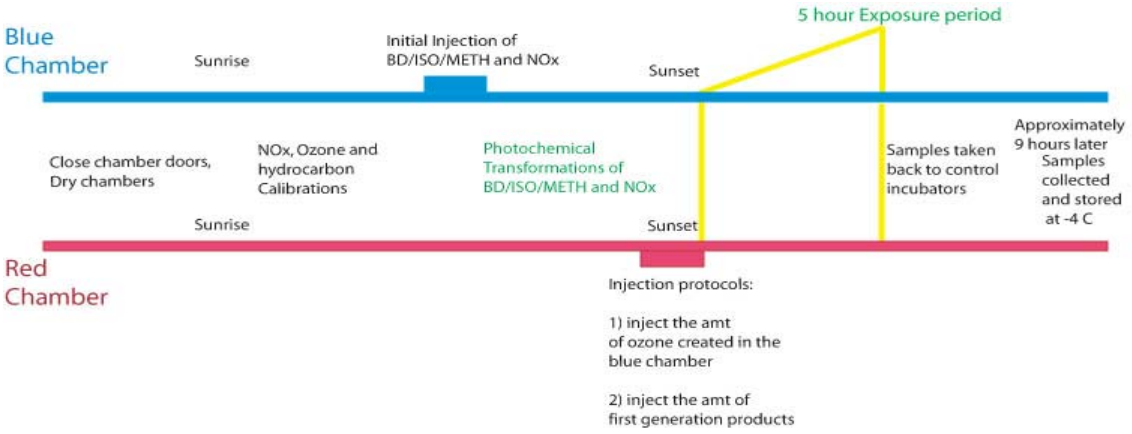



**Figure 7-2: Experimental Protocol for UNC Outdoor Environmental Chambers**

Culture samples were prepared at the UNC on the North Carolina Campus in Chapel Hill and held in culture incubators receiving breathing air and $CO_2$ until transportation to the site approximately 4 hours before sundown. An hour before the samples were due to arrive at the exposure site, modular incubator chambers held at 37° C were flushed continuously with humidified breathing air. Once the samples were taken to the site, they were held for approximately 3 hours in the control chamber until the beginning of the experiment. During the experiment, two sets of cells were placed in modular incubator chambers connected to the dual smog chamber. Post exposure, the cells were transported back to UNC and held over night in the culture incubator until processed.

## *7.6. Cytotoxicity Analysis*

Approximately 9-hours post-exposure basolateral supernatants from the exposed cells were drawn off and stored at -80°C until analysis. For the analysis of cytotoxicity, the basolateral supernatants were analyzed for the release of cell lactate dehydrogenate (LDH) using a coupled enzymatic assay (Promega, Madison, WI or Takara, Japan), as per the suppliers instructions. Cytotoxicity was expressed as LDH levels with fold increase over the individual clean air control sample.

## 7.7. Cytokine Analysis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) as per the supplier's instructions and analyzed by real-time RT-PCR for IL-8 as described previously (Jaspers et al. 2001). IL-8/GAPDH levels were measured and expressed as fold increase over the individual clean air control.

Basolateral supernatants were analyzed for IL-8 and IL-6 protein levels by ELISA (R&D Systems, Minneapolis, MN), as per the supplier's instructions. Protein levels were adjusted to account for the differences in viable cells that could produce and release IL-8 or IL-6 into the supernatant and expressed as fold increase over the individual clean air control sample.

Luminex Multiplex technology was used as a quick alternative to classic ELISA methods for the evaluation of seven other cytokines which include IL-6, IL-10, GMCSF, MCP-1, IP-10, FGF2, and VEGF. Multiplex assays "permit simultaneous cytometric quantification of multiple cytokines in solution by capturing these in structurally distinct beads" (Khan et al. 2004). The luminex system "facilitates the simultaneous evaluation of multiple immune mediators with advantages of higher throughput, smaller sample volume, and lower cost latter allowing the exploration of multiple cytokines without depleting sample supernatants". Luminex multiplex technology has "distinct advantages and is a valid alternative method to ELISA for the evaluation of the majority of cytokines tested and for the characterization of immune system status" (dupont et al. 2005), and thus was chosen to further investigate the response caused by BD, ISO, METH, and their photochemical degradation products.

# 8. Effects of 1,3-Butadiene, Isoprene, and Their Photochemical Degradation Products on Human Lung Cells

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## 8.1. Abstract

Due to potential exposure in both workplace and ambient air, the known carcinogen 1,3-butadiene (BD) is considered a priority hazardous air pollutant. BD and its 2-methyl analog, isoprene (ISO), are chemically similar but have very different toxicity, with ISO showing no significant carcinogenesis. Once released into the atmosphere, reactions with species induced by sunlight and nitrogen oxides convert BD and ISO into several photochemical reaction products. In this study, we determined the relative toxicity and inflammatory gene expression induced by exposure of A549 cells to BD, ISO, and their photochemical degradation products in the presence of nitric oxide (NO). Gas chromatography and mass spectrometry analyses indicate the initial and major photochemical products produced during these experiments for BD are acrolein, acetaldehyde, and formaldehyde, and products for ISO are methacrolein, methyl vinyl ketone, and formaldehyde; both formed less than 200 ppb of ozone ($O_3$). Post-exposure the cells were examined for cytotoxicity and interleukin-8 (IL-8) gene expression, as a marker for inflammation. These results indicate that while BD and ISO alone caused similar cytotoxicity and IL-8 response compared to the air control, their photochemical products significantly enhanced cytotoxicity and IL-8 gene expression. This suggests that once ISO and BD are released into the environment, reactions occurring in the atmosphere transform these hydrocarbons into products that induce potentially greater adverse health effects than the emitted hydrocarbons by themselves. In addition, the data suggest that based on the carbon concentration (ppbC) or per carbon basis, biogenic isoprene transforms into products with similar pro-inflammatory potential than BD.

## 8.2. Background

In this study A549 cells were exposed to ISO, BD, or their photochemical degradation products. A549 cells are a model of respiratory epithelial cells with some type II cell-like characteristics that have been extensively used to assess the toxicity of air pollutants. Both ISO and BD alone are known sensory irritants with observable effects to the eyes, nasal passages, throat and lungs (Alarie et al. 1998; Wilkins et al. 2001; US EPA 2003). Many of the photochemical products for both ISO and BD have known health effects that have been studied in animals, humans or both. For ISO, according to Rohr, et al. 2002 the inflammatory effects from the products generated by reacting ISO and ozone were greater than the effects of either ozone or ISO alone. (Wilkins et al. 2001) observed ISO products FORM, formic acid, acetic acid, MACR and MVK causing sensory irritations for mice. MACR alone causes irritation of the upper respiratory tract, painful sensation in human nasal cavities and sensitization of the trigeminal nerve endings (Delzell 1995; Larsen et al. 2000). MVK is a direct acting irritant targeting the upper respiratory tract causing nasal lesions (Cunningham et al. 2002). ACR is considered highly acutely toxic, is a known sensory and upper respiratory irritant and causes changes in respiratory function such as: decrease in respiratory rate, rhythm and amplitude (Gomes et al. 2002; WHO 1992). Studies show that FORM is a moderate sensory irritant with carcinogenic evidence from occupational exposures (Liteplo et al. 2002; WHO 1989). Exposures to acetaldehyde alone also caused irritation of the eyes and mucous membranes, reddening of the skin, pulmonary edema, headache, and sore throat in humans, and degeneration changes in the respiratory epithelium, trachea and larynx in rats and hamsters (WHO 1995) With respect to all of the products created

during photochemical reactions, ozone has been studied the longest and most in depth. Ozone toxicity includes inflammatory responses to sensory nerves, morphological injury with inactivation of alveolar macrophage secretory enzymes, epithelial cytotoxicity, changes in airway resistance, respiratory rate, epithelial permeability, bronchoactive challenges and other pulmonary functions (Krishna et al. 1997; Lippmann 1989; Mehlman and Borek 1987; NTP 1994; WHO 1978). Ozone is also a known animal carcinogen causing lesions in both F344/N rats and B6C3F1 mice (NTP 1994). Although respiratory health effects induced by some of the photochemical degradation products of ISO and BD are known, the toxicity of the entire photochemical reaction product mixture as it would occur in the atmosphere is unknown. In this study we interfaced human lung epithelial cells with a smog chamber, allowing us to assess the toxicity of ISO, BD, and their photochemical degradation products in parallel.

## *8.3. Experimental Section*

Each chamber experiment was given a unique identification name symbolizing the date the experiment was performed on. For example, AU1803 stands for the experiment performed on August 18, 2003. In addition, both chambers of the dual chambers system were used during each experiment to expose two sets of cells simultaneously to different chemical mixtures, either reacted or un-reacted, using the same environmental conditions.

For the experiments presented here, two injection protocols were used. For the first experimental protocol, hydrocarbon (ISO or BD) and NO mixtures were injected into a chamber and either allowed to react with the sunlight or injected into a chamber after sundown. This protocol permitted the cells to be exposed to either photochemically generated reaction products or to the non-reacted hydrocarbon and NO mixture. For these exposures, one chamber included a photochemically active system with 50 ppb NO and 200 ppbV of ISO or BD, while the other chamber contained the initial amount of hydrocarbon and NO, but was kept in the dark (without sunlight) and therefore unreacted. These experiments were performed on different days with slightly different environmental conditions, including temperature, amount of sunlight, and humidity. In the other experimental protocol, one chamber was operated with 200 ppbV ISO and 50 ppb NO, while the other chamber had 200 ppbV BD and 50 ppb NO. This protocol was applied to directly compare the effects of photochemical products generated with ISO or BD under the exact same environmental conditions (sunlight, humidity and photochemical reaction time).

During the experimental protocol comparing ISO or BD and their photochemical products, the initial injections of 200 ppbV ISO (99%, Sigma-Aldrich, St. Louis, MO ) or 200 ppbV BD (National Specialty Gases, Durham, NC) and 50 ppb NO were injected into one chamber at four hours before sunset (1500-1600 Eastern Daylight Time (EDT) and allowed to react in the remaining sunlight. At sundown, these same initial amounts are injected into the opposite chamber side. During the experimental protocol comparing the photochemical reaction products of ISO and BD, both 200 ppbV of ISO and BD and 50 ppb NO were added simultaneously into opposite sides of the chambers four hours

prior to sunset and allowed to react with the remaining sunlight. The exposure to the lung cells began after sundown when photochemical reactions were terminated from the absence of sunlight. This short photochemical experimental design, conducted at the end of the day, stops the photo-oxidation sequence where the first generation primary products are at their maximum concentration. Cells were exposed to the gaseous mixtures for 5 hours. After the exposures, all sets of cells were kept in the control air + 5% $CO_2$ exposure chamber until transport back to the UNC-CH campus.

## *8.4. Results*

The products generated during photochemical transformations of ISO or BD was identified by GC and confirmed using GC/MS. Table 1 summarizes the average levels and maximum concentrations produced of the known photochemical products derived from ISO or BD during the cellular exposure period on the indicated days.

During the ISO experiments, the cells were exposed to practically the same concentrations of the first generation products, primarily MACR, MVK, and FORM (within 0.030 ppm). The BD photochemical product concentrations, primarily ACR, available within the chamber during the exposure were also very similar (within 0.020 ppm). Therefore each of the toxicological results from the experiments performed on different days, but with the same hydrocarbon mixture, is comparable due to similar product concentrations generated and available during the exposure period. Figures 2 and 3 illustrate the photochemical smog chemistry within the two chamber sides during

the experiment directly comparing photochemical reaction products formed with ISO or BD. These time series concentration plots show the concentrations of the organic and inorganic species found within each chamber throughout the experiment. The formation and degradation of the inorganic species is shown in the upper graph. After the initial NO injection, $NO_2$, PAN and ozone are generated from the reactions between the hydroxyl radicals and the initial injections of the ISO or BD and NO. The bottom graphs show the photochemical degradation of the initial ISO or BD with the formation of the first generation photochemical products from the time of injection through the end of the exposure period. Figures 2 and 3 illustrate the cell exposure to the chamber mixtures occurred directly after peak concentrations of the first generation products were generated. The known ISO photochemical products formed were MACR, MVK, FORM, ozone, acetaldehyde and PAN. The products generated during photochemical transformations of ISO or BD was identified by GC and confirmed using GC/MS. Data shown in Figures 8-1, 8-2 and Table 1 indicate that while photochemical reactions using ISO or BD as hydrocarbon precursors generate some of the same products (such as ozone or formaldehyde), several products are specific for either ISO or BD. In addition, the levels of FORM and ozone produced by photochemical reactions with ISO or BD are different even though the initial carbon concentration reacted within the chamber was the same. For example, the ozone levels generated by photochemical reactions with ISO and exposed to the cells ranged from 0.118 – 0.130 ppm, while the ozone levels generated by photochemical reactions with BD ranged from 0.146-0.178 ppm.

To determine whether the products generated by photochemical reactions with ISO or BD affect cell viability, we analyzed relative cytotoxicity induced by exposure to

ISO, BD, or their photochemical product mixtures approximately 9 hours post-exposure. Results from experiments performed on different days were combined and cytotoxicity induced by ISO, BD, or their photochemical reaction products were expressed as fold increase over the respective control exposure to clean air. Figure 8-3A compares the relative cytotoxicity induced by ISO + NO or ISO + NO with photochemistry. The data show that ISO photochemical products induce a significant increase in cytotoxicity as compared to ISO+NO alone. Similar to ISO, comparing the relative cytotoxicity induced by BD + NO or BD + NO with photochemistry show that BD photochemical products are significantly more cytotoxic than BD + NO alone, as shown in Figure 8-3B. Furthermore, directly comparing the cytotoxicity induced by photochemical products generated with ISO or BD as shown in Figure 8-3C suggest that ISO photochemical products have similar effect on cell viability than BD photochemical products. The results from the repeated ISO vs. BD photochemical experiment using differentiated human bronchial cells derived from multiple individuals (Figure 8-3D) show both the ISO and BD photochemical products induced no significant change in cytotoxicity compared to the clean air exposure.

To examine whether photochemical reactions alter pro-inflammatory potential of ISO or BD, we compared the effects of ISO, BD, or their photochemical reaction products on IL-8 expression in both A549 and differentiated human bronchial cells. Figure 5A shows that ISO photochemical products induced a greater change in IL-8 expression (IL-8 protein induced) as compared to clean air control. In contrast, ISO + NO alone had no significant effect on IL-8 expression. Figure 8-4B demonstrates that BD photochemical products induced a significantly greater IL-8 expression as compared

to BD+NO alone, which also enhanced IL-8 expression as compared to the clean air control. Directly comparing the effects of photochemical products using A549 cells from either ISO or BD on IL-8 expression suggest that the products generated from ISO have a greater effect on IL-8 expression than BD photochemical products (Figure 8-4C), although these data were not statistically significant ($p=0.06$). The same experimental protocol was used to expose differentiated human bronchial cells to both ISO and BD photochemical degradation products. No significant changes in IL-8 release were seen (Figure 8-4D) in cells exposed to the photochemical mixtures as compared to the air-exposed control cells.

Along with IL-8 protein, IL-8 mRNA levels were measured for some of the experiments performed with A549 cells, as shown in figures 8-5A and 8-5B. Similar to IL-8 protein levels released into the basolateral supernatants; both ISO and BD photochemical products enhanced IL-8 mRNA levels, although the levels did not reach statistical significance.

## *8.5. Discussion and Conclusions*

Previous research has shown that many of ISO and BD's photochemical products are known sensory irritants to either animals or humans. However, the toxicities of these photochemical product mixtures produced in irradiative smog chambers using realistic atmospheric chemistry have not been previously studied. The advantage of using this approach, the smog chamber-cell exposure interface, to produce the photochemical

mixtures and conducting the toxicity studies is that all of the photochemical products are generated in the proper relative ratio, including the unspecified products. In this study, we examined cytotoxicity and IL-8 gene expression induced by these photochemical gaseous mixtures using A549 cells, a human alveolar type II-like cell line and differentiated human bronchial cells.

The data presented here demonstrate that ISO and BD photochemical products increased LDH release as well as IL-8 expression as compared to their clean air control. The IL-8 mRNA and protein data indicate that both biogenic isoprene and anthropogenic butadiene as they react within the atmosphere generate products that are more potent inducers of IL-8 gene expression than the un-reacted VOC's. While exposure to ISO and BD photochemical products generated significant levels of cytotoxicity and IL-8 expression in A549 cells, no significant effects were observed in differentiated human bronchial epithelial cells. These data indicate that differentiated human bronchial epithelial cells are less sensitive to the products generated after photochemical transformation of ISO and BD than A549 cells. Differentiated human bronchial epithelial cells release and are covered by a thin layer of mucus (Gray et al. 1996), which serves as a protectant, against xenobiotics and inhaled gases (Schlosser 1999). Interactions and partitioning of inhaled agents within the mucus layer covering the epithelium of the tracheal-bronchial region can prevent these agents from reaching the underlying cell layer to induce cellular responses (Medinsky and Bond 2001). Alveolar epithelial cells lack such a protective mucus layer and could therefore be inherently more sensitive to inhaled gaseous species. Hence the differences observed here may represent regional differences in sensitivity within the respiratory epithelium. Another reason for the observed

differences in responses to ISO and BD photochemical products in A549 cells and differentiated human bronchial epithelial cells is that A549 cells are an immortalized cell line, while differentiated human bronchial epithelial cells are primary cells with a finite life span. Previous studies have shown that exposure of primary human bronchial epithelial cells and a bronchial epithelial cell line to ozone induced greater effects in the epithelial cell line than the primary bronchial epithelial cells (Samet et al. 1992).

One concern when analyzing the results of the photochemical degradation products for both ISO and BD was the production of ozone and its known toxic health effects. These experiments were designed to produce the smallest concentration of ozone while continuing to keep the desired primary products within realistic ambient exposure concentration ranges. Previous studies have shown that A549 epithelial cells exposed to ozone concentrations less than produced in these studies did increase IL-8 production (Jaspers et al. 1997), which would suggest that ozone is the major photochemical product causing the increase in IL-8 production. However, during the side by side experiment of ISO's and BD's photochemical products, the average concentration of ozone inside the chambers during the exposure was 120 ppb for ISO and 140 ppb for BD. Hence, if IL-8 production was solely based on ozone concentrations, BD's photochemical products would produce a greater IL-8 response than ISO's photochemical products. Since this is not the case, these data indicate that ozone is not the sole inducer of IL-8 protein secretion, but that other photochemical products generated during the reactions of ISO or BD with sunlight are causing the increase in IL-8 production. In addition, this suggests that while ozone concentrations are a good indicator of the adverse health potential of

photochemical smog, one has to examine the entire photochemical mixture to estimate its toxic effect on the exposed population.

Analyses by GC-FID and GC/MS of resulting photochemical ISO and BD atmospheric reaction products showed the formation of many known products. The main ISO first generation photochemical products formed were MACR, MVK, FORM and ozone while BD formed ACR, FORM and ozone. Both experiments produced trace amounts of acetaldehyde, CO, PAN and their respective monoxides. The sample results from GC and GC/MS analysis have not been explored for unknown products. Thus, several other photochemical products, besides ozone, could have significant effects on IL-8 production in human lung cells. We will examine the pro-inflammatory and cytotoxic potential of several of these individual photochemical products. Data derived from these experiments could suggest potential agents in both the ISO and BD experiments that enhanced IL-8 production. In addition, this study described the analysis of only known photochemical products formed during the ISO and BD reactions. Extensive chemical mechanism studies are reported in the literature for both pollutants (Atkinson 1990; Atkinson et. al. 1994; Atkinson and Arey 2003; Carter 1996; Liu et al. 1999; Palen et al. 1992; Sauer et al. 1999; Tuazon et al. 1990; Wayne et al.1991; Yu et al. 1995, Yu et al. 1997) but still the complete carbon balances have yet to be identified. Some of the unspecified products include multifunctional carbonyls such as hydroxyl carbonyls, dicarbonyls, and hydroxyl dicarbonyls (Liu et al. 1999; Tuazon et al.1990; Yu et al. 1995). Besides the mechanistic history of both chemicals, other experimental limitations of both sample collection and exposure design did not allow for the lung cells to be exposed to more polar compounds nor could these compounds be quantified.

Ongoing changes in our experimental set-up will allow for these more polar products to be studied in the future.

In this study the quantification of inflammatory potential for primary photochemical products was evaluated. Within the atmosphere these primary products typically continue to react forming secondary and tertiary products. For outdoor exposures, then, the results shown here emphasize, by design, the primary pollutants. These first generation products are continuously being produced as the precursors are continuously being emitted. But these primary pollutants continue to react as they are formed, not only limiting their maximum concentration, but producing secondary and tertiary products, which themselves have toxicity characteristics. The overall toxicity of pollutants must allow inclusion of residual primary, secondary and tertiary products formed. Forthcoming experiments will include the comparison of primary, secondary and tertiary products for different toxicity endpoints.

This study demonstrated the importance of considering photochemistry when decision making for outdoor air quality guidelines. Currently, air quality models include a predictive but not complete isoprene mechanism, that is, they do not include detailed chemistry of the products formed. The data presented here demonstrate that the first generation ISO photochemical products are more toxic than isoprene itself, which indicates the need to better understand and to include the chemical mechanisms of the photochemical products in air quality models. Some studies suggest that ISO oxidation can contribute significantly to the total ozone production rate during ozone episodes (Biesenthal et al. 1997). Ozone and its known adverse effects are of great health concern when creating guidelines related to exposure and release of pollutants that have the

potential to produce a large amount of ozone once released into the atmosphere. This indicates the importance of increasing the inclusion of detailed chemistry of even chemicals known to be biogenic such as ISO in photochemical models used to investigate ozone formation and health concerns to rural and urban outdoor exposures. This would enable people within the risk assessment community to make more realistic exposure guidelines using all sources of relevant toxicity, including non-anthropogenic sources that were previously overlooked.

## 8.6. Table and Figures

**Table 1 :Concentration Analysis for Chamber Constituents for ISO and BD Photochemically Reacted Systems (Extracted from EHP – Doyle et al. 2004)**

**Table 1.** Chemical analysis of chamber constituents.

| Chamber | ISO | | MACR | | MVK | | ISO MON | | BD | | Acrolein | | Acetaldehyde | | Form | | PAN | | $O_3$ | | NO | | $NO_2$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| side | Ave | Max | Ave | Max | Ave | Max | Ave | Max | Ave | Max | Ave | Max | Ave | Max | Ave | Max | Ave | Max | Ave | Max | Ave | Max | Ave | Max |
| AU2703 ISO+NO+light vs. ISO+NO | | | | | | | | | | | | | | | | | | | | | | | | |
| Light | 0.076 | 1.138 | 0.201 | 0.209 | 0.176 | 0.228 | 0.002 | 0.024 | 0.0 | 0.0 | 0.0 | 0.0 | 0.030 | 0.037 | 0.161 | 0.177 | 0.011 | 0.011 | 0.130 | 0.155 | 0.0 | 0.0 | 0.026 | 0.030 |
| Dark | 0.870 | 1.054 | 0.032 | 0.052 | 0.005 | 0.032 | 0.001 | 0.005 | 0.0 | 0.0 | 0.0 | 0.0 | 0.023 | 0.034 | 0.018 | NA | 0.001 | 0.001 | 0.008 | 0.012 | 0.0 | 0.0 | 0.026 | 0.077 |
| ST1003 BD+NO+light vs. BD+NO | | | | | | | | | | | | | | | | | | | | | | | | |
| Light | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.042 | 0.777 | 0.197 | 0.237 | 0.013 | 0.047 | 0.096 | 0.109 | 0.001 | 0.002 | 0.178 | 0.210 | 0.005 | 0.018 | 0.012 | 0.025 |
| Dark | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.593 | 0.738 | 0.029 | 0.171 | 0.010 | 0.054 | 0.004 | 0.004 | 0.001 | 0.001 | 0.011 | 0.295 | 0.010 | 0.031 | 0.022 | 0.045 |
| ST2403 BD+NO+light vs. BD+NO | | | | | | | | | | | | | | | | | | | | | | | | |
| Light | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.125 | 0.713 | 0.184 | 0.255 | 0.023 | 0.044 | 0.083 | 0.093 | 0.001 | 0.002 | 0.154 | 0.203 | 0.001 | 0.022 | 0.001 | 0.013 |
| Dark | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.686 | 0.753 | 0.017 | 0.033 | 0.005 | 0.017 | 0.004 | 0.004 | 0.001 | 0.001 | 0.011 | 0.060 | 0.001 | 0.019 | 0.022 | 0.037 |
| OC1303: ISO+NO+light vs. BD+NO+light | | | | | | | | | | | | | | | | | | | | | | | | |
| ISO | 0.116 | 1.040 | 0.176 | 0.202 | 0.153 | 0.205 | NA | NA | 0.0 | 0.0 | 0.0 | 0.0 | NA | NA | 0.102 | 0.103 | 0.010 | 0.011 | 0.118 | 0.156 | 0.001 | 0.005 | 0.013 | 0.022 |
| BD | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.170 | 0.625 | 0.197 | 0.206 | NA | NA | 0.087 | 0.092 | 0.001 | 0.002 | 0.146 | 0.177 | 0.001 | 0.007 | 0.012 | 0.024 |

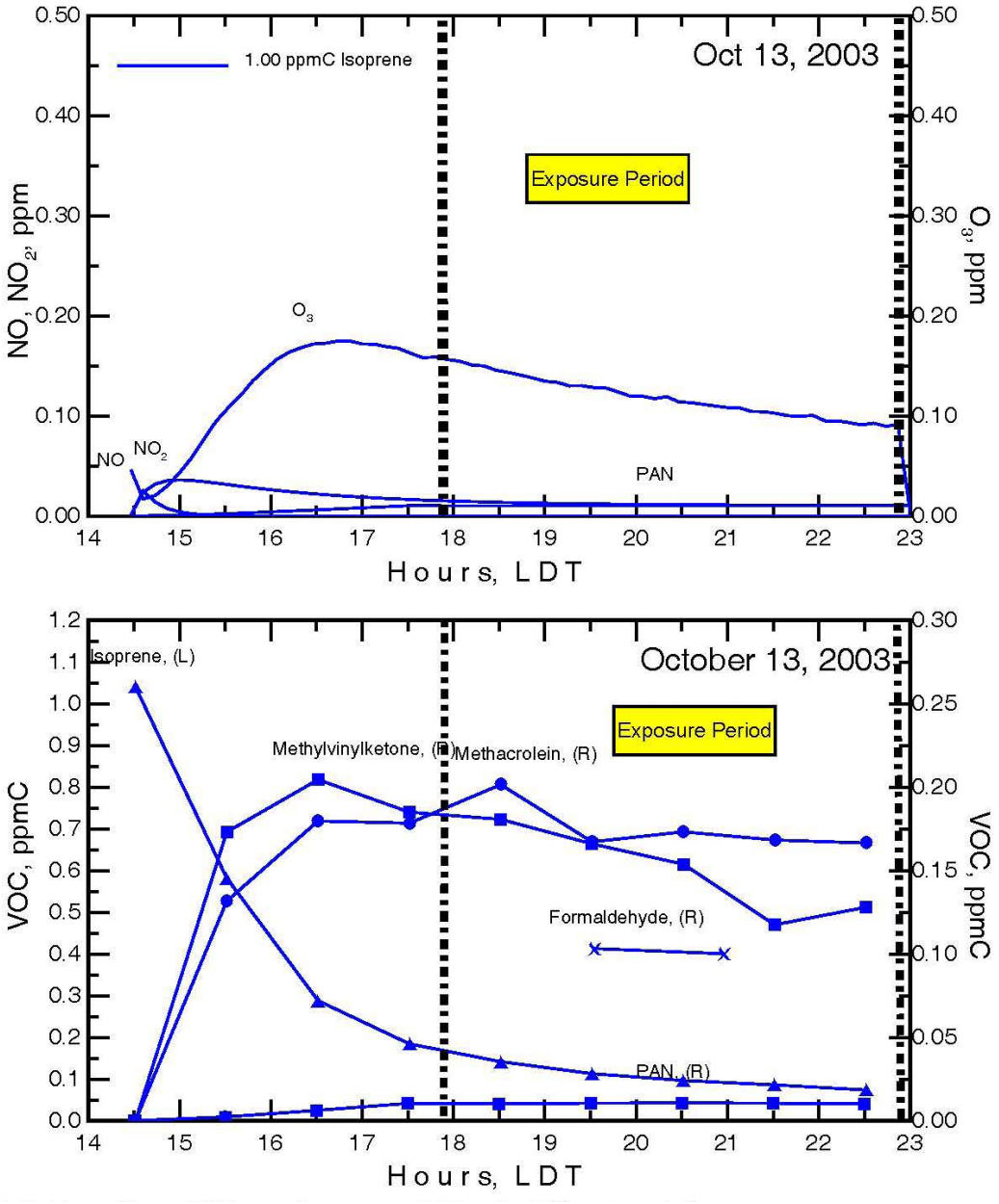


**Figure 8-1: Representative time series concentration plots for ISO photochemical reaction products.** 200ppbV ISO and 50 ppb NO underwent photochemical reactions from 4 hours before sunset until sundown producing primarily first generation photochemical degradation products. On each graph, the concentration for each chemical species is given at the specified LDT or local daylight time. Specifically, we measured isoprene (▲), methacrolein (●), and methylvinylketone (■). Concentrations for each species are given on either the left (L) or right (R) y-axis and are indicated as (L) or (R) after the respective chemical species. The dashed light represents the 5-hour period the cells were exposed to the chamber contents. Top: time course of $NO_2$ and $O_3$ formation after photochemical reaction of isoprene and NOx. Bottom: Decay of isoprene and production of methacrolein, methyl vinyl ketone, formaldehyde and PAN.

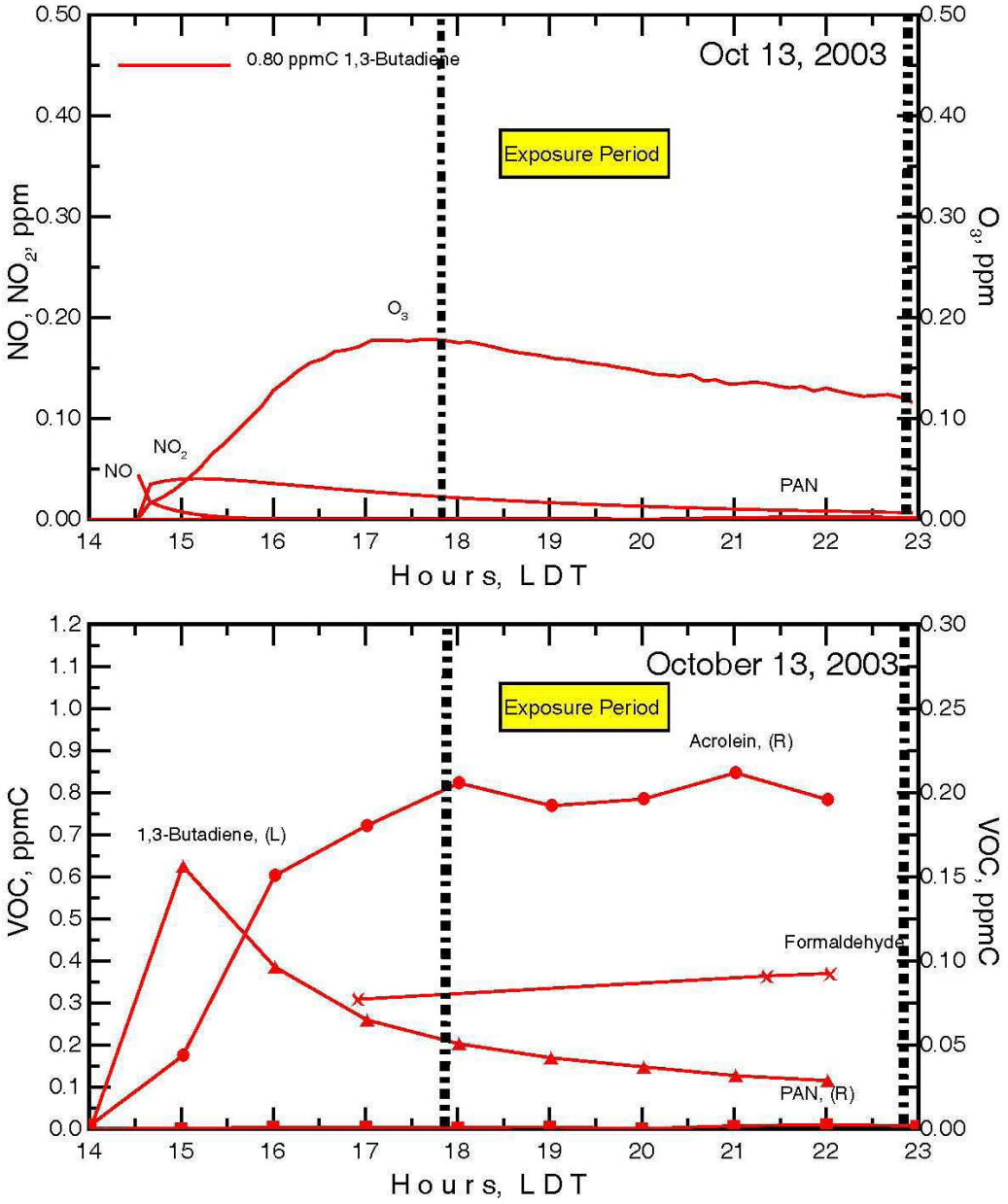


**Figure 8-2: Representative time series concentration plots for BD photochemical reaction products.** 200ppbV BD and 50 ppb NO underwent photochemical reactions from 4 hours before sunset until sundown producing primarily first generation photochemical degradation products. On each graph, the concentration for each chemical species is given at the specified LDT or local daylight time. Specifically, we measured 1,3-butadiene (▲), acrolein (●), formaldehyde (χ) and PAN (■). Concentrations for each species are given on either the left (L) or right (R) y-axis and are indicated as (L) or (R) after the respective chemical species. The dashed light represents the 5-hour period the cells were exposed to the chamber contents. Top: time course of $NO_2$ and $O_3$ formation after photochemical reaction of isoprene and NOx. Bottom: Decay of butadiene and production of acrolein, formaldehyde and PAN.

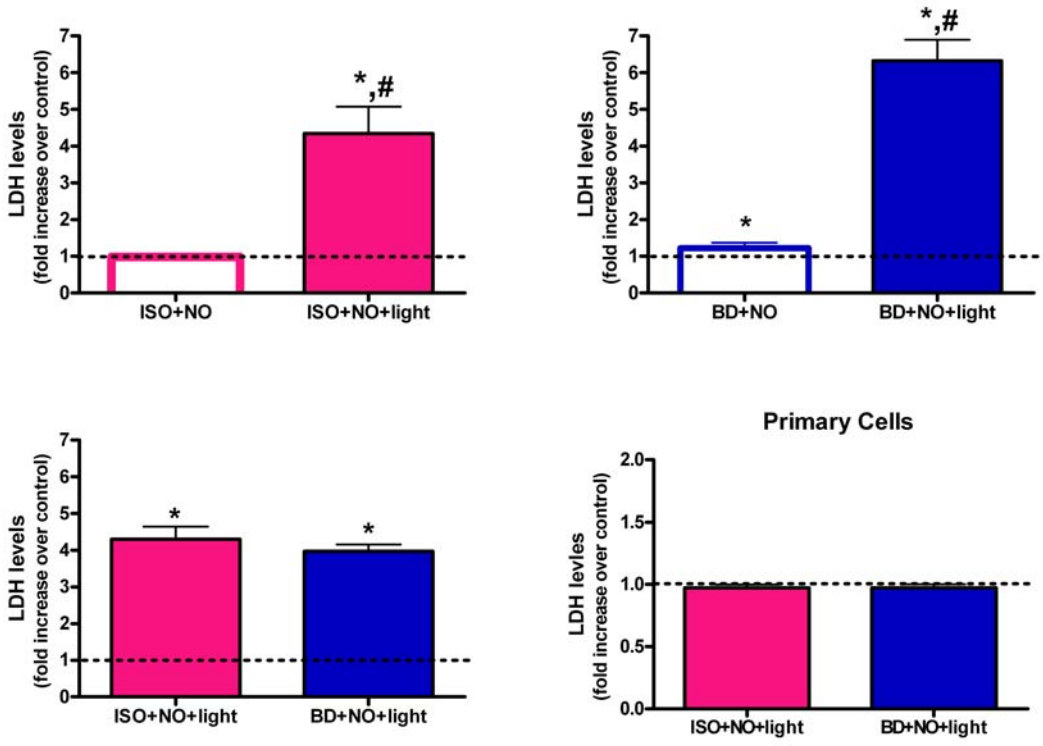


**Figure 8-3: Cytotoxicity Induced by Photochemically Reacted and Unreacted ISO and BD Mixtures.** Analysis of cytotoxicity induced by exposure to ISO, BD, or their photochemical degradation products. (A) 200 ppbV ISO and 50 ppb NO underwent photochemical reactions for approximately 5 hours or remained unreacted. (B) 200 ppbV BD and 50 ppb NO underwent photochemical reactions for approximately 5 hours or remained unreacted. (C) 200 ppbV ISO and 50 ppb NO in one chamber and 200 ppbV BD and 50 ppb NO in the other chamber underwent photochemical reactions for approximately 5 hours. (D) 200 ppbV ISO and 50 ppb NO in one chamber and 200 ppbV BD and 50 ppb NO in the other chamber underwent photochemical reactions for approximately 3 hours. At sundown, A549 cells (Experiments A, B and C) and differentiated human bronchial cells (Experiment D) were exposed to these mixtures for 5 hours. Post exposure cells were examined using collected supernatants and evaluated for cytotoxicity (LDH release). The results were expressed as fold increase over the control ± SEM. *Significantly different from the control; $P < 0.05$. #Significantly different from the other chamber side; $P < 0.05$.

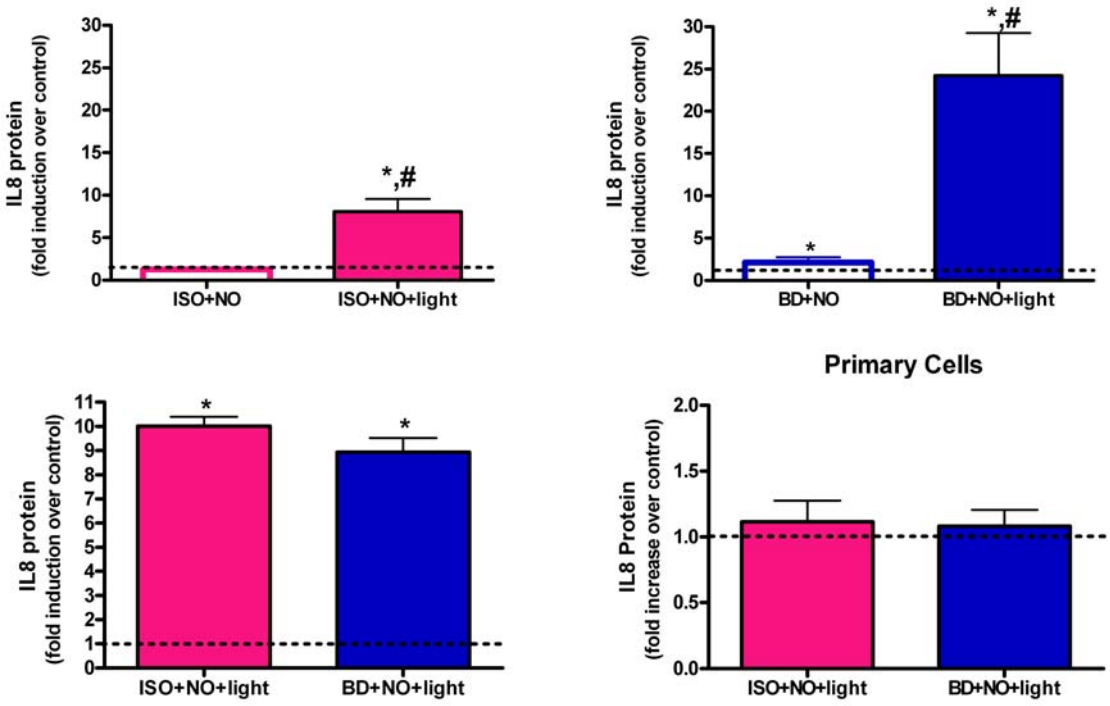


**Figure 8-4: IL-8 Protein Induced by Photochemically Reacted and Unreacted ISO and BD Mixtures.** Analysis of IL-8 protein induced by exposure to ISO, BD, or their photochemical degradation products using the supernatants secreted by A549 cells and tested with an ELISA kit. (A) 200 ppbV ISO and 50 ppb NO underwent photochemical reactions for approximately 5 hours or remained unreacted. (B) 200 ppbV BD and 50 ppb NO underwent photochemical reactions for approximately 5 hours or remained unreacted. (C) 200 ppbV ISO and 50 ppb NO in one chamber and 200 ppbV BD and 50 ppb NO in the other chamber underwent photochemical reactions for approximately 5 hours. (D) 200 ppbV ISO and 50 ppb NO in one chamber and 200 ppbV BD and 50 ppb NO in the other chamber underwent photochemical reactions for approximately 3 hours. At sundown, A549 cells (Experiments A, B and C) and differentiated human bronchial cells (Experiment D) were exposed to these mixtures for 5 hours. Post exposure, supernatants were collected from the basolateral chambers and analyzed. IL-8 protein levels were normalized to the control and expressed as mean fold increase over the control ± SEM. *Significantly different from the control; $P < 0.05$. #Significantly different from the other chamber side; $P < 0.05$.

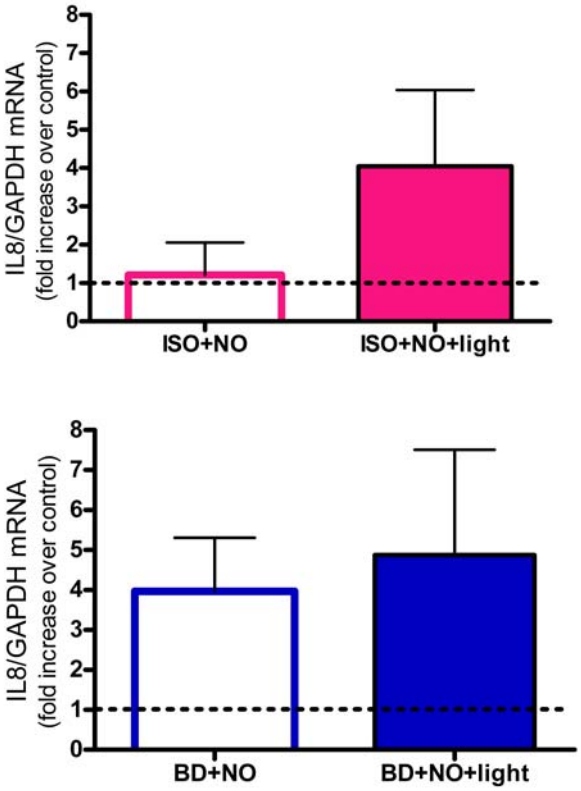


**Figure 8-5: IL-8 mRNA Induced by Photochemically Reacted and Unreacted ISO and BD Mixtures**
Analysis of IL-8 mRNA levels released by exposure to ISO, BD, or their photochemical degradation products by A549 cells. (A) 200 ppbV ISO and 50 ppb NO underwent photochemical reactions for approximately 5 hours or remained unreacted. (B) 200 ppbV BD and 50 ppb NO underwent photochemical reactions for approximately 5 hours or remained unreacted. At sundown, A549 cells were exposed to these mixtures for 5 hours. Post exposure, total RNA was isolated using Trizol as per the suppliers instructions and analyzed for IL-8 mRNA levels by real-time RT-PCR. IL-8 mRNA levels were normalized to the control and expressed as mean fold increase over the control ± SEM.

# 9. Atmospheric Photochemical Transformations Enhance 1,3-Butadiene-induced Inflammatory Responses in Human Epithelial Cells: The role of ozone and other photochemical degradation products

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CBI 5295

## 9.1. Abstract

Chemistry of hazardous air pollutants has been studied for many years, yet little is known about how these chemicals, once reacted within urban atmospheres, affect healthy and susceptible individuals. Once released into the atmosphere, 1,3-butadiene (BD) reacts with hydroxyl radicals and ozone (created by photochemical processes), to produce many identified and unidentified products. Once this transformation has occurred, the toxic potential of atmospheric pollutants such as BD in the ambient environment is currently unclear. During this study, environmental irradiation chambers (also called smog chambers), utilizing natural sunlight, were used to create photochemical transformations of BD. The smog chamber/*in vitro* exposure system was designed to investigate the toxicity of chemicals before and after photochemical reactions and to investigate interactions with the urban atmosphere using representative *in vitro* samples.

In this study, we determined the relative toxicity and inflammatory gene expression induced by coupling smog chamber atmospheres with an *in vitro* system to expose human respiratory epithelial cells to BD, BD's photochemical degradation products, or the equivalent ozone generated within the photochemical mixture. Exposure to the photochemically generated products of BD (primarily acrolein, acetaldehyde, formaldehyde, furan and ozone) induced significant increases in cytotoxicity, IL-8, and IL-6 gene expression compared to a synthetic mixture of primary products that was created by injecting the correct concentrations of the detected products from the irradiation experiments. Interestingly, exposure to the equivalent levels of ozone generated during the photochemical transformation of BD did not induce the same level of inflammatory cytokine release for either exposure protocol, suggesting that the effects

from ozone alone do not account for the entire response in the irradiation experiments. These results indicate that BD's full photochemical product generation and interactions, rather than ozone alone, must be carefully evaluated when investigating the possible adverse health effects to BD exposures. The research presented here takes into account that photochemical transformations of hazardous air pollutants (HAPs) does generate a dynamic exposure system and therefore provides a more realistic approach to estimate the toxicity of ambient air pollutants once they are released into the atmosphere.

## 9.2. Background

Although many studies have shown the adverse effects of BD and its known photochemical transformation products, considered separately, little literature could be found that studied realistic ambient concentrations, non-carcinogenic end-points, or short-term exposures. The UNC smog chambers have been used for more than 30 years to investigate and develop chemical mechanisms of atmospheric species including all of those used in regulatory air quality models. Recently, these chambers have been combined with an *in vitro* cell exposure system to better facilitate air quality research. This system was created to allow the study of effects occurring in human respiratory epithelial cells, using complete photochemical transformation mixtures naturally generated in ambient environmental conditions and without altering the natural chemical mixture state.

BD and all of its *known* primary or first generation photochemical products (primarily ACR, FORM, acetaldehyde, furan, ozone) are known respiratory irritants (Gomes and Meek 2002; Krishna et al. 1997; Lippmann 1989; Liteplo et al. 2002; Mehlman and Borek 1987; NTP 1994; WHO 1978, 1989, 1992, and 1995), but no literature could be found on what respiratory effects might occur with these irritants when an individual is simultaneously exposed to them all, for example in a neighborhood downwind of a polymer facility after an unplanned release of BD from their process system.

One large concern when working with pollutant mixtures and testing their toxicity on human respiratory epithelial cells is how to identify which toxic agent is causing the adverse effects. This paper illustrates one method to attack this problem. Here we examine the cytotoxicity and inflammatory gene expression induced from exposure 1) to the whole BD reaction system product set; 2) to ozone alone, but at concentrations equal to those generated during photochemical reactions of BD complex mixtures, and 3) to synthetic mixtures of the detected and quantified products of BD observed in the full photochemical system mixture. We can therefore assess the importance of the BD products and more importantly, assess the importance of the detected-not qualified and the undetected but present products.

This study is an extension of previous work examining the adverse effects of BD on human respiratory epithelial cells (Doyle et al. 2004). Those results indicated that photochemical transformations significantly alter the toxicity of BD mixtures when examining cytotoxicity and IL8 gene expression only. Taken together with other published findings, this study was conducted to evaluate the toxic potential of BD's

photochemical transformation products, and to possibly decipher which photochemical transformation product, if any, significantly contributed to the overall response found from the full photochemically generated product mixture.

## *9.3. Experimental Section*

Two injection protocols were used to discriminate the effects induced by different products generated during photochemically active systems representative of BD released into the environment. The chemical system requires oxides of nitrogen (NO and $NO_2$) as well as the VOC to react. Here on one side of the chamber, we injected 200 ppbV butadiene (National Specialty Gases, Durham, NC) and 50 ppb NO in the early afternoon and these were allowed to react with sunlight until sundown (approximately 5 hours). After sundown, one of two different systems were created in the second chamber: 1) a synthetic blend of BD's first generation products (acrolein, ozone, and formaldehyde) equal to the concentrations detected in the other chamber or 2) the amount of ozone alone that was created during BD's reaction. Using these two protocols, we can evaluate not only the effects due to the ozone created in the photochemically active system, but also the combined effects due to a majority of the known, first generation products.

## 9.4. Results

In this study human respiratory epithelial cells were exposed to the primary degradation products formed during photochemical transformations of BD and nitric oxides in natural systems and examined for cytotoxicity and cytokine gene expression.

The products generated by photochemical transformations of BD and nitric oxides were identified and confirmed using GC/MS. Table 1 summarizes the average and maximum concentrations produced for the known and quantifiable photochemical products derived from BD during the cellular exposure period. Although a majority of the products were quantified and used to create the synthetic product mixture (acrolein, formaldehyde and ozone), other known products were found at significantly smaller concentrations. During each of the experiments, the cells were exposed to practically the same concentrations of transformation products (within +/- 0.010 ppm). Cytotoxicity or cell viability was determined through calculation of LDH release induced by exposure to the photochemical degradation products of BD, or ozone. Figure 9-1 displays the LDH response induced by BD's generated photochemical product mixture, the synthetic blend of BD products, or ozone alone. BD's primary photochemical products (a synthetic mixture of acrolein, formaldehyde and ozone) account for a statistically significant portion of the LDH response observed from the full product mixture generated within the smog chambers, compared to the response of the ozone alone.

To examine the proinflammatory potential of BD's transformation products, we compared the effects of BD's generated photochemical products, a synthetic mixture of measured primary products (acrolein, formaldehyde and ozone), or the amount of ozone generated during the photochemical processes using A549 cells. Figures 9-2 and 9-3

show the IL-8 and IL-6 response induced by exposures to product mixtures using the smog chamber – *in vitro* system. For both mediator responses, the data indicates an increasing step-wise response from ozone, to ozone and the primary product mixture, to the full generated photochemical products. Unlike cytotoxicity, neither the ozone nor the mixture of primary products significantly account for a portion of the IL-8 or IL-6 inflammatory response found.

## 9.5. Discussion and Conclusions

Previous work using smog chambers to study environmental toxicology have demonstrated the importance of examining the photochemical transformations of pollutants when analyzing their toxic potential once released into the atmosphere (Doyle et al. 2004; Sexton et al. 2004). While research has been done on the individual products formed and their adverse respiratory effects (Krishna et al. 1997; Lippmann 1989; Liteplo et al. 2002; Mehlman and Borek 1987; NTP 1994; WHO 1978, 1989, 1992, and 1995), almost no work could be found that examined how combined exposures to these pollutants may alter their overall toxicity either through synergism or antagonism. Our system which uses outdoor irradiation chambers combined with an *in vitro* exposure unit, provides an improved method to study photochemically active pollutants in a holistic setting. This approach enables all of the photochemical transformation products, including the undetected and detected but unknown products, to be generated in proper relative ratio to one another. In this study, we examined cytotoxicity and the release of proinflammatory mediators induced by BD/NOx photochemical gaseous mixtures using

A549 cells, a human alveolar type II-like cell line. Indicators used as markers of inflammation were IL-6 and IL-8.

This unique system was used to discriminate between the responses induced by the majority of individual products formed during BD's photochemical transformations in the atmosphere. Although many products are generated during these reactions (and are previously mentioned), the synthetic product mixture representative of the known, first generation products included only acrolein, formaldehyde and ozone. These products account for a large portion of those initially generated and were measured with high certainty using GC and GCMS techniques. The limitations of the hydrocarbon measuring techniques used in this study did not allow for the quantification of other known products such butadiene monoxide, glycolaldehyde, and glycidaldehyde; however they were generated in small quantities and could be detected.

During the study, cells were exposed to BD/NOx product concentrations similar to those that could be found in the ambient environment. All three exposure protocols (BD's generated photochemical products, the synthetic mixture of BD first generation products, and the amount of ozone generated during the photochemical system) induced significant increases in LDH, IL-8 and IL-6, compared to the clean air control. While acute exposures to the combined mixture of BD's photochemical products caused significant induction of proinflammatory mediators, cell viability may be driven primarily by the concentrations of ozone generated within each system. Other products formed through BD transformations with known toxicities, i.e. furan, butadiene monoxide and PAN, have been measured during previous experiments using the outdoor environmental chambers. Although previous studies have shown these products to cause

adverse effects at higher concentrations, they were not added to the synthetic mixture because of the small amounts generated using the current study's experimental protocol. Thus, other known products generated from photochemical transformations of BD need to be evaluated for possible interactions when combined with those evaluated here. More importantly is the significant difference in the inflammatory response induced by the full photochemically generated BD products and that of the synthetic blend or mixture of primary products. This suggests that both detected but unknown and detected but not quantified products, even those formed in small quantities during the photochemical transformations of BD, play an important role in the induction of proinflammatory mediators in A549 cells.

Currently we are unable to quantify the individual toxicities of the unknown or unspecified products, therefore demonstrating the importance of additional studies to create new chemical mechanisms for photochemical transformation of hazardous pollutants once released into the atmosphere. Developing these mechanisms to identify new products could be used to discern possible interactions between the photochemical transformation products generated from BD. In addition, more controlled exposure studies are necessary to determine the potential mechanisms by which exposure to BD photochemical transformation products enhance toxicity.

Taken together, the data presented here demonstrate that exposure to BD's photochemical transformation products induce acute inflammatory responses in human respiratory epithelial cells. Although the amount of ozone generated from these conditions contributes to a portion of the given response indicators, it does not, however, account for the IL8 and IL6 response. Thus, even though ozone concentrations may be a

good indicator of the adverse health potential of photochemical smog, we must examine the entire photochemical mixtures that are produced, each of the products and their subsequent reactions, to estimate the toxicity on the exposed population.

While the mixtures caused an acute inflammatory response that could be responsible for potential adverse respiratory effects, other data evaluating cytokine expression in the A549 cells observed during these experiments indicates that these protective responses do not cause irreversible, cellular damage therefore repair, differentiation, and proliferation post inflammation is expected (data not shown). To the best of our knowledge, this technique is the only system that enables the evaluation of the adverse effects of photochemically reactive HAPs under controlled conditions while studying both the known and unknown products. This study indicates that use of outdoor environmental chambers is an essential and valuable tool when examining the toxicity of atmospheric mixtures representative of urban environments. Overall, the unique net benefit of this research approach is the integrated results from a single source of experiments. They include time-series concentration measurements for many pollutants generated during smog chamber experiments and the toxicological results from the *in vitro* exposures. Combined, these can be used to further develop air quality simulation models needed to predict the transformation products necessary for exposure analyses and relative risk assessment calculations.

## 9.6. Tables and Figures

**Table 2 : Chemical Analysis of Chamber Constituents**

| | BD | | Acrolein | | FORM | | Ozone | |
|---|---|---|---|---|---|---|---|---|
| | **Ave** | **Max** | **Ave** | **Max** | **Ave** | **Max** | **Ave** | **Max** |
| **BD+NOx+light** | 0.072 | 0.056 | 0.190 | 0.202 | 0.083 | 0.093 | 0.146 | 0.177 |
| **Syn. Pro mixture** | 0.00 | 0.00 | 0.180 | 0.198 | 0.079 | 0.087 | 0.155 | 0.185 |
| **Ozone** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.160 | 0.182 |

**Chamber concentrations (ppmC):** Ave. is the average concentration the cells were exposed to over the 5-hour period and Max. is the maximum concentration produced during the experiment. Syn. Pro mixture is the synthetic butadiene product mixture.

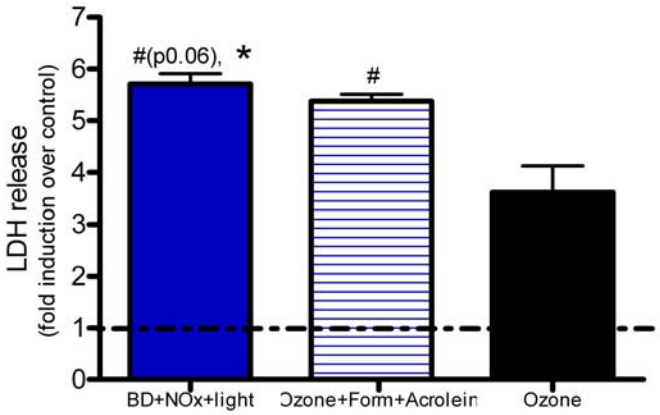



**Figure 9-1: Cytotoxicity, indicated by increased LDH release, was examined using chamber mixtures.** The mixtures compared in the study include: 1) the photochemically generated products of BD, 2) a synthetic blend of BD's first generation products (acrolein, ozone, and formaldehyde), and 3) the amount of ozone in the synthetic mixture. The results were expressed as fold increase over the control ± SEM. # Statistically significant compared to ozone p≤.05. *Statistically significant compared to synthetic BD product mixture.

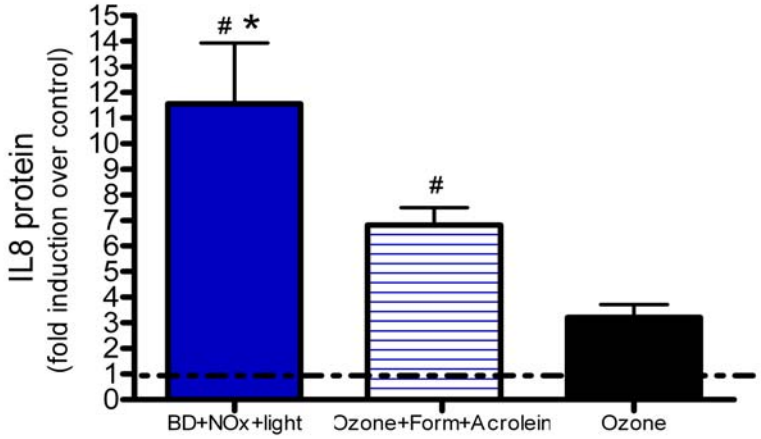



**Figure 9-2: Analysis of IL-8 was examined for BD chamber mixtures.** Analysis of IL-8, indicated by increase in protein release, was examined for BD chamber mixtures. The mixtures compared in the study include: 1) the photochemically generated products of BD, 2) a synthetic blend of BD's first generation products (acrolein, ozone, and formaldehyde), and 3) the amount of ozone in the synthetic mixture. The results were expressed as fold increase over the control ± SEM. # Statistically significant compared to ozone p≤.05. *Statistically significant compared to synthetic BD product mixture.

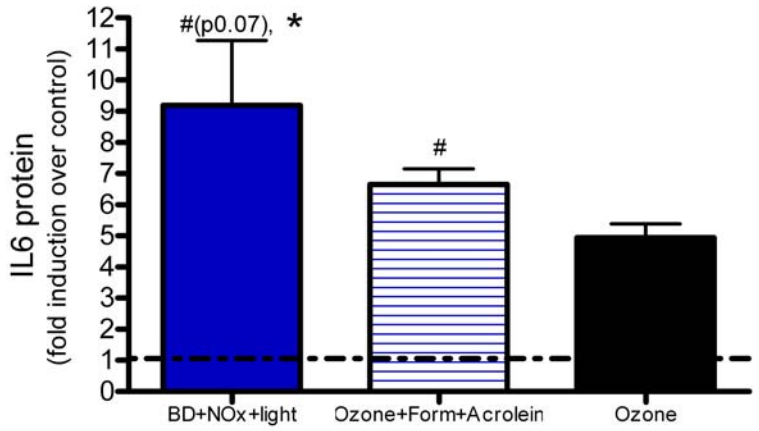



**Figure 9-3: Analysis of IL-6 was examined for BD chamber mixtures.**

The mixtures compared in the study include: 1) the photochemically generated products of BD, 2) a synthetic blend of BD's first generation products (acrolein, ozone, and formaldehyde), and 3) the amount of ozone in the synthetic mixture. The results were expressed as fold increase over the control ± SEM. # Statistically significant compared to ozone $p \leq .05$. *Statistically significant compared to synthetic BD product mixture.

# 10. Differentiating the Individual Inflammatory Response induced by Photochemical Transformation Products of Isoprene and Methanol

(in draft form)

## *10.1. Abstract*

The chemistry of hazardous air pollutants (HAPs) has been studied for many years, yet less is known about how these chemicals affect healthy and susceptible individuals once they enter *and react* in the atmosphere. Once released into the atmosphere, HAPs interact with hydroxyl radicals and ozone (created by photochemical processes), to produce products that depending on the complexity of the chemical species, may include detected and unidentified products and also undetected products (except by mass balance analysis). Once these chemical reactions occur, the toxic potential of several known HAPs in ambient environmental conditions is currently unclear. In this study, two chemical systems were chosen that represent a simplistic and a complex photochemical reaction system, each generating a range of photochemical degradation products, including formaldehyde and ozone. The objective was to determine whether the ozone produced in the reactions of these two model species was the primary cause of inflammation and cytotoxicity responses in human respiratory epithelial cells that were being exposed to HAPs mixtures undergoing photochemical reactions that create ozone and oxygenated organic products. This was accomplished by incorporating advanced smog chamber operation with toxicological techniques to

examine adverse effects on human lung cells in a realistic condition. The adverse effects were detected by measuring increased cytotoxicity (LDH release) and cytokine release (IL-8 gene expression). Exposure to the photochemically generated products of isoprene induced significant increases in cytotoxicity and IL-8 gene expression compared to exposures to just the amount of ozone generated or to exposures to a synthetic mixture of the major products (methyl vinyl ketone, methacrolein, and ozone) that were monitored during the HAP oxidation. In the case of the methanol experiments, however, the overall response generated from methanol's photochemically generated products, including ozone, were not statistically different than the effects created by just the amount of ozone generated in this system. These results indicate that in more complex atmospheric systems capable of forming complex oxygenated products, ozone alone does not significantly account for the effects. For these types of compounds, a full photochemical product evaluation may be needed to adequately characterize respiratory adverse effects arising from the emission of these types of compounds.

## *10.2. Background*

Atmospheric pollutants, e.g. isoprene (ISO) and methanol (METH), become photochemically active during the day reacting with radical sources and ozone created during photochemical processes to generate products with different toxicity than their parent pollutants. Through these reactions many identified and some specified but unidentified products are generated which commonly include the formation of ozone, FORM, acetaldehyde and $CO_2$. Although the chemistry of the atmospheric pollutants examined during this project has been studied extensively, their full photochemical

transformation mechanisms are still not fully identified. In this study A549 cells were exposed to the photochemical degradation products of METH or ISO, both generated from environmentally realistic exposure concentrations. These two systems were chosen for examination so we could compare and contrast two important chemicals found both in the environment and industry, which differ in their complexity of photochemistry therefore allowing us to study both simple and more complex mixture effects.

One large concern when working with pollutant mixtures and testing their toxicity on human respiratory epithelial cells was which toxic agent is causing the adverse effects. The focus of this part was to examine if the cytotoxicity and inflammatory gene expression induced from exposure to ozone alone, at concentrations equal to those generated during photochemical reactions of complex mixtures, cause similar responses than found during the our experiments.

This study described the first in a series of experiments to examine which of the photochemically generated products are causing the toxicity seen in previous studies examining complex mixtures in urban-like settings. Previously we have examined the cytotoxicity and IL-8 gene expression of ISO, BD, propylene and the urban smog mixture (Doyle et al. 2004; Sexton et al. 2004). The objective was to analyze whether the major products formed during the photochemical reactions of smog components significantly alters the toxicity of these mixtures more than if the cells were exposed alone to the amount of ozone generated from these systems. This would demonstrate the importance of studying not only the amount of ozone generated during pollutant episodes and its suspected adverse effects, but also the other possibly more toxic components that are generated simultaneously with ozone.

## *10.3. Experimental Design*

During this study, two types of injection procedures were used to determine whether the toxicity induced by the full photochemically generated product mixture was due to the amount of produced ozone or the other known first generation products. The experimental design is shown below in Figure 10-1. During both protocols, hydrocarbon [4.5 ppm METH (99.9%, Sigma-Aldrich, St. Louis, MO) or 200 ppbV ISO (99%, Sigma-Aldrich, St. Louis, MO)] and 50 ppb NOx mixture were injected into one side of the chamber and allowed to react with the sunlight until sundown. After sundown, two different injections were made to allow us to compare the effects of the full photochemical degradation products generated during an experiment with both the ozone made during the chemical transformations or to the mixture of the major products that are formed without having other chamber/experimental variables to have to account for during the chamber run. To compare ozone only effects, the amount of ozone that had been generated during the time ISO or METH was allowed to photochemically react was injected into the opposite chamber side. To test the effects of the major initial products, injections were made with concentrations of the first generation products equal to what was generated during the photochemically active period, i.e. MACR, MVK, and ozone.

The exposure to the lung cells began after sundown when photochemical reactions were terminated from the absence of sunlight. This short photochemical experimental design, conducted at the end of the day, stops the photo-oxidation sequence where the first generation or initial products are near their maximum concentration. Cells were exposed to the gaseous mixtures for 5 hours. After the exposures, all sets of cells

were kept in the control air + 5% $CO_2$ exposure chamber until transport back to the UNC-CH campus.

## *10.4. Results*

This study analyzed how much ozone, one of the major photochemical degradation products formed during reactions of hydrocarbons and NOx contributed to the cytotoxicity and release of the inflammatory mediator IL-8 in respiratory epithelial cells following exposures to reacted and unreacted ISO and METH chamber mixtures.

The photochemical degradation products of ISO and METH were measured using GC/GCMS methods. During each experiment, product concentrations generated inside the environmental chambers, and used for the exposure of human respiratory epithelial cells were within ± 10%, comparing the photochemically generated products to the synthetic product mixtures. During multiple experimental days, cells were exposed to mixtures consisting of: ISO products [201 ppb MACR, 176 ppb MVK, and 130 ppb $O_3$] and METH products [290 ppb $O_3$ and 160 ppb FORM]. The products measured and used for the synthetic product mixtures are not the only photochemical degradation products generated from the transformation of ISO and METH; however, they are a majority of the known, first generation products created within each system.

To determine the effects on cell viability, we analyzed the relative increase in cytotoxicity induced by exposure to the photochemical degradation products of ISO and METH, or $O_3$. Results from experiments performed on different days were combined,

and cytotoxicity induced by ISO and METH's first generation photochemical products or the respective concentration of $O_3$ generated during those experiments, were expressed as fold increase over the clean air control. Described in Figure 10-2, cell viability or cytotoxicity was measured by the release of LDH from human respiratory epithelial cells. During the three types of METH product exposures (METH's generated photochemical products, the METH synthetic product mixture, and the concentration of ozone present is the synthetic mixture) there was no significant difference in cell death via LDH induced when compared to one another. Each of the chamber mixtures induced approximately a 3.5-fold increase compared to the clean air control. Unlike the METH results, the photochemical products generated from ISO and nitric oxide mixtures induced a significant increase in LDH compared to the synthetic product mixture (MACR, MVK, $O_3$) and the $O_3$ alone.

To examine the proinflammatory potential of the photochemical products of ISO and METH IL-8 gene expression released by A549 cells was examined. The data in Figure 10-3 showed that similar to the METH cytotoxicity results, the amount of $O_3$ that was formed during METH's photochemical transformation and injected into the opposite chamber side resulted in a release of IL-8 protein that did not statistically differ from the amount induced from the naturally generated products (primarily FORM and $O_3$O3). As expected the products generated by ISO and nitric oxides induced the largest increase in IL-8 protein release. However, different from the cytotoxicity results, the synthetic ISO product mixture also induced a significant release of IL-8 protein compared to the ozone alone.

In addition to IL-8 protein, mRNA levels were measured to confirm the protein results. Similar to the previous IL-8 data, the chamber mixtures representative of the different ISO photochemical product mixtures induced a step-wise response with the naturally generated products producing the largest amount of inflammation. Unlike the protein, these differences were not statistically significant. The METH results agreed with the protein illustrating approximately a 2.5-fold increase in IL-8 mRNA compared to the control.

## *10.5. Discussion and Conclusions*

Previous work using the smog chamber / *in vitro* exposure system has emphasized the importance of examining full chemical schemes of photochemical mechanisms when dealing with atmospheric pollutants. Although photochemical transformations were shown to be areas of interest when demonstrating the respiratory health effects of pollutants, the overall importance of using ozone as a target focus area for research to examine the inflammatory response was not discussed. The main objective of this study was to determine whether ozone was the causative agent inducing cytotoxicity and inflammation from exposure to photochemically generated mixtures of atmospheric pollutants.

Two systems were chosen to represent both simple and complex photochemical reactions yielding known and unknown secondary products. These systems were chosen to compare and contrast METH and ISO, two chemicals found both in the environment and industry, which differ in their chemical complexity and known respiratory response. Overall, the data presented here suggest that in addition to the $O_3$ and FORM generated

during photochemical transformations of complex pollutants (i.e. ISO) with radical sources, the other photochemical degradation products exert significant stress on the respiratory epithelial cells inducing cytotoxicity and inflammation. Therefore, emphasizing the evaluation of toxicological profiles on all transformation products generated from photochemical systems.

The ISO results agree with Rohr et al. (2002) where they found the inflammatory effects from the products generated by reacting isoprene and ozone (which creates many of the same reaction products) were greater than the effects of $O_3$ alone. Although this was true for ISO, for METH, where the main photochemical products generated were $O_3$ and FORM, interestingly, the amount of $O_3$ induced the same IL-8 gene expression and cytotoxicity as the complete product mixture.

To understand if this trend was true for other inflammatory cytokines a Multiplex bead system was used to measure multiple cytokines using a small sample amount. Figures 10-4 illustrate the results for IL-6 and vascular endothelial growth factor (VEGF). Other cytokines (MCP-1, FGF2, IP10, and GMCSF) were measured but were below detection limits.

The IL-6 protein results for ISO were similar to the IL-8, whereas the $O_3$ did not induce a large response when compared to the generated products or the synthetic product mixture. In addition, the ISO synthetic product mixture exhibited an increase compared to the $O_3$ only exposure; however the complete photochemical mixture contributed to the greatest response. $O_3$ and FORM combined generated the same amount of inflammation via IL-6 compared to the complete photochemically generated

mixture. Different than the IL-8 results, the synthetic METH product mixture produced a greater response than the ozone alone (although not significant). This suggests that FORM plays an important role in the cellular release of IL-6 after exposure to these mixtures. Interestingly, with this mediator neither $O_3$ exposure (130 or 280 ppb) generated a response greater than the clean air control during the given rest period.

Similar to LDH, VEGF evaluates the permeability of epithelial cells (Thickett et al. 2001). The exact functional role of VEGF secretion by alveolar epithelial cells remains unclear, yet it is hypothesized by Ozawa et al. (2001) that VEGF secreted into the basolateral side acts as an ozone sensor molecule for reactive oxygen species (ROS). Similar to their findings, Kuroki et al.(1996) used anti-oxidants to demonstrate that ROS control is critical to the expression of VEGF *in vitro* and *in vivo*. Furthermore, exposures inducing strong inflammatory responses in epithelial cells leading to the release of inflammatory mediators such as IL-6 and IL-8 suggest a dose response increase in other mediators such as VEGF (Chauhan et al. 2005).

Many studies have shown that VEGF plays a significant role in the development of acute lung injury and acute respiratory diseases after various inhalation exposures (Becker et al. 2000, 2006; Kaner et al. 2000; Karmpaliotis et al. 2002). The VEGF results are comparative to LDH, both evaluating the strength of the cell membrane after exposure to environmental pollutants. In the ISO studies, the photochemically generated products induced a significantly greater release of VEGF compared to both the synthetic product mixture and $O_3$. Different from the LDH results, the photochemically generated products of METH induced a significant increase in VEGF compared to the METH synthetic product mixture and also $O_3$. Studies have shown that FORM and $O_3$ do not

cause interactive or synergistic effects within the deep lung, therefore any non-additive effects would occur in the nasal epithelium not in our model (U.S. EPA 2006). In addition, Mautz 2003 evaluated the effects induced by inhalation of FORM and found that it does not penetrate to the lower respiratory tract like the other photochemical reaction products, therefore some of the results may be higher than expected since alveolar cells are not a typical target of FORM. Therefore it may be that some product generated in a small concentration that is only found in the reacted mixture is significantly altering the response indicated.

Several groups have studied the relationship between proinflammatory mediators and endothelial permeability. Their findings suggest that increased concentrations of proinflammatory cytokines observed during periods of prolongs exposures to reactive oxygen species causes reversible changes in endothelial permeability (indicated by the release of VEGF) (Ali et al. 1999; Chua et al. 1998; Simon et al. 1998).

Taken together, these findings suggest that although $O_3$ may be a good indicator of adverse health potential of stable environments, when examining reactive pollutants, alone $O_3$ does not accurately estimate effects induced by more complex photochemical mixtures representative of those found in urban atmospheres.

## *10.6. Figures*

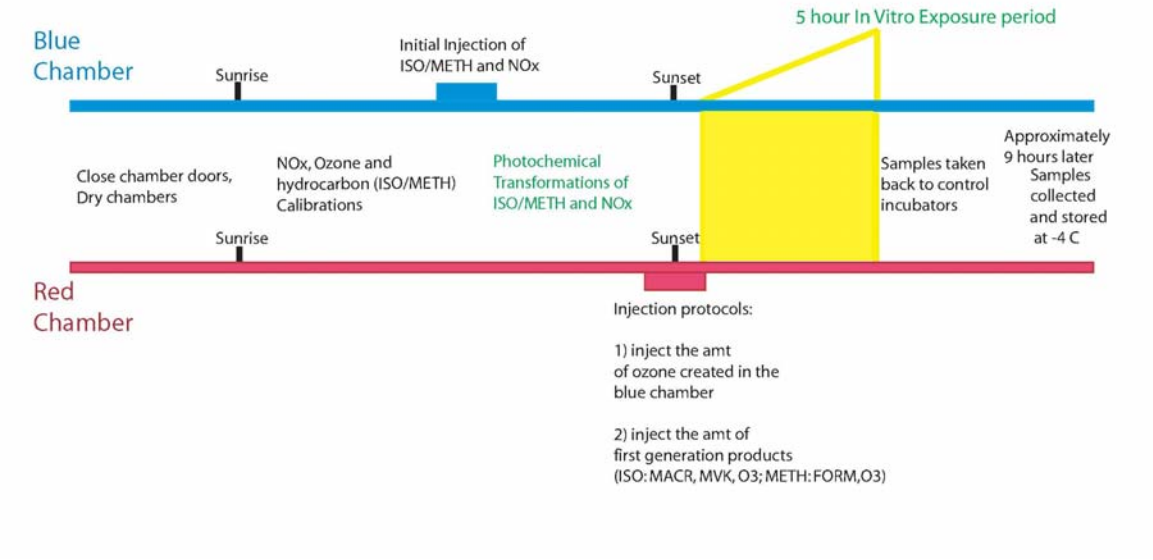



**Figure 10-1: Isoprene and Methanol Experimental Design**

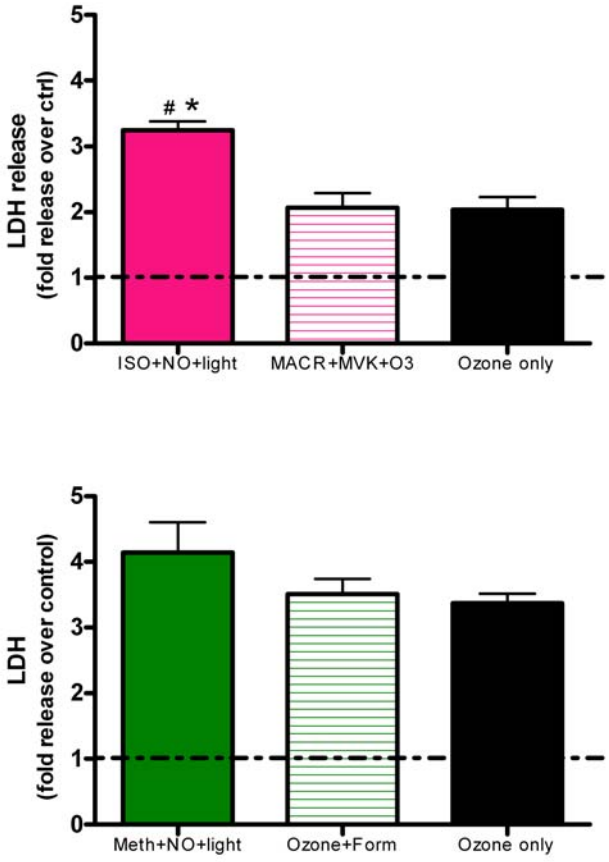



**Figure 10-2: Cytotoxicity, indicated by increased LDH release, was examined using chamber mixtures consisting of ISO and METH photochemical transformation products.**

The mixtures compared in the study include: 1) the photochemically generated products, 2) a synthetic mixture of first generation products (ISO:MACR+MVK+ $O_3$, METH:FORM+ $O_3$), and 3) the concentration of ozone in the synthetic mixture. The results were expressed as fold increase over the control ± SEM. # Statistically significant compared to ozone $p \leq .05$. *Statistically significant compared to synthetic product mixture.

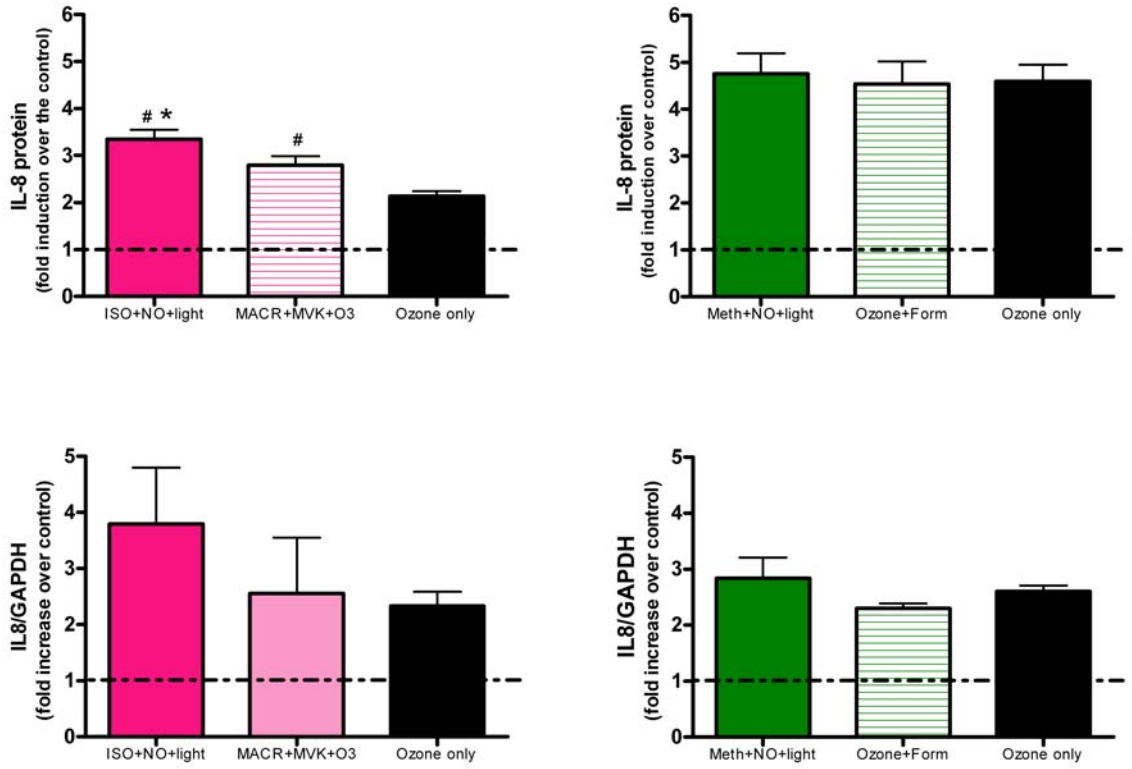



**Figure 10-3: Inflammation, indicated by increased IL-8 release, was examined using chamber mixtures consisting of ISO and METH photochemical transformation products.**

The mixtures compared in the study include: 1) the photochemically generated products, 2) a synthetic mixture of first generation products (ISO:MACR+MVK+ $O_3$, METH:FORM+ $O_3$), and 3) the concentration of ozone in the synthetic mixture. Protein and mRNA were analyzed for IL-8 production via ELISA and RT-PCR. The results were expressed as fold increase over the control ± SEM. # Statistically significant compared to ozone $p \leq .05$. *Statistically significant compared to synthetic product mixture.

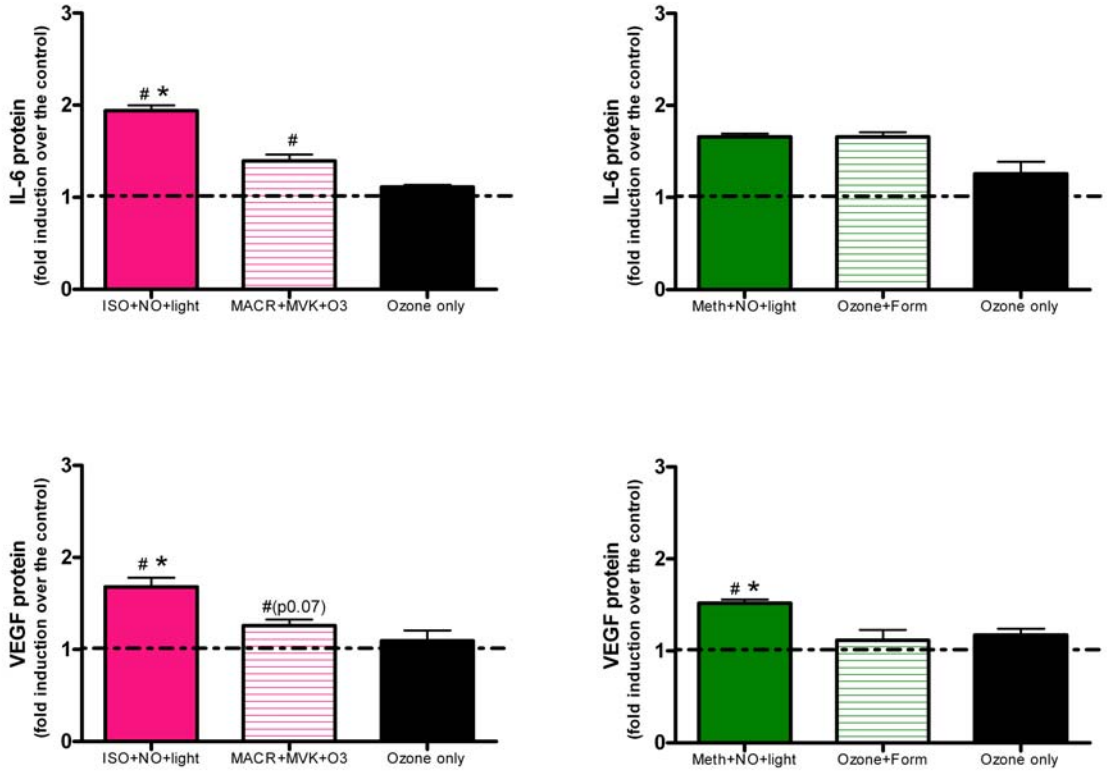



**Figure 10-4: Using Proteomics to examine cytokine release induced by exposure to chamber mixtures consisting of ISO and METH photochemical transformation products.**

Several cytokines were measured with the Multiplex system, of these, IL-6 and VEGF protein analysis is shown above. The mixtures compared in the study include: 1) the photochemically generated products, 2) a synthetic mixture of first generation products (ISO:MACR+MVK+ $O_3$, METH:FORM+ $O_3$), and 3) the concentration of ozone in the synthetic mixture. The results were expressed as fold increase over the control ± SEM. # Statistically significant compared to ozone $p \leq .05$. *Statistically significant compared to synthetic product mixture.

# 11. The Role of Repeated Ozone Exposures in Inducing Acute Inflammatory Effects

## *11.1. Abstract*

Studies have estimated that as many as 13 million healthy individuals in the US alone are routinely exposed to ozone levels that are in excess of the NAAQS. However, it is still unclear whether exposure to ozone tolerizes or sensitizes individuals to subsequent air pollution exposures. An *in vitro* exposure protocol was developed that examines how a repeated exposure to low levels of ozone modifies the response to subsequent acute pollutant challenges. Respiratory epithelial cells grown on membrane support were exposed for 2 days 5hrs/day to 200ppb ozone and subsequently challenged with either a high level of ozone (600ppb) or a more complex mixture of HAPS. These experiments examined if repeated ozone effects alters the response caused by HAPS mixtures generated as a result of photochemical transformations of isoprene (methacrolein, methyl vinyl ketone, and ozone) and 1,3-butadiene (acrolein, formaldehyde, and ozone). Approximately 20 hours after the pollutant challenge with ozone or the HAPS mixtures, cell culture supernatants were analyzed for markers of cytotoxicity and inflammatory mediator production, such as IL-8. The results demonstrate that repeated exposures to ozone alone increased the release of IL-8. In cells repeatedly exposed to air (rather than ozone) prior to challenge with the HAPS mixtures, caused both cytotoxicity and IL-8 release to slightly increase relative to the air controls. However, repeated exposure to

ozone prior to challenge with 600 ppb ozone or the HAPS mixtures significantly increased cytotoxicity and IL-8 release, relative to responses induced by ozone or HAPS mixtures alone. Taken together, these data indicate that exposures of respiratory epithelial cells to low levels of ozone, sensitizes cells to a subsequent pollutant challenges which would be commonly present in urban smog.

## 11.2. Brief Background

Ozone studies have shown that repeated exposures to concentrations around 200 ppb, a level at which is known to cause acute inflammatory responses, may or may not be capable of modifying respiratory inflammation induced by later exposures to reactive atmospheric mixtures. A complete background comparing previous studies and protocols can be found in the "Importance of Ozone" subsection.

## 11.3. Repeated Exposure Methodology

An *in vitro* exposure protocol was developed that examines how a repeated exposure to low levels of ozone modifies the response to subsequent acute exposures to pollutant challenges.

As previously described in the Materials and Methods section, respiratory epithelial cells were grown on membrane support prior to the exposures. The morning they were identified as confluent, serum-free media was replaced and exposure protocol began. There was no media exchange throughout the repeated exposure protocol; therefore an accumulated effect was measured. Cells were exposed for 2 consecutive days, 5hrs/day to 200ppb ozone and subsequently challenged with 600 ppb ozone or a complex mixture of HAPs. Cells were repeatedly exposed using the EPA indoor chambers and consisted of 3 sets of samples, 1 set exposed for 2 days to only clean air, 1 set exposed for 2 days to 200ppb ozone, and 1 set was kept in the incubator during the conditioning or repeated exposures. The exposure diagram illustrates the repeated exposure protocol used to evaluate respiratory sensitization effects induced by repeated exposures to ambient levels of ozone.

### 11.3.1. EPA indoor ozone chambers

The repeated ozone exposures and initial 600 ppb ozone experiments were conducted at the United States, Environmental Protection Agencies, Human Studies Research Building located on the University of North Carolina campus. Exposures were conducted using two indoor chambers, one chamber exposed cells only to air, while the second chamber exposed cells to a controlled concentration of ozone. Each exposure chamber is pressurized and has a total capacity of approximately 12 $ft^3$. A549 cells were repeatedly exposed to air or 200 ppb ozone for 5 hours at 5 liter/min, consecutively for 2 days, using the *in vitro* exposure chambers designed and maintained by the Human Studies Facility of the U.S. EPA. After the conditioning phase, cells were separated into four plates for the challenge-exposure plate. Each of the four plates consists of non-challenged cells (cells that remained in the incubator throughout the first 2 day conditioning period), air conditioned cells, and ozone conditioned cells. This is shown in the following exposure diagram.

## *11.4. Exposure Diagram*

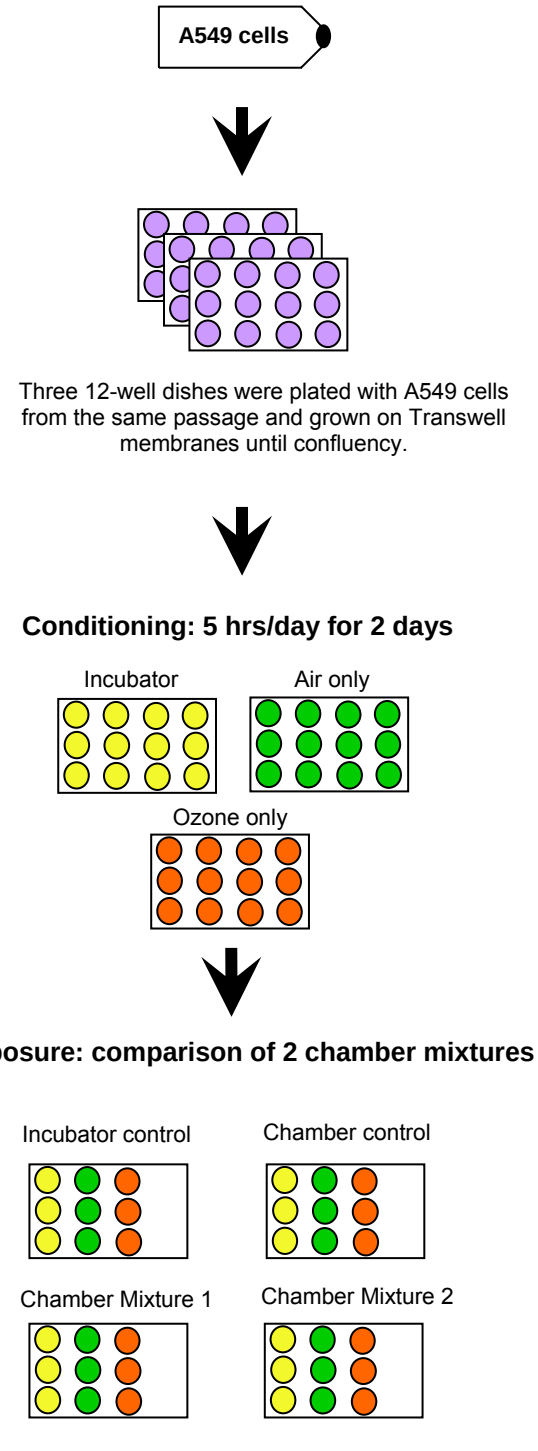



**Figure 11-1: Repeated Exposure Experimental Protocol**

## *11.5. Results*

To examine possible sensitization effects induced by repeated exposures to ozone, A549 cells were repeatedly exposed for 2 days to 200 ppb ozone, and then challenged on the 3rd day to chamber mixtures. Chamber mixtures included: 1) ozone, 2) photochemically generated mixtures of ISO and BD products, and 3) synthetic mixtures of the first generation photochemical products of ISO and BD. All bar graphs depicted in red indicate the results of preconditioning + the chamber mixture.

Each set of samples were analyzed for statistical significance using a 1-way ANOVA, the Students T-test with Welch's correction, and a Two-way ANOVA with Tukeys correction. Results were initially graphed multiple ways prior to the final results shown. They were calculated as fold increase over clean air control, fold increase over the air pre-exposed samples, and as fold increase over the air challenge only. The air repeated exposures, the air challenge only, and the combination of repeated air exposure + air challenge did not exacerbate the LDH or IL-8 protein release compared to the set of samples that never left the incubator (clean air control). Suggesting the increased air flow within the EPA indoor chambers did not modify their control response. Therefore, results were displayed as fold increase over the clean air responses.

### 11.5.1. Ozone

Optimization of the protocol included adjusting the exposure duration, the concentration of exposure, and the rest period prior to the final challenge. Initial experiments were conducted evaluating the effects induced by repeated exposures of two and three days before challenging the cells to 600 ppb ozone, a high concentration with known *in vitro* cytotoxic and inflammatory effects. Figure 11-2 illustrates the results of

exposure to 600 ppb ozone, with and without 2 days of repeated exposures. There was no significant difference in response when the 2-day and 3-day repeated exposure results were compared against one another (data not shown). Therefore, the 2-day exposure protocol was adopted. Cells repeatedly exposed with ozone prior to the air only challenge did not induce a significant increase in cytotoxicity or inflammatory mediator release (Pre only). Exposure to ozone alone caused a significant increase in LDH and IL-8 protein release compared to the clean air control ($O_3$). Pre-exposing prior to the ozone exposure enhanced both response indicators, however not significant (Pre+$O_3$).

### 11.5.2. Photochemically transformed ISO and BD

Described in Figure 11-3 are the cytotoxicity results indicated by LDH release and compare the effects of repeated ozone exposure on challenges to photochemically generated products of ISO or BD and nitric oxide mixtures. The photochemically generated products of both ISO and BD induce significant increases in cytotoxicity compared to the control. Although the mixtures alone induced an overall significant response, conditioning A549 cells prior to this exposure only caused LDH to increase during the ISO exposures. The conditioning or repeated exposures did not modify the cytotoxicity caused by the BD mixture.

The IL-8 protein results indicate that ozone conditioning enhanced the response from exposure to ISO and BD photochemically generated pollutant mixtures. The inflammatory response indicated by measuring the release of IL-8 protein increased significantly after challenges with both ISO and BD photochemically generated products (as shown in Figure 11-4).

### 11.5.3. Photochemical degradation products of ISO and BD

Synthetic mixtures containing the initial photochemical products of ISO and BD were injected into the chambers. Cells were exposed to these mixtures and analyzed for cytotoxicity and inflammatory response, with and without ozone pre-exposures. These experiments were preformed to assess the response, and evaluate if they are similar to the effects induced by exposure to the full photochemically reacted ISO and BD systems. The LDH and IL-8 protein release are shown in Figure 11-5. In contrast with the cytotoxicity results for the photochemically generated mixtures shown in Figure 11-3, ozone conditioning prior to challenge with the synthetic BD product mixture (ACR+FORM+O3) induced a significant increase in LDH release. However, pre-exposures did not enhance the cytotoxic response when cells were challenged with the synthetic ISO product mixture (MACR+MVK+O3). These results were opposite of those found from the photochemically generated mixture once combined with the conditioning regiment. On the other hand, similar to the photochemically generated mixtures, repeated ozone exposures prior to the challenge induced significant or almost significant ($p = 0.064$) increases in inflammatory release (IL-8 protein) following exposure to synthetic mixtures of BD and ISO primary, photochemical products.

## 11.6. Discussion

The present study addressed the uncertainty if conditioning human respiratory epithelial cells through repeated exposures with ozone ultimately created a sensitization model prior to exposures to reactive atmospheric mixtures. An *in vitro* exposure protocol was developed using A549 cells, a human epithelial type-II like cell-line and EPA's indoor ozone chambers. This method was testing the response induced by high concentrations of ozone, photochemically generated mixtures of ISO + NOx and BD+NOx, and synthetic mixtures containing the primary, photochemical products of ISO and BD.

It has been estimated that large populations reside in areas with continuous, high levels of ambient ozone over extended periods of time; therefore it is important to determine whether progression of ozone induced airway inflammation occurs with repeated exposures (Christian et al. 1996). Many ozone studies have shown that exposures to ozone alone induce cytotoxicity and the release of many inflammatory markers, such as IL-8. Of these studies, some have evaluated the effects induced by repeated exposures to ozone. Combined, the results of the repeated ozone exposure studies were inconclusive suggesting both tolerance and sensitization occurring within individuals, *in vivo* or *in vitro* models after repeated ozone exposure prior to challenge with subsequent air pollution mixtures.

The objective of this study was to determine whether acute, repeated exposures to an innate concentration of ozone stimulates a sensitization response in human respiratory epithelial cells thus enhancing later challenges with atmospheric pollutants.

Initial experiments were conducted to test if repeated exposures to 200 ppb ozone with an air challenge only (no later exposure to an atmospheric pollutant or mixture of pollutants) induced an increased response compared to the control. It was shown that cells conditioned with ozone, and then challenged with an air exposure, did not induce a "significant" increase in cytotoxicity or inflammatory mediator release; therefore only moderately shifting the baseline response. This indicates that the pre-exposures alone do not alter the initial background response of the cells. This was very important in the initial creation of the conditioning or repeated exposure protocol.

Combining the ozone and photochemically reacted ISO and BD mixture results, the data suggests that pre-exposing human respiratory epithelial cells with ozone enhanced the inflammatory response induced by the overall challenge. Interestingly, the ozone repeated exposures only *significantly* increased the response when challenged with the ISO or BD photochemically reacted mixtures, rather than with the ozone alone. Although the ozone challenge experiments did not reach statistical significance, they were close with $p = .07$. This difference may be due to some purely additive response occurring through ozone-only challenges; it could be due to the small sample size; or it is possible that the pathway of response indicative of injury caused by ozone exposures vs. other oxygenated species is very different and can not be compared with one another. Further studies are warranted to examine this phenomenon.

Subsequently, this enhancement may or may not be due to oxidative stress weakening the cells prior to exposure to atmospheric pollutants. Studying oxidative stress or other possible mechanisms of response were not evaluated at this time; however,

once completed will serve as important indicators in understanding the overall exposure-effects model.

## 11.7. Figures

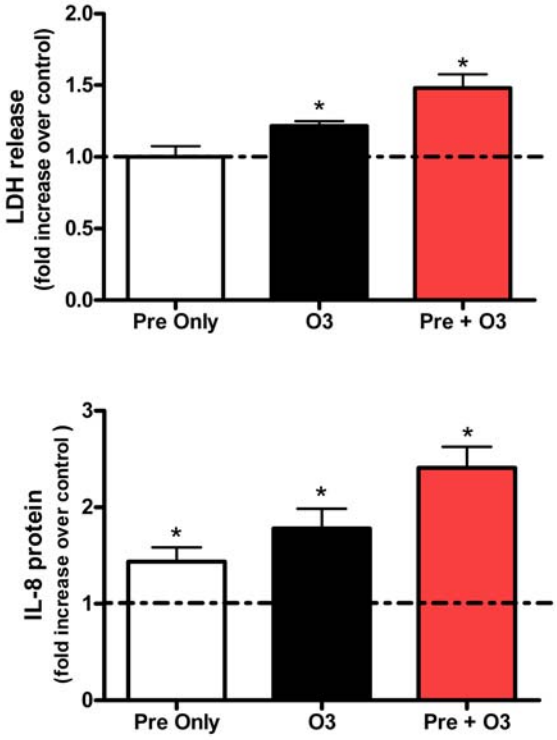



**Figure 11-2: Repeated Exposures with 600 ppb Ozone Challenge**

Using the EPA indoor exposure chambers, A549 cells were pre-exposed to ozone or air and then challenged for 5 hours with ozone only. A549 cells were exposed to 200 ppb ozone for 2 days, and then challenged with 600 ppb ozone on the 3rd day. Post exposure, basolateral supernatants were collected and analyzed for cytotoxicity (LDH release) and inflammation (IL-8 protein production). The results were expressed as fold increase over the air response. The dashed line indicated the response of the air exposures.
* statistically significant compared to air exposures.

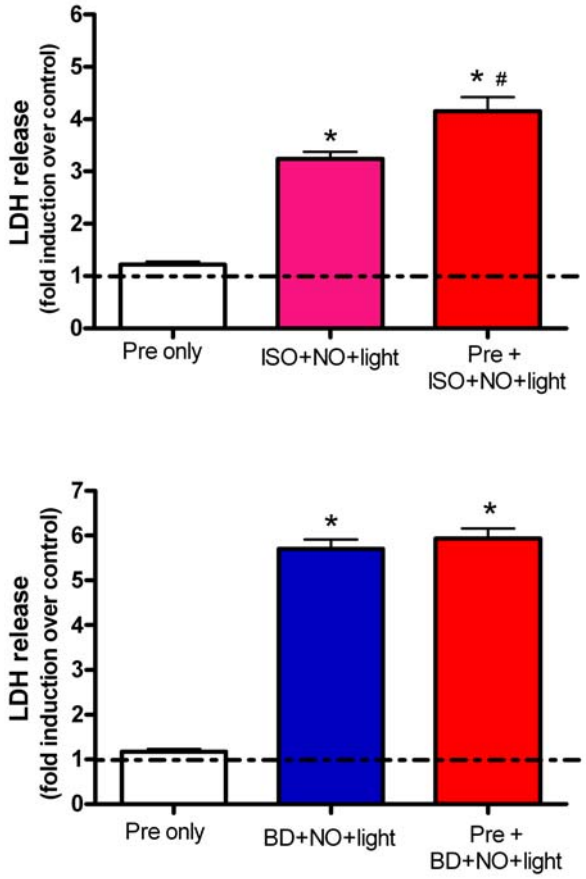



**Figure 11-3: Cytotoxicity Response induced by Repeated Ozone Exposures and Challenged with Photochemically Reacted ISO and BD Mixtures**

Analysis of cytotoxicity induced by ozone pre-exposed A549 cells challenged to photochemically reacted hydrocarbon and nitric oxide mixtures. Cells were exposed to photochemically generated product mixtures of isoprene or 1,3-butadiene and nitric oxides with or without ozone conditioning. Nine hours post-exposure, basolateral supernatants were collected and analyzed for cytotoxicity through LDH release. The results were expressed as fold increase over the air response. The dashed line indicated the response of the air exposures. * statistically significant compared to air exposures. # statistically significant compared to the hydrocarbon and nitric oxides mixtures without prior ozone exposures.

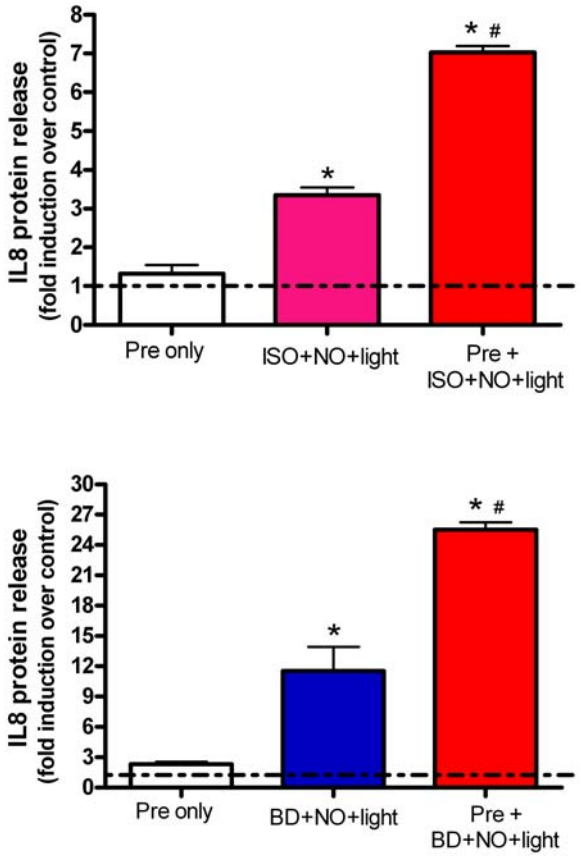



**Figure 11-4: IL-8 Response induced by Ozone Pre-exposures and Challenges to Photochemically Reacted ISO and BD Mixtures**

Analysis of inflammation induced by ozone pre-exposed A549 cells challenged to photochemically reactive hydrocarbon and nitric oxide mixtures. Cells were exposed to photochemically generated product mixtures of isoprene or 1,3-butadiene and nitric oxides with or without pre-exposing. Nine hours post-exposure, basolateral supernatants were collected and analyzed for IL-8 production via ELISA. The results were expressed as fold increase over the air response. The dashed line indicated the response of the air exposures. * statistically significant compared to air exposures. # statistically significant compared to the hydrocarbon and nitric oxides mixtures without prior ozone exposures.

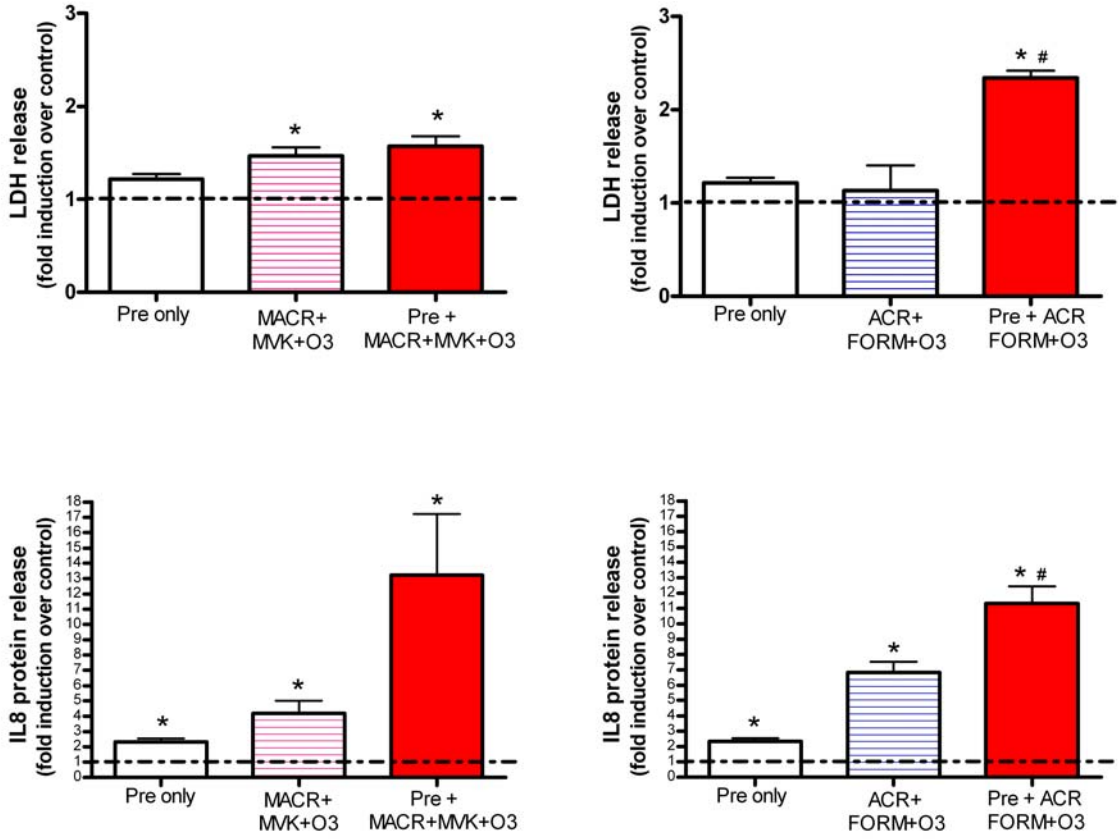



**Figure 11-5: Cytotoxicity and IL-8 Response Induced by Ozone Pre-Exposures, Synthetic Product Mixtures of ISO and BD products, and the Combination of the Two**

These experiments evaluated the effects of challenging A549 cells to synthetic product mixtures after prior repeated ozone exposures. Sets of A549 cells were conditioned with 200 ppb ozone or air, and challenged with chamber mixtures for 5 hours. The chambers consisted of synthetic mixtures of the known, primary photochemical products of isoprene and 1,3-butadiene. Post exposure, basolateral supernatants were collected and analyzed for cytotoxicity (LDH) and inflammation (IL-8). The results were expressed as fold increase over the air response. The dashed line indicated the response of the air exposures. * statistically significant compared to air exposures. # statistically significant compared to the synthetic product mixtures without pre-exposures.

# 12. The Role of Oxidative Stress

## *12.1. Background*

Many environmental pollutants, including photochemical oxidants such as HAPs, damage respiratory cells through oxidative stress (Epperlein et al. 1996). "Oxidative stress is considered to play an important role in the pathogenesis of inflammatory lung disease" (Luppi et al. 2005). This mechanism causes many types of cellular injury and plays a key role in gene expression and cell proliferation (Luppi et al. 2005).

Antioxidants provide cellular defenses against reactive oxygen species. Among all the antioxidants found within the lung, glutathione (GSH) is considered to be a main antioxidant molecule (Rahman and MacNee 1999). Buthionine sulphoximine (BSO) is a "potent and selective inhibitor of GSH synthesis" (Rajasekaran et al. 2005), and has been used to deplete intracellular glutathione levels during previous studies. Glutathione reduced ethyl ester (GSH-ET) and BSO are commonly used with *in vivo* and *in vitro* methods to study the mechanism of response induced by pollutant exposures.

"Understanding how respiratory diseases are exacerbated requires the combined evaluation of how inflammation is affected by oxidative stress, what is the initial site of injury from inflammatory cells, and how different proinflammatory mediators are affected" (Todokoro et al. 2004). In this portion of the study, oxidative stress induced by the photochemical degradation products of ISO, BD and METH were examined.

Although ozone is a known oxidative stress inducer, few studies have examined the effects of complex atmospheric mixtures that include many photochemical oxidants. The goal of this portion was to discern the cytotoxic and inflammatory effects promoted by exposure to the photochemical oxidants previously described.

## *12.2. Methods*

According to the procedure previously described in the Methods and Materials section, A549 cells were prepared for chamber exposures. The intracellular glutathione content was modified by pretreatment with BSO or GSH-ET from the basolateral side for 4 hours prior to exposure to the chamber mixtures.

### 12.2.1. BSA+BSO:

Buthionine sulphoximine (Sigma) was dissolved in sterile water and diluted to 1M solution. 1µl/ml was added to serum-free media yielding a 10µM solution.

### 12.2.2. BSA+GSH-ET:

100 mg glutathione reduced ethyl ester (Sigma, 95%) (GSH-ET) was dissolved in sterile water and diluted to 1M solution. 10 µl/ml was added to serum-free media yielding a 100 µM solution.

## *12.3. Experimental Design*

### 12.3.1. Exposure 1: Ozone

Initial experiments were performed using 200 ppb ozone and the outdoor environmental chambers. These experiments were used to test the new method evaluating the cytotoxicity and IL-8 response induced by ozone alone. Since previous studies have shown that BSO and supplemental GSH-ET alter ozone-induced inflammatory response, the GSH-ET pretreatment method should attenuate the acute ozone response while BSO should amplify the inflammatory response.

### 12.3.2. Exposure 2: Product differentiation

Experiments were performed evaluating the individual effects induced by the photochemical degradation products generated from ISO, BD, and METH reacting in the atmosphere. The main, first generation products identified from each system included: MACR and MVK; ACR and FORM; and FORM. Due to chamber limitations, only two exposures were conducted: 1) FORM and 2) MACR + MVK. It was assumed that ACR would act similarly as MACR and therefore was removed. The goal here was to evaluate if FORM, or a synthetic product mixture containing MACR and MVK would induce cytotoxicity and inflammation through oxidative stress.

### 12.3.3. Exposure 3: Photochemically generated mixtures

An experiment was performed to evaluate if altering glutathione levels in cells exposed to chambers mixtures of photochemically transformed ISO and BD modifies the level of release of cytotoxicity and inflammatory mediators. Generated within each system was

~200 ppb ozone along with the other initial oxygenated transformation products. GSH modifying techniques were used to measure oxidative stress.

## *12.4. Results*

To further examine the inflammatory response induced by HAPs, GSH-ET and BSO were used to suppress and enhance the cellular pathway that mediates the effects of oxidative stress.

### 12.4.1. Ozone

This method was tested using 200 ppb ozone, a level at which was known to cause acute inflammatory response in both humans and *in vitro* systems. The data in Figure 12-1 implies ozone without pretreatment generated approximately a 1.5-fold increase in cytotoxicity and a 2-fold IL-8 increase compared to the clean air control. Cells pretreated with GSH-ET induced a non-significant suppression of cytotoxicity and IL-8 response. BSO enhanced both cytotoxicity and IL-8 response. These effects were not significant.

### 12.4.2. Product differentiation

FORM and a synthetic product mixture containing MACR and MVK were evaluated for cytotoxicity and IL-8 response using modified media conditions. Figure 12-2 displays the results from the FORM vs. MACR and MVK synthetic product mixture experiment. Both GSH-ET and BSO did not modify the cytotoxicity or inflammatory response induced by either chamber exposure.

### 12.4.3. Photochemically generated mixtures

Glutathione modified media was used to evaluate if oxidative stress through the glutathione pathway is the primary mechanism of response induced by photochemically generated mixtures of ISO or BD and nitric oxides. After the media was pre-treated with GSH-ET or BSO, neither supplement significantly altered (increased or decreased) the cytotoxic release of LDH after exposure to ISO or BD product mixtures. Both treatment methods, GSH-ET and BSO, caused an increase in IL-8 after exposures to photochemical products of ISO. However during the BD photochemically reacted experiments, BSO caused an increase in IL-8 production, while the GSH-ET (the additional protective glutathione) induced a decrease in protein release compared to non-supplemented cell cultures.

### 12.4.4. Review of Results

In theory, oxygenated products that induce injury (cytotoxicity) or a response (inflammation) through GSH-mediated oxidative stress would increase or decrease the response similar to the pattern found for ozone in the diagram below. A **0** response implies no change from the non-treated exposure, a + implies an increased response, and a – implies a decreased response. The results measured during each of the chamber scenarios are described summarized below.

| ***Cytotoxicity*** | | | ***Inflammation*** | | |
|---|---|---|---|---|---|
| | **GSH-ET treated** | **BSO treated** | | **GSH-ET treated** | **BSO treated** |
| Ozone | **-** | **+** | Ozone | **-** | **+** |
| FORM | **0** | **0** | FORM | **0** | **0** |
| MACR+MVK | **0** | **0** | MACR+MVK | **0** | **0** |
| ISO photo. mixture | **0** | **0** | ISO photo. mixture | **+** | **+** |
| BD photo. mixture | **0** | **0** | BD photo. mixture | **-** | **+** |

## *12.5. Discussion*

The present study addressed the role of glutathione-mediated oxidative stress pathways in cytotoxic and inflammatory effects of reactive atmospheric mixtures in human respiratory epithelial cells. A549 cells were used as the exposure model combined with GSH-ET- or BSO- modified serum-free media during exposures to ozone, FORM, a synthetic product mixture containing MACR and MVK, and photochemically generated products of ISO or BD and nitric oxide mixtures.

Antioxidants play a vital role in protecting cells from oxidative damage caused by inhalation exposures to atmospheric pollutants. Although there are many antioxidants readily available in the lung, this study focused on the effects induced by modifying the intracellular glutathione concentrations. Previous studies have reported that in many cell types, including respiratory epithelial cells, "glutathione constitutes the first line of defense against oxidative injury" (Deneke and Fanberg 1989). Therefore BSO and GSH-ET were used to explore the underlying mechanism that provoked the inflammatory response reported in previous sections.

One main finding of this study suggested that although the products evaluated are photochemical oxidants generated through atmospheric transformations of ISO, BD, and METH, the entire exposure-response pathway was not significantly altered through GSH-modified exposures. Cells pretreated with either glutathione modifying method prior to exposures to the non-ozone first generation products of ISO (MACR and MVK) and METH (FORM) induced no change in LDH and IL-8 release relative to non-pretreated cells. This suggests that at the concentrations examined in this study the injury caused during the exposures to the products (oxygenated hydrocarbons formed during photochemical transformations in the atmospheres) was not initiated by glutathione mediated - oxidative stress. This was illustrated by the unchanged response. In summary, experimental conditions altering the intracellular GSH levels prior to exposure to reactive atmospheric mixtures did not alter the response induced by either FORM alone, or the ISO synthetic product mixture.

The GSH-modifying pretreatments significantly altered the response caused by both the ISO and BD photochemically generated mixtures. For both pollutants, neither

supplement caused a change in the cytotoxic response induced by the photochemically active systems. More importantly, the inflammatory response induced by exposure to the photochemically generated products of ISO and BD are not the same, that is the GSH-ET and BSO treatment acted differently under each experimental conditions.

The photochemically generated products of ISO and nitric oxides caused an increased production of inflammatory mediator, IL-8, when cells were treated with both GSH-ET and BSO. Interestingly, the GSH-ET caused an increase almost as large as the BSO-treated cells. Although the GSH-depleted cells generated the largest increase in IL-8 production, the cells supplemented with glutathione also induced an increased response which was unexpected. This may not be true with all inflammatory mediators, however only cytotoxicity and IL-8 were examined under these conditions. Currently, no ISO literature described similar results therefore the cause is unclear.

In contrast to the ISO results, the photochemically generated products of BD were similar to typical oxygenated products response for exposures evaluating the affects when intracellular GSH was altered using GSH-ET and BSO. The additional GSH caused a slight decrease in IL-8 release, while depleting these levels via BSO induced a greater induction of inflammatory cytokine production. None of these results implied significant suppression or enhancement; therefore these results suggest that only a small portion of the response is likely due to the glutathione mediated - oxidative stress pathway, and therefore other mechanisms or other oxidative stress pathways are responsible for the documented response.

Overall these results imply that production of cytokines by human respiratory epithelial cells is affected by photochemical oxidant-induced oxidative stress (indicated by changed in IL-8 production), and that glutathione in these cells may or may not modulate this production. This data suggests that although this pathway was not the main response inducer, it can not infer what other mechanism is causing the response. It may or may not be due to another oxidative stress pathway such as lipid peroxides or direct oxidative damage on the cellular surface. These results were similar to recent studies by Todokoro and researchers (2004). In summary, the upregulation of inflammatory cytokine production and cytotoxicity via lactate dehydrogenate release are not entirely mediated by oxidative stress.

## 12.6. Figures

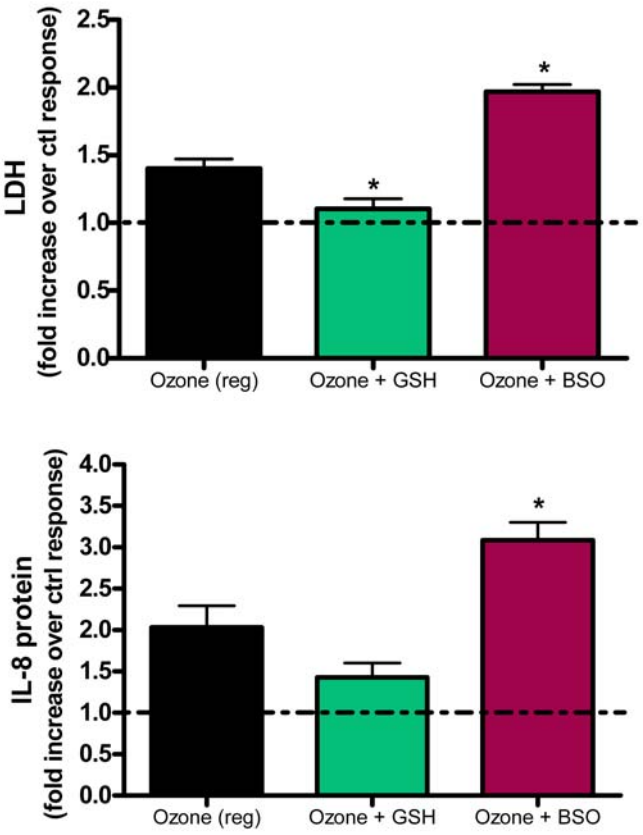



**Figure 12-1: Testing the Glutathione-modifying Method with Ozone Exposures**

Analysis of cytotoxicity and inflammatory gene expression induced by exposure to 300 ppb ozone, with and without supplemental glutathione (GSH) or buthionine sulphoximine (BSO). A549 cells were treated with regular serum-free media (BSA), media with supplemental GSH (BSA+GSH-ET) or media with BSO (BSA+BSO) 4 hours prior to 5-hr ozone exposure. Post exposure, cells were examined for cytotoxicity via LDH release and IL-8 via ELISA. The results were expressed as fold increase over the clean air with and without treatment control. The dashed line indicates the response of the control. * statistically significant compared to regular ozone exposure (BSA only), $p<0.05$.

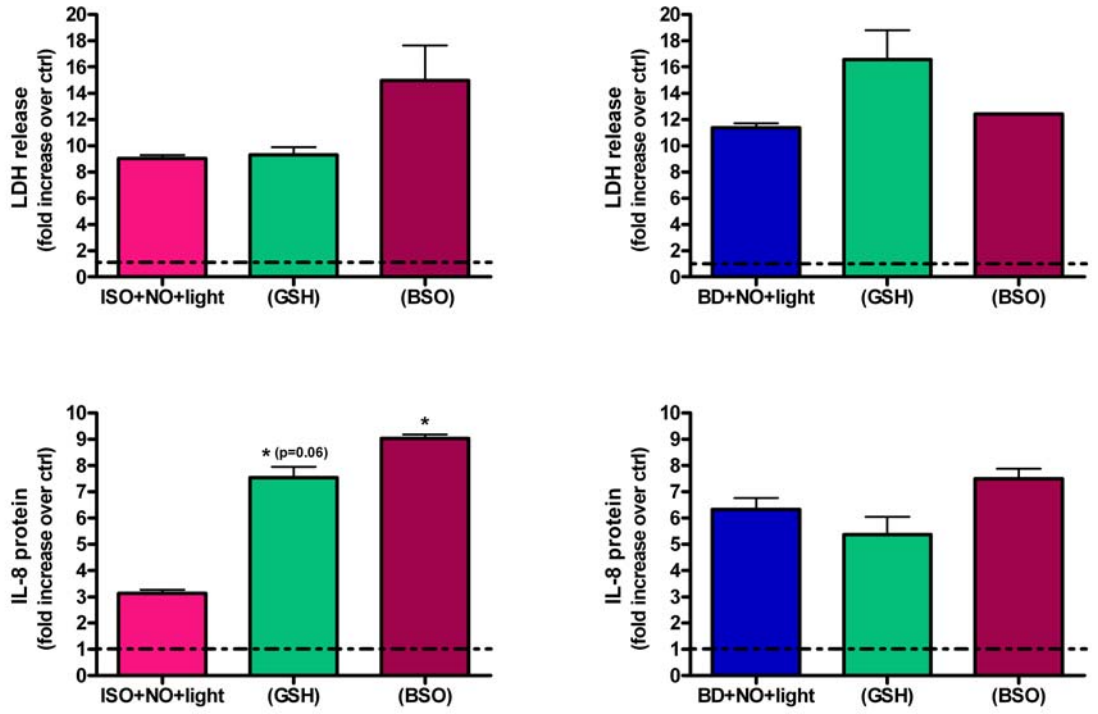



**Figure 12-2: Cytotoxicity and IL-8 Response Caused by Exposure to Photochemically Reacted ISO and BD, with and without GSH-ET and BSO treatment.**

Analysis of cytotoxicity and inflammatory gene expression induced by exposure to photochemical degradation products of isoprene or 1,3-butadiene and nitric oxide (HC+NO+light), with and without supplemental glutathione (GSH) or buthionine sulphoximine (BSO). A549 cells were treated with regular serum-free media (BSA), media with supplemental GSH (BSA+GSH-ET) or media with BSO (BSA+BSO) 4 hours prior to exposure. After sundown BSA, BSA+GSH-ET, and BSA+BSO treated cultures were exposed for 5 hours to chamber mixtures. Post exposure, cells were examined for cytotoxicity via LDH release and IL-8 via ELISA. The results were expressed as fold increase over the clean air with and without treatment control. The dashed line indicates the response of the control. * statistically significant compared to HC+NO+light (BSA only), $p<0.05$.

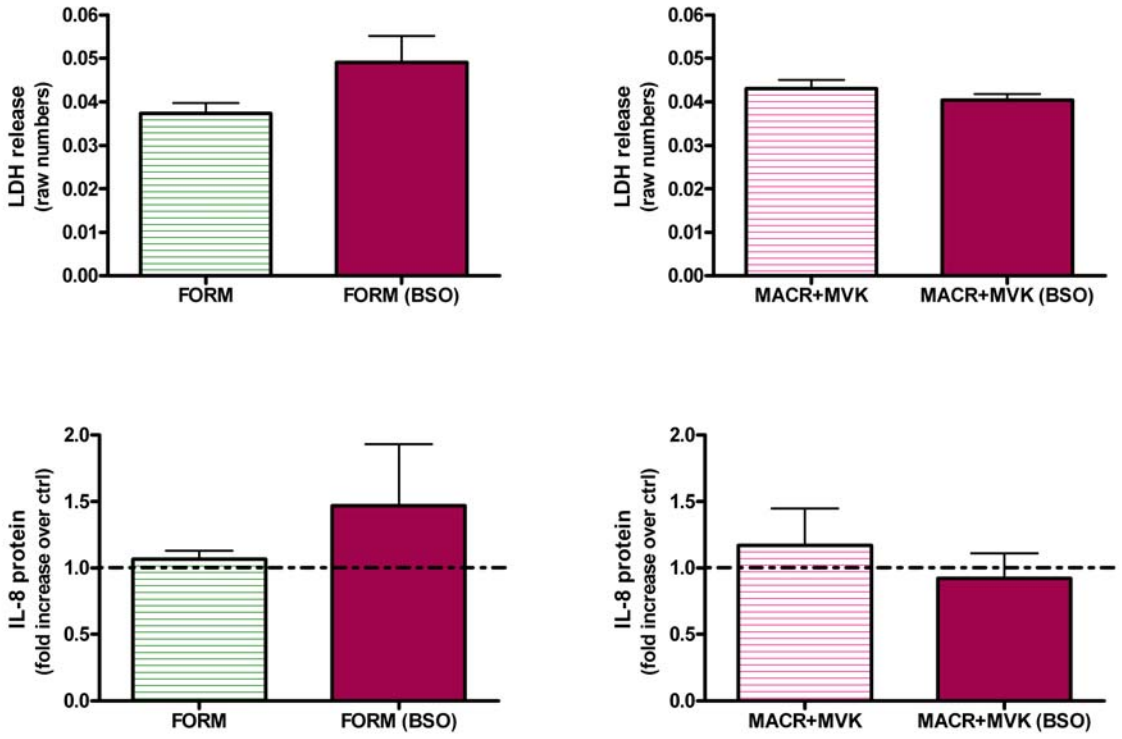



**Figure 12-3: Cytotoxicity and IL-8 Response Caused by FORM, and MACR+MVK mixtures, with and without GSH-ET and BSO treatment.**

Figure 12-3: Analysis of cytotoxicity and inflammatory gene expression induced by exposure to synthetic product mixtures, with and without depleted GSH due to buthionine sulphoximine (BSO). Synthetic product mixtures were created to analyze the effects induced by isoprene and methanol photochemical degradation products other than ozone, and to examine induced oxidative stress. A549 cells were treated with regular serum-free media (BSA) or media with BSO (BSA+BSO) 4 hours prior to exposure. After sundown BSA, and BSA+BSO treated cultures were exposed for 5 hours to chamber mixtures (FORM, or MACR+MVK). Post exposure, cells were examined for cytotoxicity via LDH release and IL-8 via ELISA. LDH results are shown as raw values, while IL-8 is expressed as fold increase over the clean air with and without treatment control. The dashed line indicates the response of the control. * statistically significant compared to HC+NO+light (BSA only), $p<0.05$.

# 13. Study Conclusions:

Atmospheric pollutants are constantly transforming in the environment. People are constantly being 'bathed' in this atmosphere and cannot limit their intake to clean sources as they might for water, thus making it difficult to examine possible health effects occurring during inhalation exposures. These characteristics make air pollution a unique health concern. In this work a novel experimental approach was developed combining the UNC outdoor environmental chambers to create complex reacting atmospheric environments with an *in vitro* exposure system to detect potential harmful effects on living human lung cells induced by exposure to the gases in these photochemical reactive atmospheric mixtures.

The three parts presented in this dissertation examine several issues related to studying hazardous atmospheric pollutants, including the role of individual photochemical degradation products in inducing cytotoxicity and inflammatory cytokine production released from human respiratory epithelial cells. This chapter summarizes major findings and contributions of this work to the larger literature, discusses some of the limitations of the overall body of work, and raises some questions for future research.

## 13.1. Findings and Contributions

The first part of my study hypothesis was

> Exposure to a photochemically transformed air pollutant mixture always has more respiratory health effects than exposure to the VOC precursors, or the ozone generated during the reactions, when these effects are measured by gene expression *in vitro*.

The first contribution of this work is the creation and testing of a method for the effective evaluation of the role of photochemical transformations in modifying the *in vitro* toxicity of atmospheric pollutants. As an alternative method to study the adverse effects of gaseous 1,3-butadiene and isoprene, a smog chamber – *in vitro* exposure system was developed to permit an active air-to-lung cell exchange during each exposure. These results indicate that products created from photochemical transformations exhibit significantly greater toxicity when they are permitted to contact human respiratory epithelial cells. This supports my stated hypothesis.

This method was also used for studies of methanol and toluene toxicity. Similar to the isoprene and 1,3-butadiene work, the results from both compounds indicate that photochemical transformations induced a significant enhancement in cytotoxicity and inflammatory cytokine release compared to the un-reacted parent pollutant. Thus, confirming the stated hypothesis even for relatively simple compounds.

A major contribution of the second objective is the differentiation of individual roles that products generated from photochemical transformations play in the induction of cytotoxicity and inflammatory mediator response. Most importantly, the ozone contribution when comparing the overall adverse effects of reactive atmospheric mixtures; this has been a great concern of the health community for many years. From

the literature, ozone has been long postulated as one of the most important species relating to smog production and its adverse health effects. Alone ozone is known to increase IL-8 production in A549 cells at levels that were below those achieved and compared against during these experiments (Jaspers et al. 1997), however, the intriguing question remains, could this induction account for a statistically significant portion of the overall inflammatory response?

To evaluate atmospheric pollutants found in the environment, compounds with both simple and complex chemical reactions needed to be considered. Although the chemistry of isoprene and 1,3-butadiene has been studied for many years, still only ~75% of the total products are known. Therefore, they were chosen as the "complex" pollutants while methanol was studied to be representative of the "simple" pollutant. The first generation products were chosen to study the initial photochemical transformations that occur following immediate release into the environment. Synthetic mixtures of these products were used during the experiments.

Experiments studying the effects induced by mixtures of photochemical transformation products found that for simple pollutants with known chemical mechanisms, the complete toxicity was calculated using the synthetic product mixture consisting of only ozone and formaldehyde. And interestingly, ozone was primarily the cause of the effects. However, this was not the case for isoprene and 1,3-butadiene. The synthetic mixture did not yield results similar to the complete photochemical products generated during atmospheric transformations using the outdoor smog chambers. This is likely due to small concentrations of either unknown products or those found known but not quantified during this study. Subsequently, the additional cytotoxicity or

inflammatory response may be due to some synergistic response on initated when the photochemical transformation products are generated aside one another rather than compositely blended together. Although no interactive response was observed in previous studies, it still could be one possibility why the photochemically reacted METH mixture was more responsive than its synthetic product mixture.

The second part of my study hypothesis was

> Further, "preconditioning" *in vitro* samples by prior ozone exposures always shows an increased response compared to "new", unconditioned cells exposed only to the challenge mixture.

In addition, the effects of repeated exposure or conditioning prior to a challenge with atmospheric pollutants were examined. A pre-exposure protocol was developed to simulate changes human respiratory epithelial cells undergo once exposed to innate concentrations of pollutants over a short period of time. These changes were identified by their response to pollutants previously studied using the smog chamber – *in vitro* exposure system. Studies evaluating the photochemical products of isoprene and 1,3-butadiene found that pre-exposures to ozone significantly modifies the cytotoxic and inflammatory response. These results could contribute to the epidemiology studies discussed earlier that found sensitization-like responses after acute exposures to atmospheric mixtures.

Lastly, how photochemical transformations actually induce the response was evaluated; methods examining injury due to oxidative stress were examined. The data demonstrates that exposure to the photochemically generated products of 1,3-butadiene increased markers of oxidative stress, and that modifying cellular GSH levels by adding

reduced glutathione ethyl ester only reversed a small portion of the effect. Furthermore, similar responses were found when buthionine sulphoximine was added to deplete intracellular GSH levels prior to exposure. This suggests that although the BD products generated from the parent compounds are oxygenated species, the entire response is not mediated by oxidative stress.

In contrast, the ISO results were very different when intracellular GSH levels were modified prior to the exposure. The buthionine sulphoximine treatment exacerbated the cytotoxic response and the inflammatory response. On the other hand, the glutathione ethyl ester did not alter the cytotoxicity but also increased the release of the inflammatory mediator IL-8.

Kida et al. (2005) studied the anti-oxidant role of IL-6 within the lung and its protective properties on resident cells, in particular the effects of ROS-cell death on alveolar epithelial cells. They found that IL-6 is a multifunctional cytokine with specific mechanisms that inhibits some inflammation mediated through an anti-oxidant defense. Xing et al. 1998 found similar results with IL-6 altering inflammatory responses in addition to mediating temporal changes in endothelial permeability. This may explain why isoprene and 1,3-butadiene reacted so differently with the GSH-modifying treatments. Furthermore, many first members of a chemical family react differently than their subsequent members, e.g. methane chemical reactions vs. ethane chemical reactions. We could speculate that reactively 1,3-butadiene and isoprene (2-methyl-1,3-butadiene) are toxicologically different due to the same rationale as their reaction specifications.

In summary, combined these studies indicate that implementation of a smog chamber – *in vitro* exposure system with multiple toxicological assessment methods is an essential and invaluable tool when examining adverse respiratory effects of reactive atmospheric mixtures representative of urban environments.

## 13.2. Limitations

To understand fully the findings and contributions of this work, it is helpful to understand its limitations relative to the methodologies and current science available.

By focusing only on *in vitro* models, systemic effects and possible alterations in effects found during this study were overlooked. A "major limitation of *in vitro* is the cells are removed from their normal environment. There are no neighboring cells or tissues to interact with, and no blood to supply, potentially important factors or nutrients" (Devlin et al. 2005). A second limitation relative to the method was the choice of using only A549 cells, a model of human respiratory epithelial cells, for a majority of the work rather than another immortalized cell line representative of sensitive populations or different regions of the respiratory tract affected during inhalation exposures.

The development of the exposure protocol included the understanding that exposures were set-up during stable experimental conditions; that is when the chemical reactions had reached a limit due to lack of sunlight. This enabled the creation of simple synthetic product mixtures, which nevertheless, due to the incomplete science, did not include all the products generated during these transformations. Just looking at isoprene and 1,3-butadiene results, only approximately 75% of the photochemical products have

been identified. The chemical mechanisms of more complex environmental pollutants are even more incomplete overall. These facts indicate the need for further chemical research prior to advanced synthetic mixture work to evaluate the toxicity of photochemical degradation products.

## *13.3. Further research questions*

Not surprising, these studies generate more questions. Some of these questions are related to perceived shortcomings of the present research. The questions include:

- How does the smog chamber – *in vitro* exposure system compare to previous methods to evaluate the toxicity of reactive atmospheric pollutants? Although the system described here is the first to permit the study of dynamic atmospheric mixtures in direct contact with living cells, it was not directly tested against classic methods. Other members of our research group are conducting a limited set of such tests.

- Would preconditioning by exposure to a reacting representative urban smog mixture instead of just to 200 ppb ozone induce similar sensitization results? A majority of the studies evaluating the effects of repeated exposure to atmospheric pollutants included the effects of ozone. Ozone was used in this model to precondition human respiratory epithelial cells because it is omni-present in most urban ambient environments. However, the synthetic mixture representative of the pollutants in urban smog would better represent a natural environment individuals would be exposed to on a day-to-day basis. Limited testing using such a mixture

does show that it produces more of an effect than ozone alone. The positive effect results of pre-exposure sensitization shown in my experiments do suggest that the next more complex system tests may be worth the significant time and effort to perform.

- Would the use of other types of cells or cell models simulate similar results when evaluating the effects induced by exposures to reactive atmospheric mixtures created inside the outdoor environmental chambers? We know from early experiments that primary bronchial cells react very differently when exposed to photochemically generated ISO and BD mixtures. However, the bronchial region of the respiratory tract is not the primary target for gaseous pollutants therefore that could explain some of the differences. At that time primary alveolar cells were unavailable. Studies comparing non-immortalized alveolar cells are warranted.

- Would the use of an *in vivo* model in place of the *in vitro* model produce similar responses? Both with respect to the photochemistry and conditioning studies. Previous studies have shown similar responses when they compared the two models and examined the effects induced by ozone exposures. I believe, the smog chamber toxicology system and the repeated ozone exposure method would generate similar responses if an in vivo model representative of a healthy individual were used.

What processes and techniques can be used to help facilitate integrated research necessary to evaluate the toxicity of natural atmospheric mixture pollutants

consisting of both gaseous and particulate species? (Similar to isoprene which produces some particulate matter during photochemical transformations in the environment.)

# Appendices

## *Appendix A: Smog Chamber – In Vitro Exposure Experiments*

1. **July 1, 2002**: Ozone test using new modular incubator chambers. A549 cells were exposed to 300 ppb ozone for 3 hours and then analyzed for cytotoxicity via Trypan blue exclusion method.

2. **July 16, 2002**: Second ozone test using new modular incubator chambers. A549 cells were exposed to 300 ppb ozone for 3 hours and then analyzed for cytotoxicity via Trypan blue exclusion method.

3. **July 23, 2002**: Wet vs. dry air. Two sets of A549 cells were exposed to 95 % medical grade breathing air + 5 % $CO_2$, one set had 50 % humidified air while the other was given only the breathing air + $CO_2$ mixture.

4. **August 6, 2002**: Matched cell exposure with humidified air. Two sets of A549 cells were exposed to a 50 % humidified mixture of air comprised 95 % of medical grade breathing air and 5 % $CO_2$. The A549 cells were analyzed for cytotoxicity via Trypan blue exclusion method and IL-8 mRNA via RT-PCR.

5. **August 13, 2002**: Second matched cell exposure with wet air. This was a repeat of the August 6, 2002 exposure design.

6. **August 14, 2002**: Third matched cell exposure with wet air. This was a repeat of the August 6, 2002 exposure design.

7. **August 27, 2002**: Fourth matched cell exposure with wet air. This was a repeat of the August 6, 2002 exposure design.

8. **August 28, 2002**: Fifth matched cell exposure with wet air. This was a repeat of the August 6, 2002 exposure design.

9. **September 3, 2002**: Tested HC loss in modular incubator chambers.

10. **June 10, 2003**: Matched ozone test. Two sets of A549 cells were exposed to 200 ppb ozone using two modular incubator chambers, one in the blue incubator and the other in the red incubator. The A549 cells were then analyzed for cytotoxicity via Trypan blue exclusion method and IL-8 mRNA via RT-PCR.

11. **July 7, 2003**: Two new modular incubator chambers were exposed to 500 ppb ozone overnight and analyzed for loss to the plastic coating. A 7% ozone loss was found when the concentration of ozone entering the modular incubator chamber and the concentration flowing out of the modular incubator chamber was compared.

12. **July 18, 2003**: Comparison between the old and new modular incubator chambers. Two sets of A549 cells were exposed to 400 ppb ozone for 3 hours. One set of cells were in the old modular incubator chamber while the other set was in the new modular incubator chamber. All samples were analyzed for cytotoxicity and cytokine expression.

13. **July 25, 2003**: Matched ozone test. Two sets of A549 cells were exposed to matched 211 ppb ozone for 5 hours and then analyzed for cytotoxicity and cytokine expression. The ozone was injected into both sides of the dual chambers and exposed simultaneously using the two incubator systems: the blue and red set-up.

14. **August 18, 2003**: Isoprene + NOx full day photochemistry + night time exposure. 100 ppb ISO + 100 ppb NOx were injected into the chamber and allowed to photochemically react from sunrise to sundown (0636-1952, ~13.5 hours). At sundown, 100 ppb ISO + 100 ppb NOx were injected into the empty chamber. Two sets of A549 cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

15. **August 21, 2003:** Isoprene + NOx full day photochemistry + night time exposure. 200 ppb ISO + 72 ppb NOx were injected into the chamber and allowed to photochemically react from sunrise to sundown (0632-2002, approximately 13.5 hours). At sundown, 200 ppb ISO + 72 ppb NOx were injected into the empty chamber. Two sets of A549 cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

16. **August 27, 2003**: Isoprene + NOx half day photochemistry + night time exposure. 200 ppb ISO + 50 ppb NOx were injected into the chamber approximately 5 hours before sundown, and allowed to photochemically react until sundown. At sundown, 200 ppb ISO + 50 ppb NOx were injected into the empty chamber. Two sets of A549 cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

17. **September 3, 2003**: Ozone match test, 1 vs. 4 L/min. Two sets of A549 cells were exposed to 200 ppb ozone for 4 hours and analyzed for cytotoxicity and cytokine expression. One set was exposed at 1 L/min, while the second set was exposed at 4 L/min. Both modular incubator chambers were set up and connected to the outdoor smog chamber facility. Incubator temperatures, relative humidity, and flow rates were recorded.

18. **September 10, 2003**: 1,3-Butadiene + NOx half day photochemistry + night time exposure. 200 ppb BD + 50 ppb NOx were injected into the chamber approximately 5 hours before sundown (1438-1900), and allowed to photochemically react until sundown. At sundown, 200 ppb BD + 50 ppb NOx were injected into the empty chamber. Two sets of A549 cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

19. **September 17, 2003**: HC calibrations in outdoor smog chambers. Isoprene, 1,3-butadiene, methacrolein, acrolein, methyl vinyl ketone, acetaldehyde, formaldehyde, propylene, butadiene monoxide, and isoprene monoxide.

20. **September 24, 2003**: 1,3-Butadiene + NOx half day photochemistry + night time exposure. 200 ppb BD + 50 ppb NOx were injected into the chamber approximately 4 hours before sundown (1526-1801), and allowed to photochemically react until sundown. At sundown, 200 ppb BD + 50 ppb NOx were injected into the empty chamber. Two sets of A549 cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

21. **September 29, 2003**: Isoprene + NOx half day photochemistry + night time exposure, regular and pre-exposed cells used. 200 ppb ISO + 50 ppb NOx were injected into the chamber approximately 4 hours before sundown (1505-1904), and allowed to photochemically react until sundown. At sundown, 200 ppb ISO + 50 ppb NOx were injected into the empty chamber. A set of A549 cells were exposed to 200 ppb ozone for 5 hours, while a second set of A549 cells were exposed to air only for 5 hours. The next day, both air and ozone pre-exposed cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

22. **October 6, 2003**: Isoprene + Ozone dark aging + night time exposure, regular and pre-exposed cells used. 200 ppb ISO + 200 ppb Ozone were injected into the chamber approximately 7 hours before sundown. This was performed on a cloudy day to examine isoprene oxidation products, rather than the photooxidation products. At sundown (1800), 200 ppb ISO was injected into the empty chamber. Three sets of A549 cells were exposed to a) one set was kept in the incubator, b) one set was exposed to 200 ppb ozone for 5 hours, c) one set was exposed to clean air for 5 hours. 24 hours later the air, ozone and no pre-exposed cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

23. **October 8, 2003**: Ozone test in EPA chambers, 200 ppb ozone. Two sets of A549 cells were exposed to 200 ppb ozone or clean air for 5 hours and analyzed for cytotoxicity and cytokine expression.

24. **October 13, 2003**: Isoprene + NOx vs. 1,3-Butadiene + NOx half day photochemistry + night time exposure. 200 ppb ISO + 50 ppb NOx were injected into one side of the chamber, while 200 ppb BD + 50 ppb NOx were injected into the other side of the chamber. Both chamber mixtures were injected at 1436 and allowed to photochemically react for 4.5 hours (1900). Two sets of A549 cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

25. **October 22, 2003**: Methanol + NOx full day photochemistry + night time exposure. 4.5 ppm METH + 50 ppb NOx were injected into one side of the chamber at 0830 and allowed to photochemically react until sundown (1800). At sundown, 4.5 ppm METH + 50 ppb NOx were injected into the empty chamber. Two sets of A549 cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

26. **November 3, 2003**: Isoprene + NOx half day photochemistry vs. Isoprene + Ozone, night time exposure. 200 ppb ISO + 50 ppb NOx were injected into one side of the chamber at 1130 and allowed to photochemically react until sundown at 1720. In the other chamber, 200 ppb ISO + 200 ppb ozone were injected at 1137. This experiment is directly comparing the oxidation with the photooxidation products of isoprene. Two sets of A549 cells were exposed for 5 hours to the two chamber mixtures and analyzed for cytotoxicity and cytokine expression.

27. **May 12, 2004**: Matched ozone test. 200 ppb ozone was injected into both sides of the outdoor dual smog chambers. Two sets of A549 cells were exposed for 5 hours to the ozone, while a third set of A549 acted as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

28. **May 16, 2004**: Matched ozone and tracer test. 200 ppb ozone and $CCl_4$ tracer were injected into both sides of the outdoor smog chambers. Two sets of A549 cells were exposed for 5 hours to the ozone, while a third set of A549 acted as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

29. **June 17, 2004**: Matched ozone test. 300 ppb ozone was injected into both sides of the outdoor smog chambers. Two sets of A549 cells were exposed for 4 hours to the ozone, while a third set of A549 acted as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

30. **June 29, 2004**: Isoprene + NOx vs. 1,3-butadiene + NOx half day photochemistry , night time exposures using primary bronchial epithelial cells. 200 ppb ISO + 50 ppb NOx were injected into one side of the dual chambers and 200 ppb BD + 50 ppb NOx were injected into the other side of the dual chambers at 1709. Both mixtures were allowed to photochemically react until sundown at 2002. At sundown, two sets of differentiated bronchial epithelial cells were exposed for 5 hours to the mixtures inside the chambers, and analyzed for cytotoxicity and cytokine expression.

31. **September 22, 2004**: Methanol + NOx vs. Ozone half day photochemistry + night time exposure. 4.5 ppm METH and 50 ppb NOx were injected into one side of the chambers at 0630 and allowed to photochemically react until sundown at 1945. At sundown, the equivalent amount of ozone that was generated in the METH system was injected into the empty chamber. Two sets of A549 cells were exposed for 5 hours to the chamber mixtures, while a third set of A549 cells acted as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression

32. **September 30, 2004**: Methanol + NOx vs. Ozone half day photochemistry + night time exposure. This is a repeat of the September 22, 2004 experiment. 4.5 ppm METH + 50 ppb NOx were injected into one side of the chambers at 0624 and allowed to photochemically react until sundown at 1943. At sundown, the equivalent amount of ozone that was generated in the METH system was injected into the empty chamber. Two sets of A549 cells were exposed for 5 hours to the chamber mixtures, while a third set of A549 cells acted as a control receiving only medial grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

33. **October 21, 2004**: MACR+MVK+Ozone vs. Ozone, night time exposure. This is the first of the product confirmation experiments. October 21, 2004 was a very cloudy day so the dark experiment began before sundown. The product concentrations or amounts injected and compared during this run were equivalent to the amounts generated during the previous isoprene and NOx half day photochemical experiments. At 1809, 201 ppb MACR, 176 ppb MVK, and 130 ppb ozone were injected into one side of the chamber. Into the empty side, 130 ppb ozone was injected. Two sets of A549 cells were exposed for 5 hours to the chamber mixtures, while a third set of A549 cells acts as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

34. **October 27, 2004**: ACR+Ozone vs. Ozone, night time exposure. This is the 2nd product confirmation experiment. At 1649, 220 ppb ACR + 200 ppb ozone were injected into one side of the chamber. Into the empty chamber, 200 ppb of ozone was injected. Two sets of A549 cells were exposed for 5 hours to the chamber mixtures, while a third set of A549 cells acts as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

35. **November 10, 2004**: Isoprene + NOx half day photochemistry vs. Ozone, night time exposure. 200 ppb ISO + 50 ppb NOx were injected into one side of the chamber at 1200 and allowed to photochemically react until sundown at 1713. At sundown, the equivalent amount of ozone generated in the other chamber was injected the empty chamber. Two sets of A549 cells were exposed for 5 hours to the chamber mixtures, while a third set of A549 cells acts as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

36. **May 4, 2005**: Matched ozone test. Matched amounts of approximately 200 ppb ozone were injected into both sides of the chamber. Two sets of A549 cells were exposed for 5 hours to the chamber mixtures, while a third set of A549 cells acts as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

37. **May 10, 2005**: 2 ppm ISO and BD + 0.66 ppm NOx. This high concentration run was performed to analyze the photochemical degradation products that may not be traceable at the concentrations at which the cellular exposures take place. 2 ppm ISO + 0.66 ppm NOx were injected into one side of the chamber. In the other chamber, 2 ppm BD + 0.66 ppm NOx were injected. Both mixtures were allowed to photochemically react approximately 5 hours until sundown. Throughout the experiment GC and GC-MS samples were analyzed for product generation.

38. **June 13, 2005**: Ozone+Form vs. Ozone, night time exposure. This is the 3rd product confirmation experiment. 166 ppb formaldehyde and 314 ppb ozone were injected into one side of the chamber at 2032. 314 ppb ozone was injected into the other chamber at 2036. Two sets of A549 cells were exposed to the chamber mixtures for 5 hours, while a third set of A549 cells acts as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression. At the end of this experiment, the $CO_2$ was disconnected from one of the modular incubator chambers therefore the experiment needs to be repeated.

39. **June 22, 2005**: Ozone+Form vs. Ozone, night time exposure. This experiment is a repeat of the June 13, 2005 experiment. 150 ppb formaldehyde and 280 ppb ozone was injected into one side of the chamber at 2000. At 2010, 280 ppb ozone was injected into the other chamber side. Two sets of A549 cells were exposed for 5 hours to the chamber mixtures, while a third set of A549 cells acts as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

40. **July 7, 2005**: Matched ozone test with new incubator underneath chamber. An incubator containing 1 modular incubator chamber was placed beneath the outdoor dual smog chamber and connected to one of the sides. Equivalent mounts of approximately 200 ppb ozone was injected into both sides of the chambers. Three sets of A549 cells were exposed for 5 hours to the chamber mixtures, two sets inside the lab and one set underneath the chamber. A fourth set of A549 cells acts as a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

41. **July 20, 2005**: 1,3-Butadiene + NOx vs. Ozone half day photochemistry, night time exposure. 200 ppb BD + 50 ppb NOx were injected into one chamber side at 1650-1702 and allowed to photochemically react until sundown. 200 ppb ozone was injected into the empty chamber at 2000, after sundown. Two sets of A549 cells were exposed to the chamber mixtures for 5 hours, while a third set of A549 cells acts a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

42. **August 17, 2005**: ACR+FORM+Ozone vs. Ozone, night time exposure. This is the fifth product confirmation experiment. After sundown, 200 ppb ACR, 81 ppb formaldehyde and 171 ppb ozone were injected into one side of the outdoor smog chambers. The equivalent amount of ozone, 171 ppb ozone, was injected into the empty chamber. Two sets of A549 cells were exposed to the chamber mixtures for 5 hours, while a third set of A549 cells acts a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression

43. **September 28, 2005**: MACR+MVK+Ozone vs. ACR+FORM+Ozone, air and ozone preconditioned cells. After sundown, 201 ppb MACR, 175 ppb MVK and 130 ppb ozone were injected into one side of the chamber. 200 ppb ACR, 81 ppb formaldehyde, and 171 ppb ozone were injected into the other side of the smog chambers. Each set of A549 cells are comprised of non-preconditioned cells, air preconditioned cells and ozone preconditioned cells. Two sets of samples were exposed to the chamber mixtures for 5 hours, while a third set of samples acts a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression

44. **October 20, 2005**: Isoprene + NOx and 1,3-butadiene + NOx half day photochemistry, air and ozone preconditioned cells. 200 ppb ISO + 50 ppb NOx were injected into one side of the chamber, while 200 ppb BD + 50 ppb NOx were injected into the other side of the chamber at 1330. Both chamber mixtures were allowed to photochemically react until sundown at 1830. Each set of A549 cells are comprised of non-preconditioned cells, air pre-conditioned cells and ozone preconditioned cells. Two sets of samples were exposed to the chamber mixtures for 5 hours, while a third set of samples acts a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

45. **April 19, 2006**: Matched ozone test. Matched amounts of approximately 200 ppb ozone were injected into both sides of the chamber. Two sets of A549 cells were exposed for 5 hours to the chamber mixtures, while a third set of A549 cells acts as a control receiving only medical grade breathing air during the 5 hour exposure. Each set contained cells with 3 types of media to study oxidative stress; regular BSA media, BSA + 1/1000 BSO, BSA + 1/100 GSH. All samples were analyzed for cytotoxicity and cytokine expression.

46. **May 9, 2006**: MACR+MVK vs. FORM, night time exposure. This experiment was performed to evaluate the effects of ISO and METH primary products other than ozone. This is the final experiment for the ISO/METH product confirmation manuscript. After sundown, 81 ppb formaldehyde was injected into one side of the outdoor smog chamber, and 201 ppb MACR and 176 ppb MVK were injected into the adjacent empty chamber. Two sets of A549 cells were exposed to the chamber mixtures for 5 hours, while a third set of A549 cells acts a control receiving only medical grade breathing air during the 5 hour exposure. Each set contained cells with 3 types of media to study oxidative stress; regular BSA media, BSA + 1/1000 BSO, BSA + 1/100 GSH. This was cancelled due to low ambient temperatures and condensation in the chambers.

47. **May 30, 2006**: MACR+MVK vs. FORM, night time exposure. This is a repeat of the May 9, 2006 experiment. After sundown, 81 ppb formaldehyde was injected into one side of the outdoor smog chamber, and 201 ppb MACR and 176 ppb MVK were injected into the adjacent empty chamber. Two sets of A549 cells were exposed to the chamber mixtures for 5 hours, while a third set of A549 cells acts a control receiving only medical grade breathing air during the 5 hour exposure. Each set contained cells with 3 types of media to study oxidative stress; regular BSA media, BSA + 1/1000 BSO, BSA + 1/100 GSH. All samples were analyzed for cytotoxicity and cytokine expression.

48. **June 7, 2006**: ISO+NO+light vs. BD+NO+light, half day photochemistry with BSA regular, BSA+GSH, and BSA+BSO. 200 ppb ISO + 50 ppb NOx were injected into one side of the chamber, while 200 ppb BD + 50 ppb NOx were injected into the other side of the chamber at 1425. Both chamber mixtures were allowed to photochemically react until sundown at 2015. Each set contains A549 cells prepared with BSA media, BSA + 1/100 GSH media, and BSA + 1/1000 BSO media. Two sets of samples were exposed to the chamber mixtures for 5 hours, while a third set of samples acts a control receiving only medical grade breathing air during the 5 hour exposure. All samples were analyzed for cytotoxicity and cytokine expression.

## *Appendix B: Additional Results*

### B.1 SynUrban Experiments

During the summer of 2002, the smog-chamber – in vitro-exposure-system was designed and built interfacing the UNC dual outdoor environmental chambers in Chatham County and a modified modular incubator chamber unit. In addition to matched ozone experiments, comparing the effects caused by photochemically reacted hydrocarbon mixtures were examined. The UNC synthetic Urban Mix was evaluated before and after full day photochemical transformations. Subsequently, ozone and NO2 were added to the chamber mixtures and reanalyzed. The new system and initial results were published in Inhalation Toxicology, Sexton et al. 2004.

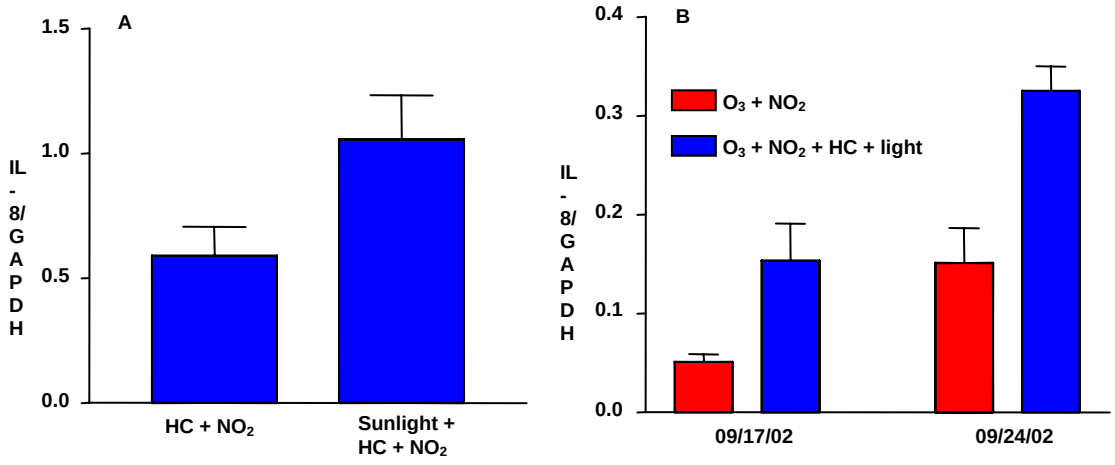



**Figure 13-1: Syn Urban Experiments**
2 ppmC Synthetic Urban Mix and NOx underwent photochemical reactions from sunrise until sunset, producing ozone, formaldehyde, and other carbonyls and PAN. A.) At midnight NO was added to titrate the ozone (0.27 ppm), forming $NO_2$; at midnight, the control chamber was injected with the same amount of $NO_2$ and the VOC precursors (HC). B.) At midnight, the control chamber was injected with ozone, and $NO_2$ equal to the levels formed through the photochemical reactions of the hydrocarbon mixture. Data are shown from experiments performed on two different days. A549 cells were exposed to these mixtures for 5 hours and total RNA isolated 4 hours post-exposure was analyzed for IL-8 mRNA levels using real-time RT-PCR. (Sexton et al. Inhalation Toxicology 2004)

## B.2 High Concentration Isoprene Experiments

Experiments were performed examining how photochemical transformations of ISO affect its toxicity on human respiratory epithelial cells. 500 ppb ISO and 100 ppb NO were injected into one side of the dual outdoor smog chamber before sunrise and reacted with natural sunlight until sundown. After sundown, the precursors (the initial ISO and NO concentrations) were injected into the adjacent, empty chamber. A549 cells were exposed to unreacted and reacted ISO mixtures for 5 hours. Post exposure, cells were placed back into a control chamber and left at the exposure site until morning. Approximately 9 hours post-exposure cells were brought back to the UNC campus and processed. Cells were damaged during the first stage of processing therefore no cytotoxicity analysis was performed. RNA was collected and later analyzed for IL-8.

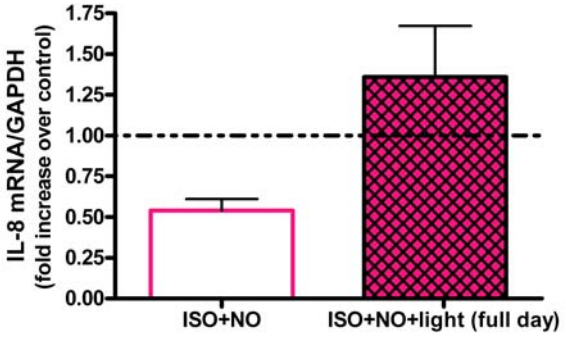



**Figure B-2: Full day ISO photochemical Experiment**

The ISO mixture photochemically reacted throughout the day (approximately 15 hours) and produced secondary and tertiary products. These products were identified and quantified using GC and GC/MS.

One goal of this project was to be able to produce synthetic product mixtures with high certainty, and examine the effects of those products found in the mixtures vs. those not in the mixture. Unfortunately, as ISO photochemically reacts the certainty of product

identification weakens therefore creating a need for a more identifiable product set. The exposure time was adjusted through multiple experiments to allow for only first generation reactions. These photochemical reactions generated initial products, many of them known and quantifiable. Therefore, the new exposure protocol examined and quantified the toxicity of only initial transformations of HAPs.

## B.3 Toluene Photochemistry Experiment

On November 15, 2005 an experiment was run comparing the previous ISO and BD results with a toluene mixture. 200 ppb Toluene (TOL) and 50 ppb NO were injected into one side of the dual outdoor chamber and reacted with sunlight for approximately 5 hours. After sundown, the precursors or parent compounds were injected into the adjacent, empty chamber. A549 cells were exposed to unreacted and photochemically reacted TOL mixtures for 5 hours. Post exposures, cells were held at the site in the control incubator until morning. Approximately 9 hours post exposure, cells were brought to the UNC campus for processing. Basolateral supernatants and RNA were collected and stored until analysis.

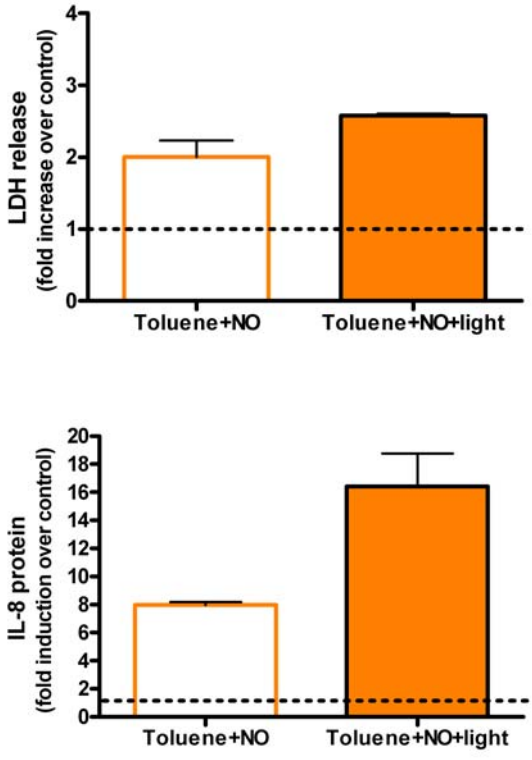



**Figure B-3: Toluene Photochemical Experiment**

Similar to the ISO, BD and METH results, the photochemically reacted mixture induced a significantly greater inflammatory response compared to the unreacted parent compounds. More interestingly is, these results were caused without a large increase in LDH or cytotoxicity. Additional studies need to be examined evaluating the products formed during the photochemical transformations, and quantification of other cytokine mediators.

## B.4 Liquid Exposure Techniques

To examine the effects of individual products generated during photochemical transformations of ISO, a common liquid exposure technique was used. First, MACR was diluted with DMSO into six concentrations. A second set of exposures included two previously tested MACR concentrations, and identical MVK concentrations. This was performed to test the repeatability of the liquid method. A549 cells were grown on membranous support and exposed apically to the liquid mixtures for 5 hours to match the previous ISO experiments. Post exposure, apical and basolateral supernatants were discarded and cytotoxicity was evaluated by the Trypan Blue Exclusion method.

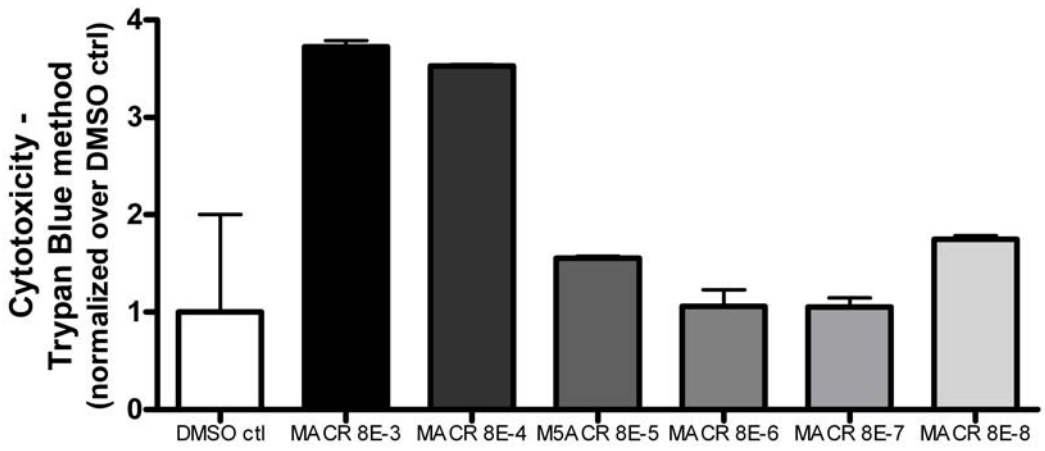



**Figure B-4: Methacrolein Exposure via Liquid Technique**

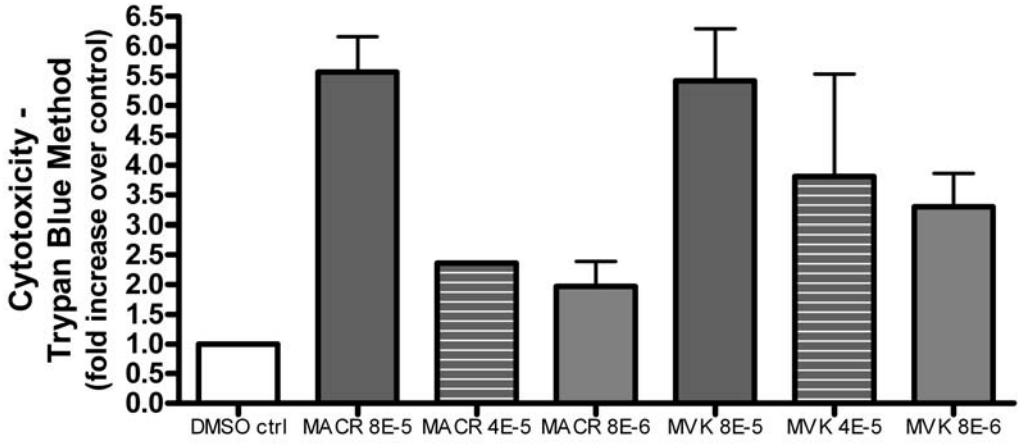



**Figure B-5: Methacrolein and Methyl Vinyl Ketone Exposure via Liquid Technique**

The results from the two of MACR mixtures were not similar, with the second experiment causing a significantly greater amount of cell death compared to the previous experiment.

The MACR and MVK exposures were repeated, using a 1:1 MACR:MVK mixture exposure added.

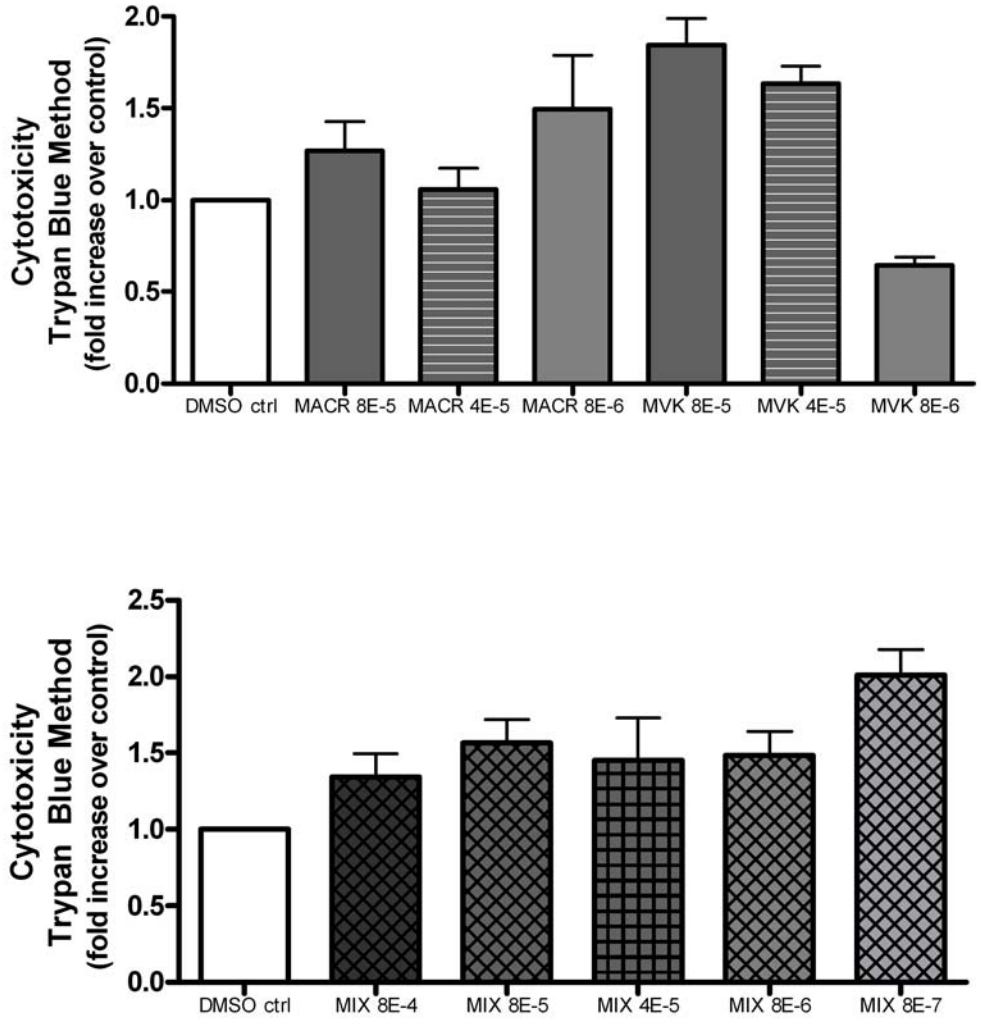
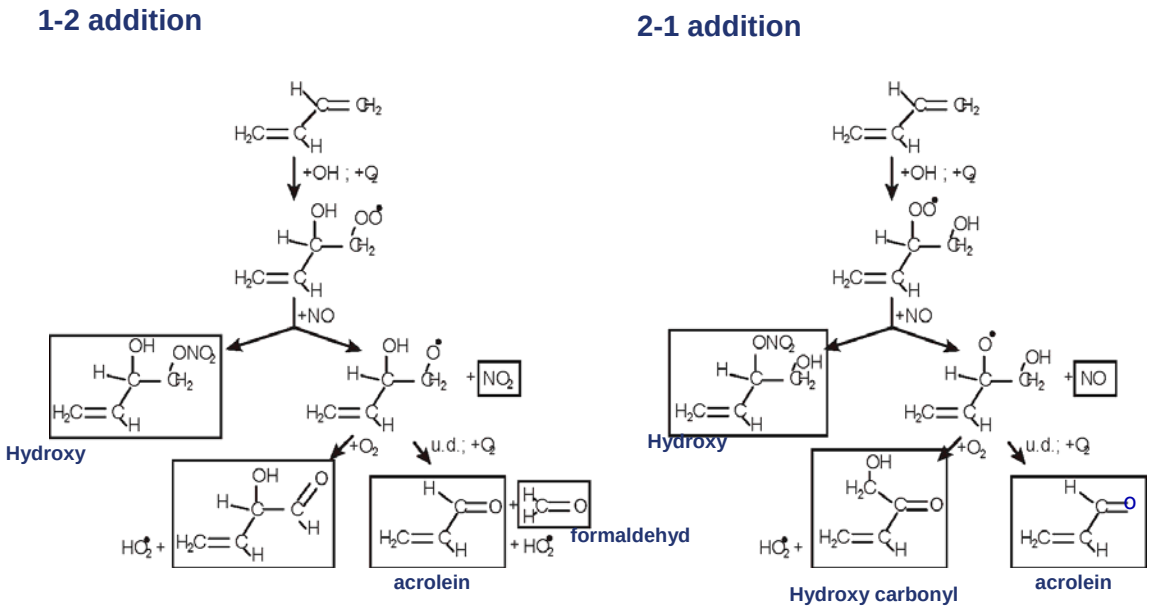



**Figure B-6: Methacrolein, Methyl Vinyl Ketone, and a Combined Mix using Liquid Techniques**

Once again, the matched MACR and MVK experiments did not produce a similar response for concentrations that were repeatedly studied. Furthermore, the mixture results were inconclusive. After these results the liquid technique was discarded.

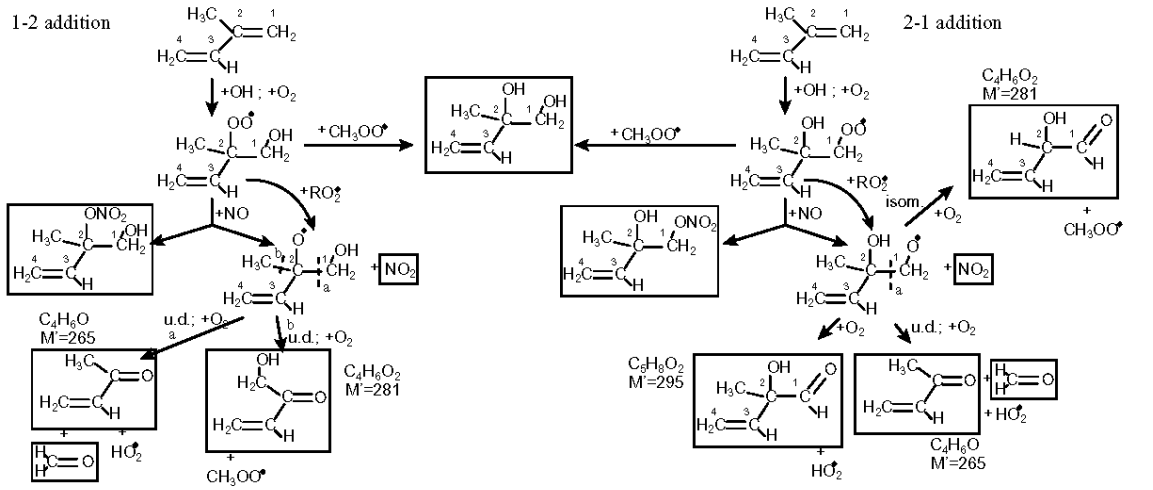
## *Appendix C: OH Reaction Mechanisms for 1,3-Butadiene and Isoprene*

### C.1 Partial OH reaction scheme for 1,3-butadiene




(Jeffries et al.)

### C.2 Partial OH reaction scheme for isoprene




(Jeffries et al.)

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