ABSTRACT

MANZO, NICHOLAS DAVID. Effects of Diesel Exhaust Particle Exposure on Inflamed Murine Lung Epithelial Cells. (Under the direction of Dr. Janice Dye and Dr. Jerry Law.)

This dissertation set out to develop and understanding of what makes individuals with pre-existing pulmonary inflammatory diseases more susceptible to particulate matter (PM) air pollutant exposure. The data presented here describes (1) the development of an *in vitro* system that models the epithelial inflammatory microenvironment in inflammatory lung diseases, (2) the differential cellular effects of pulmonary epithelial exposure to traffic related PM in the setting of health or an "inflammation", and (3) the contributing mechanism(s) involved in these differential cellular effects.

Pulmonary epithelial cells are exposed to both inhaled PM and pro-inflammatory mediators, and thus are appropriate to investigate the effects of PM exposure. First, an *in vitro* model of pulmonary epitheail cells grown in a inflammatory microenvirnment was created. Primary mouse tracheal epithelial (MTE) cells grown at an air-liquid interface characteristic of the conducting airways, and LA-4 epithelial cells characteristic of alveolar type II cells were exposed to pro-inflammtry cytokines (cytomix: TNFα + IL-1β + IFNγ). Cytomix treatment of MTE and LA-4 cells resulted in increased release of neutrophillic (MIP-2) and eosinophillic (RANTES) chemokines 24 hr after treatment. Cytomix treatment of LA-4 cells resulted in increased mRNA and protein of iNOS, with the subseqent release of nitric oxide (NO), a reactive oxygen species (ROS**)** invoved in inflammtion.

Using this *in vitro* model, the differential effects of PM exposure were evaluated and compared to normal cells. Control and cytokine-treated LA-4 and MTE cells were exposed

to diesel exhaust particles (DEP), abundantly present in urban environments and rich in organic carbon, as well as particles with low (SRM 2975) or no (carbon black) organic carbon content. Exposure of control and cytokine-treated MTE cells to DEP (20 $\mu g/cm^2$; 24 hr) was seemingly without effect on cell injury (LDH release) and antioxidant status (GSH). However, exposure of inflamed MTE cells resulted in increased epithelial solute permeability. LA-4 cells exposed to DEP (25 $\mu g/cm^2$; 24 hr) induced adaptive cytoprotective (HO-1), antioxidant (GSH) responses with no apparent cell injury (LDH). In contrast, DEP exposure of cytokine-treated LA-4 cells resulted in oxidative stress culminating in significant cytotoxicity that was seemingly related to the organic carbon fraction of the particles, with particles with little or no organic carbon eliciting no significant changes.

To further investigate the oxidative stress elicited in cytokine-treated LA-4 cells exposed to DEP, the generation of the ROS superoxide ($O_2^{\cdot -}$) was evaluated, as well as its ability to cooperate with NO to form peroxynitrite ($ONOO^-$), a more potent radical involved in cell injury. LA-4 cells exposed to DEP (25 $\mu g/cm^2$, 24 hr) resulted in ROS production, including $O_2^{\cdot -}$. However, DEP exposure of cytokine-treated LA-4 cells resulted in even greater ROS production that could be inhibited with an iNOS inhibitor (1400W) and an $O_2^{\cdot -}$ antioxidant (SOD). The resulting cell injury of cytokine-treated LA-4 cells exposed to DEP could be reduced by treatment with FeTMPyP, an $ONOO^-$ scavenger. Furthermore, *in vivo* pulmonary administration of pro-inflammatory cytokines followed by DEP inhalation resulted in increased ROS production that was effectively abrogated by treatment with FeTMPyP.

Collectively, these findings show that cytokine-treated lung epithelial cells are more susceptible to the damaging effects of organic rich DEP exposure through the cooperative effects of NO and $O_2^{\cdot -}$. Whereas multiple processes may be involved in the adverse health effects of PM exposure in susceptible populations, the above mechanism may contribute to the adverse health effects of DEP exposure of individuals with chronic inflammatory lung diseases.

Effects of Diesel Exhaust Particle Exposure on Inflamed Murine Lung Epithelial Cells

by
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DEDICATION

This dissertation is dedicated to my family, my wife, and my children for enduring with me throughout my doctorate.

BIOGRAPHY

Written by Sarah Cleary Manzo

Nicholas David Manzo has two passions in life: his family and science. He grew up in Brooklyn, NY in a family of six children, and eventually attended Union College, earning a degree in Biology and honors for his research on the regeneration of Salamander hearts in 2000. Shortly after graduating, he moved to Boston to begin his career in pulmonary research at New England Medical Center. There in Boston, he also met and began dating his future wife, Sarah. After two years of research at NEMC, his work was relocated to Massachusetts General Hospital. In the summer 2004, he and Sarah were married. Shortly after, they relocated to the Raleigh, North Carolina area, so that Nick could begin the graduate program in Comparative Biomedical Sciences at North Carolina State University.

Nick began his graduate program as a Master's student under the direction of Dr. Janice Dye and Dr. Linda Martin, attending classes at the University and doing his research at the EPA in Research Triangle Park. Eventually, he became so involved in his work that he applied to become a doctoral candidate. He greatly enjoyed what he was doing and knew that a Ph.D. would eventually give him a stronger voice in the field of science. His request was approved and he continued his research and studies under the supervision of Dr. Janice Dye.

During this time of study, he and his wife also bought their first home, adopted two dogs, and became proud parents to daughters Abigail and Ella. He also worked part time as a veterinary technician at a low cost spay and neuter clinic for cats to help support his growing

family. Throughout his time of study, Nicholas did a tremendous job of balancing his family life and his research, both of which are very important to him. As he prepares to finish his time at North Carolina State University, he looks forward to beginning his next scientific journey as he will soon begin a post-doctoral position involving pulmonary research at Duke University.

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To my committee members, Dr, Jerry Law, Dr. Philip Sannes, and Dr. Jody Gookin, I am truly thankful for your thoughtful criticism, encouragement, and time as you navigated me through my doctorate.

As well, I have had the pleasure of working with a number of very talented and knowledgeable people both at the U.S. EPA and NCSU. To all of you, thank you for sharing your time and help not only in the lab but also for being willing to listen to me and offering advice about everything from school to life in general.

Finally, I would also like to thank my wife Sarah, our daughters Abigail and Ella, and my parents, brothers, and sister, for all the encouragement, love, and support that you have always proved over the years. Although too young for school, Abigail and Ella have always provided the perfect reason to take a break and play with blocks. Last, but not least, I would like to thank my wife Sarah for her understanding, love, and my source of strength over the past few years. This degree is as much as testament to her endurance as it is to mine and I certainly would have not made it this far without her by my side.

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CHAPTER 1:

Introduction

This dissertation set out to develop an understanding of what makes individuals more susceptible to air pollutant exposure. More specifically, its purpose was to examine contributing mechanisms of particulate pollutant-mediated adverse respiratory effects in individuals with pre-existing inflammatory pulmonary disease. In these studies, cultured pulmonary epithelial cells pre-treated with selected pro-inflammatory mediators, as well as a corresponding *in vivo* mouse model, were exposed to particulate matter (PM) representative of a traffic-dominated urban air shed.

The first chapter of this dissertation will provide background information on PM air pollution and its effects on the lung. First, PM air pollution and its physical and chemical characteristics will be examined and discussed. Then, exposure levels and deposition patterns of PM relevant to humans will be considered. Finally, the adverse health effects and possible mechanism(s) of PM exposure of healthy individuals will be compared to individuals with other respiratory disorders.

The three chapters that deal with experimental data consist of: first, development of an *in vitro* model of pre-existing inflammation in which epithelial cells representative of the airways and deep lung are treated with pro-inflammatory cytokines; second, characterization of the adverse cellular events that occur when cytokine-treated epithelial cells are exposed to well characterized PM of varying composition; and third, examination of the mechanism(s) involved in epithelial injury observed when cytokine-treated (i.e. inflamed) epithelial cells are exposed to diesel exhaust particles (DEP). This is followed by a concluding chapter which places this research in the context of the broader field of PM toxicology and susceptibility to air pollutants.

AMBIENT PARTICULATE MATTER AIR POLLUTION

The air we breathe is far from clean, but rather polluted by a wide variety of agents from many different sources, both natural and anthropogenic. Air pollution is of increasing concern worldwide because of its associated adverse effects on the environment, human health, and its resulting economic impact. Despite current government regulations, there are still adverse health effects associated with exposure to air pollutants, necessitating a better understanding of how air pollutants exert their effects and who is at risk.

Under the authority of the Clean Air Act, the United States Environmental Protection Agency (US EPA) sets National Ambient Air Quality Standards (NAAQS) for six common air pollutants (termed "criteria pollutants") considered present nationally and harmful to public health and the environment. The six "criteria" pollutants are carbon monoxide (CO), lead (Pb), nitrogen oxides (NOx), ground level ozone (O_3), sulfur oxides (SOx), and PM (US EPA 2004, 2002). Air pollution PM, unlike the other criteria pollutants (O_3, CO, SOx, NOx, and Pb), is a dynamic mixture of particles from different sources, both natural and anthropogenic, with equally diverse physical and chemical properties. PM is a mixture of large and small particles that are classified into three categories depending on their aerodynamic diameter: coarse (> 2.5 μm), fine (≤ 2.5 μm), and ultrafine (< 0.1 μm) (US EPA 2004). On a mass basis, the majority of ambient PM is distributed into coarse and fine particles. However, ultrafine particles account for the majority of particle numbers of ambient PM (US EPA 2002, 2004).

The sources and atmospheric lifetime of PM is highly dependent on their size. Coarse particles are primarily formed by the mechanical disruption (grinding, erosion, and

re-suspension by the wind) and are composed of both natural (soil, bacteria, pollen, mold, fungus spores, and plant/animal debris) and composite (tire, break pad, and road debris) materials (Sioutas, Delfino, and Singh 2005; US EPA 2004; Wichmann 2007). Coarse particles are removed from the atmosphere rapidly (via dry deposition and rainfall) due to their large size (Sioutas, Delfino, and Singh 2005; US EPA 2004). Conversely, fine and ultrafine particles are mostly anthropogenic in origin, produced primarily as a by-product of combustion which can persist in the atmosphere for up to several weeks and travel great distances (Sioutas, Delfino, and Singh 2005; US EPA 2004). Their chemical composition consists of sulfate, nitrate, ammonium, organic carbon (OC), elemental carbon (EC), as well as a variety of trace metals.

DIESEL EXHAUST PARTICLES

In recent years, several studies have shown that a large portion of urban PM is the result of fossil fuel combustion from mobile sources like automobiles (Sioutas, Delfino, and Singh 2005; US EPA 2004, 2002). Emission from diesel motors has been of particular concern since it contributes significantly to ambient air pollution (Pandya et al. 2002; Riedl and Diaz-Sanchez 2005; Ris 2007; US EPA 2004, 2002; Wichmann 2007). Due to their excellent fuel economy and durability, diesel engines are typically in operation for more years and travel greater miles than their gasoline-powered counterparts. Thus, the diesel engine is preferred in locomotives, ships, trucks, busses, agricultural or other off-road equipment. Produced by the combustion of diesel fuel, diesel exhaust consists of a complex mixture of PM, acid aerosols, volatile organic compounds, various hydrocarbons, transition metals, and gases (Ris 2007; Singh et al. 2004; US EPA 2002; Wichmann 2007). Owing to

differences in operating conditions such as engine and vehicle type, engine speed and load, fuel formulations, and air temperature and humidity, the composition of diesel exhaust can be variable. Diesel exhaust particles (DEP) account for a large portion of ambient fine and ultrafine particles, and thus have historically been used as a surrogate to measure human exposure to diesel exhaust emissions (Ris 2007; US EPA 2002; Wichmann 2007). Whereas DEP are classified as fine PM with a mean aerodynamic diameter of approximately 0.2 μm, a large number (~ 80%) of freshly emitted DEP are classified as ultrafine particles with aerodynamic diameters ≤ 0.1μm. These particles consist of an inert elemental carbon core with a large surface area onto which many organic carbon compounds, as well as minute amounts of sulfate, nitrate, metals, and other trace metals become adsorbed. Although the exact chemical composition of DEP can vary; in general DEP contain 33-90% elemental carbon (EC), 7-49% organic carbon (OC), 1-5% metals and elements, 1-4% sulfate and nitrate, and 1-10% other (Hesterberg et al. 2009; Ris 2007; Singh et al. 2004; US EPA 2002). In addition, many of the organic compounds adsorbed to the surface of DEP possess mutagenic and carcinogenic properties and are listed as hazardous air pollutants by the US EPA (Hesterberg et al. 2009; Pandya et al. 2002; Ris 2007; Singh et al. 2004; US EPA 2002).

AMBIENT & OCCUPATIONAL DEP EXPOSURE

The US EPA conducts national-scale assessments of DEP, as well as 33 other air pollutants, as part of the National Air Toxics Assessment activities. Assessment of ambient DEP concentrations has relied on collection and characterizing of ambient samples, modeling studies that recreate emissions and atmospheric conditions, as well as the measurement of surrogate species, such as elemental carbon. Whereas the national ambient DEP

concentrations range from 1.4-2.06 μg/m^3, there exist differences between rural and urban settings dependent on the amount of diesel vehicle traffic (Hesterberg et al. 2009; Ris 2007; US EPA 2002). In urban areas, where people spend a large portion of their time outside in close proximity to major roadways, DEP concentrations average 1.6-2.4 μg/m^3, and as high as 4.0 μg/m^3 in some locations, unlike rural environments with relatively low ambient DEP concentrations (0.6-0.74 μg/m^3) (Hesterberg et al. 2009; Ris 2007; US EPA 2002). Furthermore, in urban environments off-road sources of DEP contribute roughly twice the amount of PM, as compared to on-road sources. While current air quality regulations and improvements in diesel engine technology have resulted in national decreases in ambient DEP concentrations, occupational monitoring indicates that DEP levels remains high, 100-400 μg/m^3, for miners, railroad workers, public-transit workers, airport crew, mechanics, dock workers, and truck drivers (Hesterberg et al. 2009; Ris 2007; US EPA 2002).

DEP DEPOSITION & CLEARANCE

The average adult human inspires about 500 mL of air per breath, which at a respiratory rate of 12 b.p.m. means that on a daily basis we inhale over 8,500 L of air. Inhalation of DEP will result in particle deposition throughout the lung, but the exact DEP dose to pulmonary tissues is related not only to the extent of its deposition but also to its clearance. Particles that enter the lung during inhalation show specific deposition patterns that are dependent on particle size and shape. Physical characteristics of particles determine if it will deposit by impaction, sedimentation, or diffusion (Salvaggio 1994; West 1995). As particles are carried into the pulmonary system at a high velocity, their resulting momentum resists sharp changes in airflow and the particles impact onto the mucus covered epithelium.

The nose and bifurcations of the large airways possess dramatic changes in air flow direction and thus many particles, especially particles > 2.5 μm, impact on these surfaces. Nearly all particles that are greater than 20 μm are not inhaled past the conducting airways because of impaction at the nose or airway bifurcations (Salvaggio 1994; West 1995). As airflow reaches the terminal bronchioles, the end of the conducting airways, and enters the respiratory zone the resulting cross-sectional area dramatically increases due to the successive branching of respiratory bronchioles and alveolar ducts leading to the alveolar sacs (West 1999). The resulting airflow velocity becomes very slow, with particle movement being achieved by diffusion, thus medium sized particles of 1-5 μm in diameter frequently deposit in the bronchoalveolar duct. Lastly, extremely small particles, less than 0.5 μm, behave like gasses and may either be exhaled or deposit in the small airways and alveoli (Salvaggio 1994; West 1995). Deposition of DEP in the lung is considered to have a polydispersed distribution. Although the median aerodynamic diameter of DEP is 0.2 μm, a large portion of DEP is < 0.1 μm, and due to agglomeration, there are even larger particles. Thus while the majority of DEP will deposit in the alveolar region, deposition of DEP will also occur in the tracheobronchial and extrathoracic regions of the lung (Oberdorster and Utell 2002; Riedl and Diaz-Sanchez 2005).

The pulmonary epithelium represents a primary site for the deposition of inhaled particles, like DEP. As the initial line of defense, the pulmonary epithelium effectively separates the luminal (or apical) surface from the submucosal (or basolateral) region. This barrier is accomplished by the presence of tight junctions that not only preclude particles from accessing the underlying tissue, but also prevent plasma leakage into the airway lumen

or alveolar space. In addition to providing a physical barrier, the pulmonary epithelium can respond to exogenous insults and produce a variety of active compounds, including lipid mediators, growth factors, and cytokines/chemokines, which are involved in innate immunity and modulators of pulmonary inflammation.

Clearance of deposited particles is primarily achieved by two mechanism: mucociliary clearance and/or alveolar macrophage phagocytosis. Secreted by goblet cells and submucosal glands, the airways are cover by a thick mucus bilayer that traps and prevents contaminants from reaching and damaging the underlying epithelium. The underlying ciliated cells of the epithelium beat in a coordinated, unidirectional fashion, moving the mucus and its trapped contaminants towards the larynx to be either expelled by coughing or swallowed. While these coordinated actions, known as mucociliary clearance, are very efficient, they do not extend to small airways (Salvaggio 1994; West 1995). Particles that reach the distal airways are primarily removed by the resident phagocytic cells, the alveolar macrophages. Upon ingestion, particle-laden macrophages may migrate to be removed from the lung via mucociliary clearance or lymphatic vessels. Macrophages also possess several mechanisms to neutralize ingested contaminates, including production of toxic nitric oxides, superoxides, and hydrogen peroxides, production of antimicrobial defensins, the competitive binding of essential iron cofactors, and fusion of the phagasome with vesicles containing enzymes such as lysozyme resulting in acidification of the ingested contaminates (Janeway et al. 2006; Salvaggio 1994). Occasionally, if the invading material is too large to be phagocytized or it resists the neutralizing actions of the macrophage, it will be walled off from the surrounding tissue by the coordinated actions of multiple

macrophages in the form of a granuloma. In addition to mucociliary clearance and alveolar macrophage phagocytic actions, the lung utilizes several immune mediators, such as immunoglobulins, complement, and cytokines/chemokines to hopefully aid the inactivation/destruction of the inhaled material (Janeway et al. 2006; Salvaggio 1994). These defense mechanisms can become overwhelmed resulting in inflammation when (i) the lung is exposed to high or chronic particle burden, (ii) the inhaled particle becomes permanently lodged due to its shape, (iii) the inhaled particle is toxic, or (iv) the result of ensuing disease.

DEP HEALTH EFFECTS

Epidemiological Studies

Epidemiological studies have linked air pollution to human morbidity and mortality. Increased PM has been linked to observed increases in respiratory health effects. Numerous studies have shown associations between increased symptoms of cough, bronchitis, asthma, and chronic obstructive pulmonary disease (COPD) to increases in air pollutants (Jaffe, Singer, and Rimm 2003; Riedl and Diaz-Sanchez 2005). The most profound adverse effects of PM exposure were published in 1993, in which increased mortality was associated with increasing air pollution concentrations across six US cities representative of the range of particulate air pollution in the United States. The results of this study showed a statistically significant association between increased mortality and increased fine particle ($PM_{2.5}$) concentrations, even after controlling for several other risk factors such as age, sex, cigarette smoking, education level, body-mass index, and occupational exposure. Furthermore, the effects of $PM_{2.5}$ exposure were positively associated with mortality due to cardiopulmonary diseases (Dockery et al. 1993). Further analysis of the "six city" study combined with

elemental composition of size fractionated particles showed that emissions from motor vehicles were associated with increased mortality (Laden et al. 2000). The results of the "six city" study were later replicated and confirmed in the larger American Cancer Society (ACS) study in 1995 which sampled over 500,000 US adults matched to site specific $PM_{2.5}$ data, showing a dose-dependent increase in adjusted mortality associated with increased $PM_{2.5}$. Further analysis of the ACS study revealed that for each 10 $\mu g/m^3$ increase in ambient $PM_{2.5}$ there was an associated 6% increase risk of cardiopulmonary mortality (Pope et al. 2002). The link between $PM_{2.5}$ concentrations and adverse health outcomes were further strengthened by an extended 8 yr follow-up of the "six city" study as well as the examination of county-specific life expectancy in 50 metropolitan areas of the US that showed reduced mortality risk and increased life expectancy with the concomitant decrease in $PM_{2.5}$ concentrations (Laden et al. 2006; Pope, Ezzati, and Dockery 2009).

Whereas the preponderance of epidemiological studies have shown adverse effects of PM exposure on health, the majority of these studies are based on central pollutant monitoring sites. More recently, researchers have begun to consider the local spatial variability on pollutant levels related to vehicle emissions that are dependent on the proximity to road traffic. Since DEP account for a significant portion of traffic-related PM, traffic-related studies have been used as proxies to determine the effects of diesel emission exposure. And conversely, experimental studies using DEP have been used as proxies for investigating traffic-associated health effects. The studies have shown that the proximity to busy roads is associated with adverse health outcomes, including respiratory symptoms, asthma, COPD, atopic disease, allergic sensitization, and rhinitis (Cesaroni et al. 2008; Kim

et al. 2008; Kim et al. 2004; McConnell et al. 2006; Morgenstern et al. 2008; Renzetti et al. 2009; Schikowski et al. 2005). For example, in one southern California study, asthma and wheeze were strongly associated with residential locations adjacent to major roadways, whereas the risks decreased at locations 150-200 meters from the road (McConnell et al. 2006). A similar study conducted in the East Bay of California showed high correlations between asthma and bronchitis for children living within 150 meters of a major roadway, whereas children living 300 meters from a major roadway had weaker associations (Kim et al. 2008). Comparable strong associations between living close to main roads and atopic disease and allergic sensitization in children have also been reported, with the strongest effects being evident for children living less than 50 meters from busy streets (Morgenstern et al. 2008). Interestingly, in one study rapid improvement in both airway inflammation and lung function were observed when asthmatic children living in highly polluted urban environments were relocated to less polluted rural environments (Renzetti et al. 2009).

Human Exposure Studies

In addition to epidemiological studies, human controlled exposure studies have also been used to investigate the health effects of pollutants including diesel exhaust. Compared to epidemiological studies, human exposure studies enable the direct effect on humans to be studied under well define PM exposure concentrations and durations, and in the absence of confounding exposures to other air pollutants. The clinical effects of short-term DEP exposure have been explored in both healthy subjects as well as individuals with inflammatory lung diseases like asthma and COPD. In general, exposure of healthy subjects to short-term DEP of relatively high concentrations (100-300 $\mu g/m^3$), results in pulmonary

and systemic inflammatory responses, without corresponding change in pulmonary function (Pourazar et al. 2004; Salvi et al. 1999; Salvi et al. 2000; Stenfors et al. 2004). For example, whereas exposure of healthy subjects to short term (1 hr) diesel exhaust (300 μg/m3) did not alter pulmonary function, there was a significant increase in acute inflammatory response, with increased pulmonary inflammatory cells (neutrophils, B cells, T cells, mast cells) (Salvi et al. 1999). The DEP-induced airway leukocyte infiltration was further enhanced by increases in cell adhesion molecules on the pulmonary endothelium (ICAM-1, VCAM-1) and recruited cells (LFA-1) as well as increases in inflammatory chemokines (IL-8, GRO-a, IL-5) (Salvi et al. 1999; Salvi et al. 2000). A similar effect of DE-induced pulmonary inflammation in healthy subjects was also observed when exposed to lower concentrations (108 $\mu g/m^3$, 2 hr) (Stenfors et al. 2004).

Based on epidemiological evidence suggesting that individuals with pre-existing inflammatory lung diseases, like asthma and COPD, are more susceptible to the respiratory effects of PM exposure, it would seem logical that human controlled studies would show similar results. However, controlled DEP exposure of individuals with mild asthma has yielded conflicting results. For example, asthmatics exposed to DEP (300 $\mu g/m^3$, 1 hr) showed significant increases in pulmonary levels of the pro-inflammatory cytokine IL-6 as well as significant increases in both airway resistance and hyper-responsiveness (Nordenhall et al. 2001). In contrast, studies of healthy and asthmatic patients exposed to lower levels of DEP (108 $\mu g/m^3$) for 2 hr showed no difference between healthy and asthmatic patients for any the parameters evaluated (Stenfors et al. 2004). Although not a controlled exposure study, a recent panel study of 60 participants with mild to moderate asthma demonstrated

adverse respiratory effects when exposed to real-world traffic emissions. In this study, adults with asthma walked for 2 hr in an area where only diesel-powered vehicles were permitted. The results showed significant reductions in lung function and increased inflammatory changes in sputum as compared to asthmatics that walked in a nearby park (McCreanor et al. 2007).

MECHANISMS OF DEP-INDUCED EFFECTS

Reactive Oxygen Species

The lungs exist in a high oxygen environment, which in conjunction with its large surface area, makes it especially vulnerable to oxidant damage. The pulmonary epithelium is constantly exposed to oxidants generated by normal cellular metabolism as well as to oxidants present in ambient air. In the lung there exist a variety of cellular sources of reactive oxygen species (ROS), including leukocytes (neutrophils, eosinophils, macrophages), as well as epithelial (bronchial, bronchiolar, and alveolar) and endothelial cells. Endogenous biological production of ROS can occur during reduction of molecular oxygen to water during normal metabolic reactions such as aerobic respiration in the mitochondrial electron transport chain, as well as the by-product of enzymatic reactions involving cyclooxygenases, cytochrome P_{450} oxidases, xanthine oxidases, and NADPH oxidases (Rahman, Biswas, and Kode 2006). Exposure of the lung not only to exogenous stimuli but also to endogenous inflammatory mediators present in disease can result in the generation of ROS that includes: superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2).

There is increasing evidence implicating the important physiological role of ROS as secondary messengers in the cellular response to stimuli. Specifically, redox sensitive molecular targets include signaling molecules (MAPK, Akt, PKC, PLA_2), transcription factors (NFκB, AP-1, HIF-1), and gene expression through chromatin modeling (histone acetylation/deacetylation/methylation/demethylation) (Park, Kim, and Lee 2009; Rahman 2002). However, oxidative stress can occur when high levels of ROS are produced without a corresponding increase in antioxidants, thus creating an oxidant/antioxidant imbalance in favor of oxidants ultimately causing the oxidation of proteins, DNA, and lipids and resulting in altered function (Park, Kim, and Lee 2009).

Like many ROS, the endogenous free radical nitric oxide (NO) is a ubiquitous intracellular messenger, which at low concentrations regulates several physiological activities, including airway tone. NO is synthesized enzymaticly by the oxidation of L-arginine to L-citrulline. The reaction is catalyzed by NO synthases (NOS), which exists in three isoforms: nNOS (neuronal), eNOS (endothelial), and iNOS (inducible) (Ricciardolo et al. 2005; Ricciardolo et al. 2006). Whereas nNOS and eNOS are constitutively expressed predominantly in neurons and endothelial cells, respectively, iNOS is present in macrophages and lung epithelial cells and when induced under pro-inflammatory conditions, is capable of producing large quantities (nmol concentrations) of NO (Martinez and Andriantsitohaina 2008; Redington et al. 2001; Redington 2006). However, NO can become deleterious when it reacts with other free radicals, like $O_2^{\cdot -}$, to form the more potent oxidant peroxynitrite ($ONOO^-$), resulting in functional alteration of proteins and lipids, damaging DNA, and

inducing cell death (Martinez and Andriantsitohaina 2008; Ricciardolo et al. 2005; Ricciardolo et al. 2006; Ricciardolo, Nijkamp, and Folkerts 2006; Xiao, Nel, and Loo 2005).

DEP-Induced Oxidants

Several *in vivo*, *in vitro*, and acellular investigations into the effects of DEP have shown the formation of ROS to be significantly increased after DEP exposure (Dellinger et al. 2001; Han, Takeshita, and Utsumi 2001; Knaapen et al. 2002). The production of DEP-induced oxidative stress can be the result of a variety of mechanisms, and has generally been thought to be due to its adsorbed chemicals and compounds contained on the particle surface. In addition to the direct generation of ROS from free radicals and oxidants on the particle surface, DEP contain organic carbon compounds as well as minute amounts of transition metals that can also generate ROS. For instance, transition metals such as iron, copper, chromium, and vanadium have been found in trace amounts on DEP and can generate ROS, such as the extremely reactive $OH^{\cdot}$. There exists increasing evidence that the organic fraction of DEP induces oxidative stress by mechanisms including the redoxy-cyling of quinones to produce $OH^{\cdot}$, and the metabolic activation of organic compounds resulting in the induction of pulmonary P_{450} enzymes that in turn produce ROS. For example, exposure of human pulmonary epithelial cells to either DEP or the organic extract of DEP, can result in increased ROS production as well as increased expression of phase I (P_{450} 1A1) and phase II (NQO-1) xenobiotic metabolization enzymes (Baulig et al. 2003).

Antioxidants of the lung

In order to combat various endogenous and exogenous oxidant stressors, the lung is armed with a well developed system of non-enzymatic and enzymatic antioxidants. In

addition to vitamin C and E, the tripeptide glutathione is the predominant non-protein thiol in cells, and serves to maintain normal cellular redox status. Glutathione exists in either its reduced (GSH) or oxidized/disulfide form (GSSG). Whereas the GSH content in a cell can range from 5-10 mM, GSSG occurs at much lower levels (Biswas and Rahman 2009; Sugiura and Ichinose 2008). The presence of a sulfhydryl group in GSH allows it to function as an antioxidant. Alternatively, the lungs possess several different enzymatic antioxidants, such as superoxide dismutase (SOD) and heme oxygenase-1 (HO-1) that prevent ROS accumulation. Present in every cell, SOD is found in three forms, CuZnSOD (cytosolic), MnSOD (mitochondrial), and ECSOD (extracellular). All of these SOD forms serve to protect against $O_2^{\cdot -}$ by dismutating it to H_2O_2 (Rahman, Biswas, and Kode 2006; Sugiura and Ichinose 2008). Likewise, HO-1 plays an important protective role against oxidative stress. HO-1 catalyzes the degradation of heme into bile pigments and carbon monoxide, both of which have been shown to possess free radical scavenging properties.

SUMMARY

Epidemiological studies have noted an association between levels of urban traffic-related particulate air pollutants, like DEP, and increased mortality. Individuals with pre-existing inflammatory lung diseases, like asthma and COPD, are uniquely susceptible to the adverse health effects of traffic-related PM. Furthermore, human experimental PM exposure studies, in either controlled or real-world settings, have also shown that individuals with pre-existing inflammatory lung disease have greater inflammatory and functional lung responses as compared to health control subjects. Due to the respirable size and the deposition characteristics of DEP, the pulmonary epithelium is a primary target for DEP to elicit

oxidative stress. However, the pulmonary epithelium of individuals afflicted with diseases, such as asthma and COPD, exists in a state of persistent inflammation prior to DEP exposure that may render them at further risk of adverse health effects. Therefore, this dissertation will focus on understanding and elucidating further why individuals with pre-existing inflammatory lung diseases are more susceptible to the effects of DEP exposure. Specifically, in the chapters that follow, data will be presented that (a) describes and characterizes an *in vitro* model of pulmonary epithelial inflammation achieved by administration of key pro-inflammatory cytokines (Chapter 2), (b) utilizes this model to further explore if DEP exposure, and its particle characteristics, results in increased adverse effects (Chapter 3), and (c) to determine whether the differential effects observed are the result of the cooperation of contributing oxidants induced by inflammation and DEP exposure (Chapter 4).

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CHAPTER 2:

Development of an *In Vitro* Model of Lung Inflammation Utilizing Murine Airway and Alveolar Epithelial Cells Treated with Exogenous Cytokines

ABSTRACT

Thirty-five milion people in the US are afflicted with chronic pulmonary inflammatory disorders, like asthma and chronic obstructive pulmonary disease (COPD). Epidemological evidence suggests that these individuals are at increased risk of the adverse health effects from particulate matter (PM) exposure. Whereas most of the epidemiological evidence linking PM exposure to health effects is derived from individuals with pre-existing inflammatory lung diseases, the majority of experimental *in vitro* studies are conducted in normal healthy cells and do not mimic the inflammatory microenvironment representative of these disease settings. To address this, two different mouse cell preparations were employed modeling specific lung regions: (1) primary mouse tracheal epithelial cells grown at an air-liquid interface characteristic of the conducting airways, and (2) the LA-4 cell line characteristic of alveolar type II cells. Cells were exposed to pro-inflammatory cytokines (TNFα, IL-1β, and IFNγ) to mimic the inflammatory microenviroment and the resulting increase in pro-inflammatory enzymes (iNOS) and release of chemokines (MIP-2 and RANTES) was evaluated. In both cell types, exposure to either IL-1β or TNFα, individually and in combination, resulted in increased mRNA expression and release of neutrophillic (MIP-2) and eosinophillic (RANTES) chemokines. RANTES mRNA expression and production was further increased by the addition of IFNγ. Cytokine-treated LA-4 cells resulted in increased mRNA and protein expression of iNOS and nitric oxide (NO) production. Together, the present study describes an *in vitro* epithelial system that models the inflammatory microenvironment found in individuals with inflammatory lung diseases.

KEY WORDS: TNFα, IL-1β, IFNγ, nitric oxide, MIP-2, RANTES

INTRODUCTION

Human exposure to particulate matter (PM) air pollution, produced by the incomplete combustion of fossil fuels, can result in increased mortality, most notability in individuals with pre-existing inflammatory lung diseases (Dockery et al. 1993). The use of *in vitro* models have helped further our understandings of the mechanisms and modes of action of PM-induced effects. However, there exist few *in vitro* models that are representative of the inflammatory microenvirnment representative of inflammatory lung dieseases like asthma or chronic obstructive pulmonary disease (COPD).

Exposed to the external environment the pulmonary epithelium acts as a protective barrier to the underlying tissue. However, the pulmonary epithelium of individuals suffering from diseases, such as asthma and COPD, exists in a state of persistent inflammation orchestrated and seemingly maintained by a complex network of cellular and soluble mediators, such as the pro-inflammatory cytokines tumor necrosis factor-α (TNFα) (Howarth et al. 2005) interleukin-1β (IL-1β) (Bjornsdottir and Cypcar 1999), and interferon-γ (IFNγ) (Di Stefano et al. 2004; Kumar et al. 2006). When present in this inflammatory microenvironment the pulmonary epithelium can become activated, producing secondary mediators such as oxidants, lipid mediators, and cytokines/chemokines that further perpetuate the inflammatory response (Casola et al. 2002; Martin et al. 1997; Redington 2006; Saperstein et al. 2009). Whereas most of the epidemiological evidence linking PM exposure to health effects is derived from individuals with pre-existing cardiopulmonary conditions, like asthma and COPD, the majority of experimental *in vitro* studies are conducted in normal cells.

In the present study, we sought to develop an *in vitro* epithelial cell model that mimicked the generic inflammatory microenvironment found in individuals with pre-existing inflammatory lung diseases. Accordingly, murine lung epithelial cell cultures representative of the conducting airways as well as the alveolus were treated with differing combinations of exogenous pro-inflammatory cytokines (TNFα, IL-1β, and IFNγ). The resulting epithelial activation of cytokine-treated cells was assessed by the induction of secondary inflammatory mediators, such as the release of the neutrophilic (MIP-2) and eosinophilic (RANTES) chemokines, as well as the expression of the enzyme iNOS responsible for producing the pro-inflammatory free radical nitric oxide (NO).

MATERIALS AND METHODS

Cell Culture and Cytokine Treatment

Primary mouse tracheal epithelial (MTE) cells were harvested from female BALB/c mice (Charles River Labs, Wilmington, MA), 12–16 weeks of age (19–23 grams), and grown on Transwell membranes in Ham's F12:DMEM as a 50:50 mixture containing 10% fetal bovine serum (FBS), amphotericin-B (250 ng/mL), nystatin (44 IU/mL), gentamicin (100 mg/mL), bovine pituitary extract (BPE; 104 mg/mL), insulin (5 mg/mL), transferrin (5 mg/mL), mouse epidermal growth factor (mEGF; 5 ng/mL), dexamethasone (0.1 μM), cholera toxin (20 ng/mL), and retinol (0.01 μM). The isolation, cultivation, and characterization of MTE cells using this method has been described previously (Lankford et al. 2005). Initially fed from both the apical and basal compartment, MTE cells were transferred to an air-liquid interface (ALI) once they reached 80% confluence, by removing the apical medium and fed solely basolaterally. Once confluent, approximately 3-4 days post

ALI, the MTE cells were fed basally for 1 day with FBS-free medium supplemented with bovine serum albumin (BSA; 0.5 mg/mL) prior to treatment. All protocols were approved by the Institutional Animal Care and Use Committee of the US EPA.

LA-4 cells (American Tissue Culture Collection, Manassas, VA), a murine alveolar type II-like epithelial cell line (passages 49 to 55), were grown to 95-100% confluence in Ham's F12K supplemented with 15% FBS, streptomycin (50 μg/mL), penicillin (50 U/mL), and amphotericin B (0.5 μg/mL) in 5% CO_2 at 37°C. Prior to treatment, the medium was changed to FBS-free Ham's F12K supplemented with BPE (25 μg/mL), insulin (2 μg/mL), mEGF (10 ng/mL), and BSA (0.5 mg/mL) purchased from Sigma (St. Louis, MO). Total live cell counts of LA-4 cells was determined by Trypan blue dye exclusion (0.04%) using a hemocytometer.

LA-4 and MTE cell cultures were treated basally via their respective medium with exogenous cytokines (TNFα, IL-1β, IFNγ; R&D Systems, Minneapolis, MN) at 0.2 ng/mL for LA-4 cells, and 2 ng/mL for MTE cells. These concentrations were based on preliminary dose-response studies in which cytokine treatment consistently resulted in reproducible increases (at least 5-fold) of neutrophilic MIP-2) or eosinophilic (RANTES) chemokines, without causing overt cytotoxicity. Inhibition of phosphorylated p44/42 (P-p44/42) was accomplished by pre-treating the LA-4 cells for 30 min with U0126 (10 μM; Cell Signaling Technology, Danvers, MA), a pharmacologic inhibitor of MEK1/2 the active kinases responsible for p44/42 phosphorylation.

Cell Injury

Release of lactate dehydrogenase (LDH) was used as a measure of cell injury in MTE and LA-4 cultures. LDH activity was assayed using a commercially available kit (Thermo Electron Corp., Louisville, CO), modified and adapted for use on the KONELAB 30 clinical chemistry analyzer (Thermo Clinical Labsystems, Espoo, Finland). Cell-free conditioned medium and cell lysates collected from cells treated with cold phosphate-buffered saline (PBS) containing 0.1% Triton-X 100 (Sigma, St. Louis, MO) for 30 min were used in the assay.

Chemokine Measurements

MIP-2 and RANTES content was assayed using murine-based enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) from both the conditioned basal medium of MTE and LA-4 cells, as well as the apical surface of MTE cells gently washed with 500 μL PBS.

Real-Time RT-PCR

Total RNA was isolated using RNeasy (Qiagen, Valencia, CA). cDNA synthesis and real-time PCR using gene-specific primers was then performed using SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, California). Primer/probe sets for MIP-2, RANTES, iNOS, and β-actin were purchased from Applied Biosystems (Foster City, CA). After determining that β-actin mRNA expression was not altered by our experimental treatments, the level of mRNA expression was normalized to β-actin using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Western Blot Analysis

Whole cell lysates were prepared by lysing the cells in RIPA buffer (50 mM Tris, 0.5% Deoxycholic acid, 150 mM Sodium Chloride) containing 0.1% Sodium Dodecyl Sulfate (SDS), 1% Triton X-100, and protease inhibitors (leupeptin, aprotinin and sodium orthovanadate). Protein extracts (10 μg) were separated on E-PAGE 8% gels (Invitrogen, Carlsbad, California), under reducing conditions, using an E-BASE electrophoresis device (Invitrogen, Carlsbad, California). Following electrophoresis, the proteins were electrophoretically transferred to PVDF iBlot Transfer stacks (Invitrogen, Carlsbad, California) using the iBlot dry blotting system (Invitrogen, Carlsbad, California). The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20. After blocking, the membranes were incubated overnight at 4˚C with specific antibodies to iNOS (1:500; BD Transduction Laboratories, San Jose, CA), p44/24 (1:1000; Cell Signaling Technology, Danvers, MA), Phospho-p44/42 (1:1000; Cell Signaling Technology, Danvers, MA), STAT1 (1:1000; Cell Signaling Technology, Danvers, MA), Phospho-STAT1 (1:1000; Cell Signaling Technology, Danvers, MA), and β-actin (1:5000; Sigma, St. Louis, MO). The membranes were then washed with TBS containing 0.1% Tween 20 and incubated for 1 hr at room temperature with the appropriate secondary antibody in 5% nonfat dry milk in TBS containing 0.1% Tween 20. Bands were visualized using chemiluminescence (LumiGlo; Cell Signaling Technology, Danvers, MA) and acquired using an Alpha Innotech 8900 imaging station (San Leandro, CA) and Fluorchem software (Alpha Innotech, San Leandro, CA).

Statistical Analysis

Data are expressed as means ± SEM. Data were analyzed using a one-way or two-way analysis of variance (ANOVA) and where relevant, followed by Bonferroni post-hoc tests for comparison between groups. A value $p < 0.05$ was considered significant.

RESULTS

MIP-2 and RANTES Release in Cytokine-Treated MTE Cells

First, the inflammatory microenvironment present in asthmatics and patients with COPD was modeled *in vitro* using primary MTE cells grown at ALI. These cells reflect more physiologic cell populations within the airways, in which they become polarized with discrete basal/apical surfaces, and possess the ability to form true ciliated and goblet cells. The early response cytokines TNFα and IL-1β are often found elevated together in the lungs of individuals afflicted with asthma or COPD, and result in the release of secondary inflammatory mediators. To mimic this setting, MTE cultures grown at ALI were treated basolaterally via their growth medium with non-cytotoxic concentrations of TNFα and IL-1β (data not shown), individually or in combination, and the resulting release of MIP-2 and RANTES was evaluated in the basal medium (Figure 1). Whereas under control conditions MTE cells release little MIP-2 (Figure 1a) or RANTES (Figure 1b) , exposure to IL-1β resulted in significant MIP-2 and RANTES release that was unaffected by the addition of TNFα.

Although known for its role in cell-mediated immunity, IFNγ is a potent mediator that plays an active role in inflammatory responses in a number of diseases. Increased IFNγ levels are often found in conjunction with increased TNFα and IL-1β in the bronchoalveolar

lavage fluid (BALF) of individuals with asthma and COPD (Barnes 2008). The potential contributing role of IFNγ in TNFα + IL-1β induced MIP-2 and RANTES release to the baslolateral compartment was then evaluated. Interestingly, while the addition of IFNγ to TNFα + IL-1β had no effect on MIP-2 secretion (Figure 2a), MTE co-exposure of TNFα + IL-1B with IFNγ potentiated and prolonged the release of RANTES to 48 hr even after the removal of the cytokine stimulus (Figure 2b).

Vectoral Secretion of MIP-2 and RANTES in Cytokine-Treated MTE Cells

When maintained at ALI and cultured in the appropriate conditions, MTE cell cultures can reflect physiologic cell populations of airway epithelial cells with distinct basal and apical (i.e. luminal) surfaces. Taking advantage that MTE cells grown in this cell culture setting become polarized, the ability of MTE to vectorally secrete MIP-2 and RANTES in response to the cytokine-treatment (cytomix: TNFα + IL-1β + IFNγ) was evaluated. MTE cells were exposed basally to cytomix for 24 hr and the resulting conditioned basal supernatant and apical washes were evaluated for MIP-2 and RANTES release. Interestingly, as shown in Figure 3 cytokine-treated MTE cells resulted in the vectoral secretion of both RANTES and MIP-2 to the basolateral compartment.

Optimization of LA-4 Cell Culture Medium

To further extend our *in vitro* model of pulmonary inflammation to represent alveolar epithelial cells, which make up the majority of deep lung epithelial cells, the model was next adapted to the murine LA-4 cell line. Whereas primary MTE cells grown in ALI is a powerful tool for studying airway biology, offering cell populations that more appropriately reflect the airway, their usefulness is limited by the increased time and expense required to

cultivate them as well as the inherent variability form one cell harvest to the next. Unlike MTE cells which are cultivated in the absence of FBS, LA-4 cells are grown in the presence of FBS (15%) which can ultimately interfere with our endpoints. To closely mimic the serum-free conditions in which MTE cells were grown, we first examined the necessity of serum on LA-4 growth and homeostasis. The resulting cell injury, as determined by LDH leakage, was evaluated in LA-4 cells grown to near confluence and fed daily with either normal medium containing 15% FBS, or 0% FBS, or 0% FBS but supplemented with BPE, insulin, EGF, and BSA (Figure 4). When deprived of FBS, LA-4 cells are unable to maintain cellular homeostasis and incur significant cell injury. This effect was significantly reduced by supplementing the FBS-free medium with critical growth factors normally present in FBS. Furthermore, LA-4 cells can be cultivated for several days in this supplemented FBS-free medium without any significant change in cell injury (Figure 4) or cell number (Figure 5).

Release of Inflammatory Mediators Cytokine-Treated LA-4 Cells

To further examine the induction of secondary inflammatory mediators of cytokine-treated LA-4 cells, the mRNA expression and protein levels of MIP-2, RANTES, and iNOS, the inducible NO synthase enzyme responsible for producing nitric oxide (NO) a important signaling molecule in asthma and COPD, was assessed (Redington 2006). LA-4 cells grown in supplemented serum free medium were treated with TNFα, IL-1β, and IFNγ either individual or in combination, and the resulting time dependent mRNA changes in MIP-2, RANTES, and iNOS revealed all to be significantly increased at 6 hr (Figure 6). Cytokine treatment of LA-4 cells resulted in the rapid, and transient, MIP-2 mRNA expression at 6 hr that was nearly undetectable at 24 hr (Figure 6a). Whereas IL-1β alone induced a mild but

significant induction of MIP-2 mRNA, its combined exposure with TNFα resulted in a synergistic increase that was not further enhanced by the addition of IFNγ (Figure 6a). Likewise, RANTES mRNA expression was rapidly, but transiently, increased by cytokine treatment (Figure 6b). Treatment with TNFα and IL-1β alone induced significant RANTES expression, which was further increased by their combined treatment. Furthermore, combined treatment of LA-4 cells with TNFα + IL-1β + IFNγ (cytomix) resulted in even further RANTES expression that remained significantly elevated at 24 hr when compared to control cells. Similarly, expression of iNOS mRNA was rapidly increased as early as 6 hr, and significantly maintained to 24 hr, by IL-1β as compared to untreated control cells (Figure 6c). As well combined treatment of TNFα and IL-1β resulted in significantly greater iNOS expression at both points, which was further increased by the addition of IFNγ.

Under control conditions LA-4 cells constitutively release minute amounts of MIP-2 and RANTES, that was significantly increased by cytokine treatment (Figure 7). Although individual exposure to these specific cytokines did not result in significant increase in MIP-2 release, the combined treatment of LA-4 cells with TNFα + IL-1β resulted in a synergistic increase in MIP-2 release at 6 hr, that persisted for 24 hr, and returned to near below detection at 48 hr (Figure 7a). MIP-2 release was unaffected by further co-incubation with IFNγ (Figure 7a). Under analogous conditions, RANTES release appeared as early as 6 hr, persisting for up to 48 hr (Figure 7b). In contrast to the effects observed on MIP-2 release, TNFα and IL-1β alone induced significant RANTES release at 24 hr that resulted in additive effects when combined (Figure 7b). As observed in MTE cells, when LA-4 cells were

treated with IFNγ in combination of TNFα + IL-1β, we observed synergistic increases in RANTES release (Figure 7c).

Protein expression of iNOS followed the same trend witnessed in its mRNA expression, with IL-1β increasing iNOS protein at 6 hrs, which was further elevated by TNFα, and IFNγ (Figure 8a). Interestingly, the protein expression of iNOS remained elevated in cytomix-treated LA-4 cells at 24 hr (Figure 8b). In addition, using the NO-sensitive fluorescent probe DAF-FM diacetate, the production of NO was shown to be significantly increased in cultured LA-4 cells treated with cytomix for 24 hr, which could be effectively inhibited by pre-treatment with 1400W, a iNOS-specific inhibitor (Figure 8c).

Signaling Pathways in Cytokine-Treated LA-4 cells

Exposure of LA-4 cells to the pro-inflammatory cytokines TNFα, IL-1β, IFNγ activates numerous signaling mechanisms that result in the increased expression and production of the secondary inflammatory mediators like iNOS, MIP-2, and RANTES. The mitogen-activated protein kinases (MAPK) family consists of several signaling molecules (p44/42, JNKs, p38) involved in cell proliferation/differentiation and stress responses. Whereas p44/42 is activated by phosphorylation in response to extracellular signals like TNFα and IL-1β, IFNγ is known to activate STAT1 signaling, a member of the signal transducers and activators of transcription (STAT) family of transcription factors. To determine the extent of MAPK and STAT1 activation, protein extracts from LA-4 cells treated for 6 hr with TNFα, IL-1β, IFNγ, individually and in combination, where evaluated (Figure 9). Interestingly, while expression of JNK and p38 were unaffected by cytokine treatment (data not shown), IL-1β treatment resulted in the activation of the p44/42 signaling

pathway via phosphorylation, which was unaffected by the combined treatment with TNFα, IL-1β or IFNγ (Figure 9a). As well, IFNγ treatment resulted the activation of the STAT1 signaling pathway, via its phosphorylation, that was not affected by the addition of TNFα or IL-1β (Figure 9b).

Effects of MAPK Inhibition on Chemokine Release in Cytokine-Treated LA-4 Cells

The signaling pathways involved MIP-2 and RANTES expression and secretion are largely depended on that of MAPK signaling. Pre-treatment of cytokine-treated LA-4 cells with U0126, a specific p44/42 inhibitor, the role of MAPK signaling was evaluated in cytomix-stimulated MIP-2 and RANTES release (Figure 10). Interestingly, whereas MIP-2 was completely blocked by U0126, RANTES release was only partially inhibited, by approximately 50%.

DISCUSSION

In the US nearly 35 million individuals are afflicted with asthma or COPD collectively (Mannino, Homa, Akinbami, Ford et al. 2002; Mannino, Homa, Akinbami, Moorman et al. 2002). Furthermore, these individuals are at increased risk of the adverse health effects from exposure to air pollutants like PM (Kim et al. 2009; Pope and Dockery 2006; Sint, Donohue, and Ghio 2008). While considerable advances in our understanding of these diseases, their mechanisms, and responses to PM have been accomplished through whole animal studies, comparable *in vitro* models are still lacking. With the understanding that certain cytokines play a key role in orchestrating the chronic inflammation of asthma and COPD, the present study set out to create an *in vitro* system that models the inflammatory microenvironment of generic inflammatory lung disease. Using both pulmonary epithelial

cells representative of conducting (i.e. airways) and deep lung (alveolus), we show that treatment with key cytokines (cytomix: TNFα + IL-1β + IFNγ) resulted in significant release of secondary inflammatory chemokines (MIP-2 and RANTES) and induction of enzymes (iNOS) responsible for producing inflammatory free radicals (NO).

Cytokines play a key role in orchestrating the chronic inflammation associated with asthma and COPD by recruiting, activating, and promoting the survival of multiple inflammatory cells in the lung. The pro-inflammatory cytokines TNFα and IL-1β, which have been shown to be increased in the sputum and BALF of individuals with asthma and COPD, are produced by a number of cells and play an important role in orchestrating and maintaining pulmonary inflammation (Barnes 2008; Bjornsdottir and Cypcar 1999; Kips and Pauwels 1996). Furthermore, inhibition studies of TNFα and IL-1β, through the administration of specific binding proteins or receptor agonists have supported the importance of these cytokines in humans patients and mouse models of asthma and COPD (Berry et al. 2006; Rosenwasser 1998). In addition to TNFα and IL-1β, the T_H1 cytokine IFNγ also plays an important role in pulmonary inflammation, and is increased in the serum and BALF of asthmatics and patients with COPD (Barnes 2008). Together these cytokines exert their effects on resident phagocytic cells, recruited white blood cells, and pulmonary epithelial cells to further amplify inflammation through the activation of numerous signaling pathways, gene activation, and secondary mediator release.

The pulmonary epithelium forms a physical barrier against deleterious airborne substance such as allergens, particles, pollutants, and gasses. In addition, the pulmonary epithelium is an active participant in both generating and perpetuating pulmonary

inflammation. However, disruption of the normal functioning epithelium, as witnessed in inflammatory lung diseases like asthma or COPD, could result in greater adverse effects when exposed to deleterious airborne substances like PM. Thus, since the pulmonary epithelium serves as a target of local inflammatory cytokines and inhaled PM, and can actively participating in the inflammation response, it was chosen to create an *in vitro* system that models the inflammatory microenvironment. In the present study, we have developed *in vitro* cell culture systems that models, in part, the acellular inflammatory microenvironment in which epithelial cells are continually exposed to in such inflammatory lung diseases like asthma and COPD. Treatment of both airway (MTE) and alveolar (LA-4) lung epithelial cells with a pro-inflammatory cytokines (cytomix: TNFα + IL-1β + IFNγ) resulted in increased production of the neutrophilic (MIP-2) and eosinophilic (RANTES) chemoattractants as well as iNOS, the enzyme responsible for producing large quantities of nitric oxide (NO). Chemokines play an important part in the recruitment of inflammatory cells from the circulation to the lungs in both asthma and COPD. In patients with asthma and COPD, the expression and secretion of RANTES is highly increased in the airway epithelium, smooth muscle, BALF, and sputum (Barnes 2008). In addition to serving as a mediator to attract and activate eosinophils at the site of inflammation, RANTES can also serve as a chemokine for several other cells types that possess its binding receptor, CCR3, such as basophils, mast cells, and T cells (Appay and Rowland-Jones 2001). Likewise, MIP-2 (the mouse orthologue to the human chemokine IL-8), a strong chemoattractant primarily for neutrophils, is also increased in the BALF and sputum of COPD patients as well as individuals with severe asthma (Barnes 2008). Furthermore, *in vitro* studies on lung

epithelial cells derived from healthy and diseased donors have provided further evidence that the lung epithelium can actively secrete chemokines like MIP-2 and RANTES. First, normal human bronchial epithelial cells treated with cytokines (including TNFα and IL-1β) have been reported to induce the expression of numerous chemokines involved in inflammation, including RANTES and IL-8 (Takizawa 2005). Alternatively, bronchial epithelial cells from patients with chronic inflammation, such as asthma, have been shown to produce greater amounts of MIP-2 and RANTES under basal (unstimulated) conditions (Takizawa et al. 2000).

In the present study we show that RANTES mRNA expression and protein release was significantly and rapidly increased both by TNFα and IL-1β treatment, alone and in combination. Interestingly, whereas IFNγ treatment alone was without effect on RANTES mRNA expression and release, when in the presence of TNFα and IL-1β, RANTES expression and release was significantly increased. The synergistic role of IFNγ on RANTES production when present with TNFα and IL-1β has been documented in several different cell types, including renal epithelial cells, endothelial cells, conjunctival epithelial cells, as well as alveolar epithelial cells (Casola et al. 2002; Deckers et al. 1998; Marfaing-Koka et al. 1995; Stahl et al. 2003). The mechanisms of this effect have in part been elucidated and are largely attributed to activation of NFκB, via TNFα and IL-1β stimulation, as well as RANTES mRNA stabilization attributed to IFNγ (Casola et al. 2002). The potentiation of inflammation by IFNγ when in the presence of TNFα and IL-1β may explain why asthmatic are particularly susceptible to respiratory viral infections.

To further define the signaling mechanisms activated in LA-4 cells treated with cytokines, we examined MAPK and STAT signaling and showed that while p44/42 was primarily activated by IL-1β treatment, STAT1 was activated only by IFNγ. Since the signaling pathways involved in MIP-2 and RANTES expression and secretion are largely depended on that of p44/42 MAPK signaling (Henriquet et al. 2007; Krunkosky and Jarrett 2006; Schuh and Pahl 2009), we further examined its role in the release of MIP-2 and RANTES, using a specific p44/42 inhibitor. Interesting, whereas MIP-2 release was completely abolished by p44/42 inhibition, the present data show that RANTES release was only partially inhibited further suggesting multiple signaling pathways, such as IFNγ, in RANTES activation and release.

Since MTE cells grown at ALI form a tightly polarized barrier, we studied the potential of MTE cells to secrete MIP-2 and RANTES in a polarized fashion. The results presented here show that MTE cells treated with cytomix resulted in the vectoral secretion of both MIP-2 and RANTES predominately to the basolateral surface. These findings are similar to other investigations of the airway epithelial cells grown at ALI (Auger et al. 2006; Mellow et al. 2004). The proportionately greater basal chemokine secretion could allow sequestering of systemic inflammatory cells from the interstitium to the airways, while the additional, albeit smaller, apical secretion helps to "home" those inflammatory cells to the lumen of the airway itself. In addition, with their extremely high surface area, PM is capable of differentially binding soluble mediators, such as MIP-2 and RANTES, thus confounding their analysis (Seagrave et al. 2004). Taking advantage of the transwell cell culture system and the ability of MTE cells to differentially secrete chemokines to the basolateral surface,

future experiments involving PM will be able to reduce the risk of PM interferences by allowing basally sampled medium to be physically separated by the semi-permeable membrane on top of which the MTE cells will be grown and exposed to PM.

In addition to chemokines, in the present studies, cytokine-treated LA-4 epithelial cells increased both iNOS mRNA and protein production as early as 6 hr after treatment. Nitric oxide (NO) is produced from the oxidation of L-arginine to L-citrulline by nitric oxide synthases (NOS) (Jiang et al. 2009; Redington 2006). Whereas low concentrations of NO are produced by constitutively active NOS isoforms (eNOS, nNOS) and may possess bronchoprotective properties, induction of the inducible NOS isoform (iNOS) produces high concentrations of NO that can react with other radicals (i.e. superoxide) to cause further inflammation, vascular permeability, and cytotoxicity (Han et al. 2004; Heiss et al. 1994; Redington 2006). Furthermore, increased amounts of iNOS have been reported in asthmatics as well as patients with COPD and thus play a contributing role to pulmonary inflammation (Brindicci et al. 2009; Ichinose et al. 2000).

In summary, the present study demonstrates that cytokine-treatment (cytomix: TNFα + IL-1β + IFNγ) of airway and alveolar epithelial cells results in the activation of a inflammatory enzyme as well as the release of secondary inflammatory chemokines. This *in vitro* model of the pulmonary inflammatory microenvironment mimics the generic exposure that lung epithelial cells experience is chronic pulmonary inflammatory diseases, such as in asthma and COPD, and thus provides a means to further investigate differential effects of pulmonary toxicant exposure (i.e. PM) in the setting of health or disease. Such *in vitro*

models could be incorporated into high-throughput approaches enabling the effects of many environmentally relevant agents, in health and diseased settings, to be evaluated.

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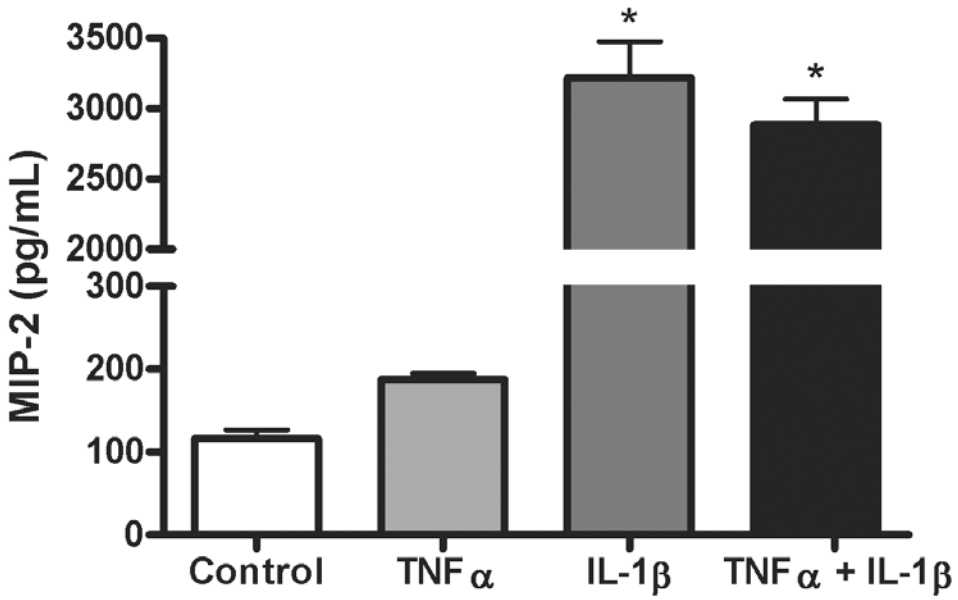
A)



B)

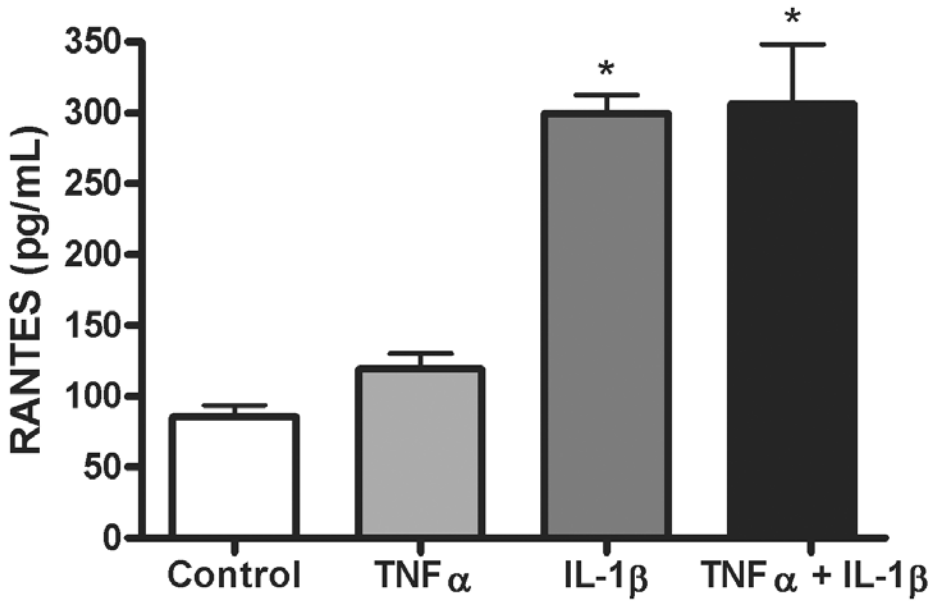


Figure 1. Release of MIP-2 and RANTES in TNFα and IL-1β treated MTE cells. MTE cells were treated with saline (control), TNFα, IL-1β, or TNFα + IL-1β at 2 ng/mL in the basal medium for 24 hr. Conditioned basal medium was analyzed for MIP-2 and RANTES concentrations by ELISA. Data are expressed as the concentration (pg/mL ± SEM) of either MIP-2 (A) or RANTES (B). Significance ($p < 0.05$) is indicated by: * vs. control, and TNFα.

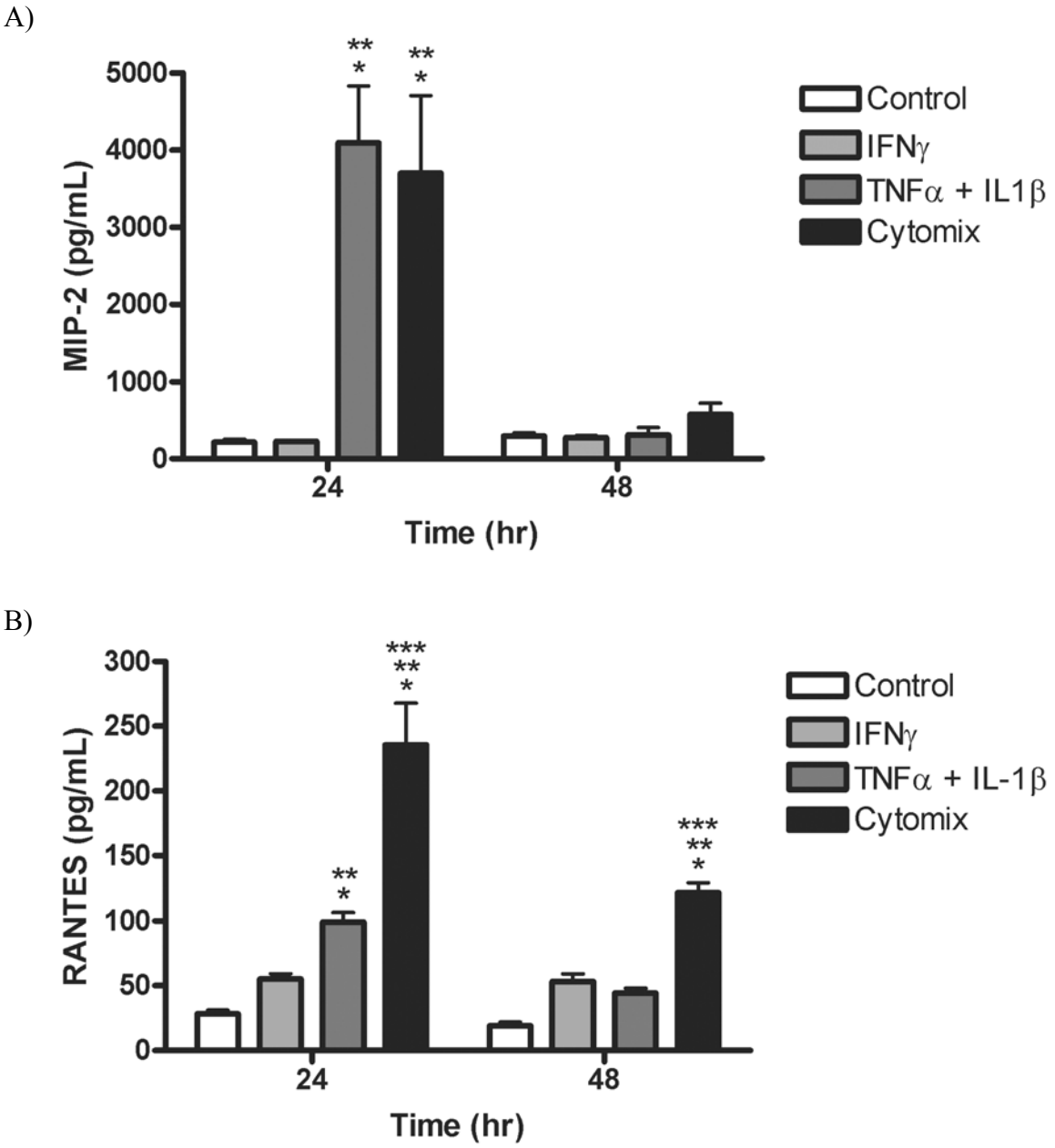


Figure 2. MIP-2 and RANTES release in cytomix-treated MTE cells. MTE cells were treated with saline (control), IFNγ, TNFα + IL-1β, or cytomix (TNFα + IL-1β + IFNγ) at 2 ng/mL in the basal medium for 24 hr. Conditioned basal medium was analyzed for MIP-2 and RANTES concentrations by ELISA at 24 and 48 hr after initial cytokine treatments. Data are expressed as the concentration (pg/mL ± SEM) of either MIP-2 (A) or RANTES (B). Significance ($p < 0.05$) is indicated by: * vs. control, ** vs. IFNγ, *** vs. TNFα + IL-1β.

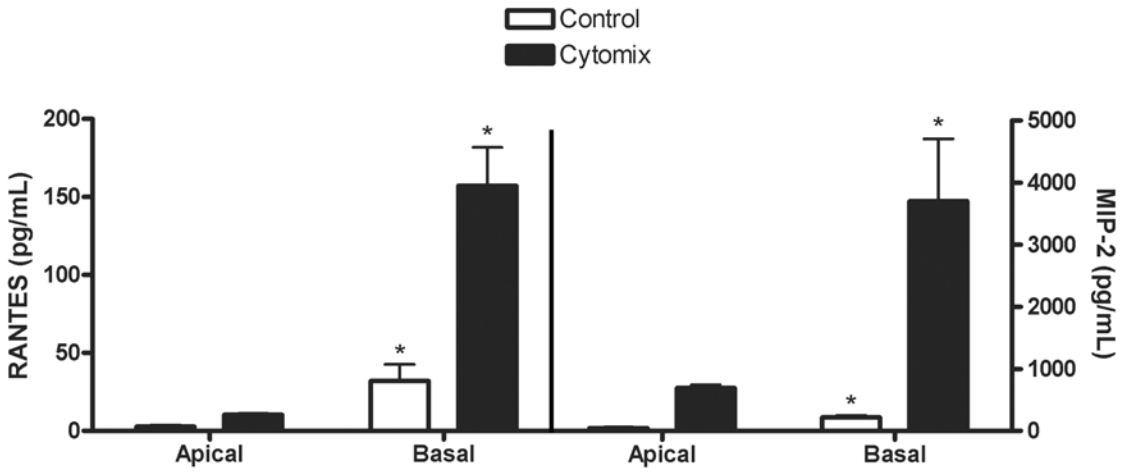


Figure 3. Vectoral secretion of MIP-2 and RANTES in cytokine-treated MTE cells. MTE cells were treated basolaterally to saline (control) or cytomix (TNFα + IL-1β + IFNγ) at 2 ng/mL for 24 hr. Conditioned basal medium and apical washes were analyzed for MIP-2 and RANTES release by ELISA at 24 hr after cytomix treatment. Data are expressed as the concentration (pg/mL ± SEM) of either MIP-2 or RANTES. Significance ($p < 0.05$) is indicated by: * vs. corresponding supernatant.

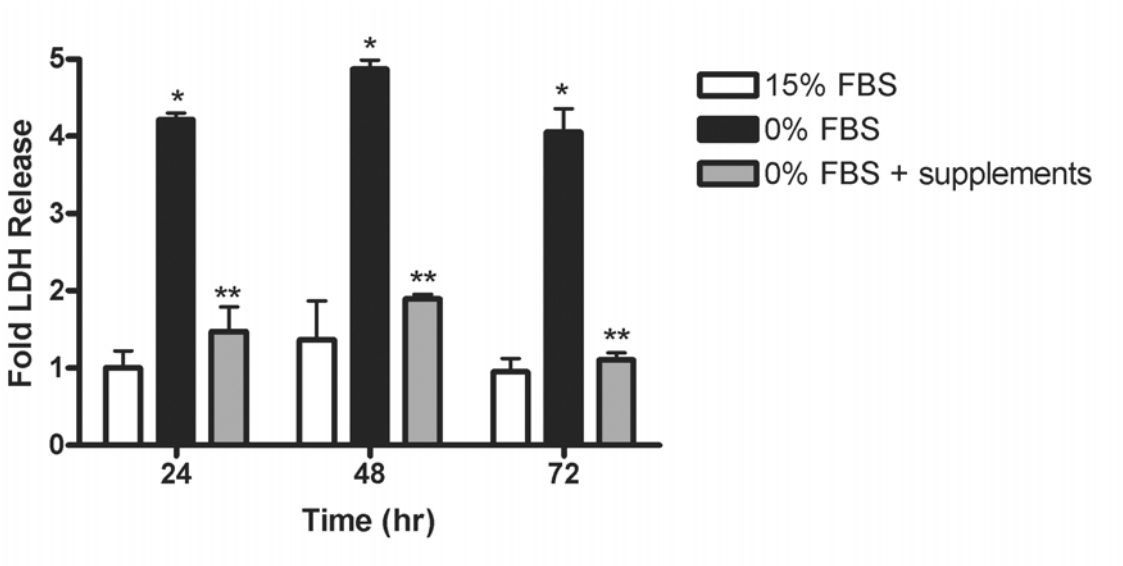


Figure 4. Cell injury of LA-4 cells grown in optimized serum-free medium. LA-4 cells were grown either in normal medium (Ham's F12k) containing 15% FBS, medium containing 0% FBS, or medium with 0% FBS supplemented with BSA + insulin + mEGF (0% FBS + supplements) for 24 hr. Conditioned medium and cell lysates were analyzed for LDH released. Data are expressed as mean fold increase over control (± SEM) of LDH release (normalized to total LDH). Significance ($p < 0.05$) indicated by: * vs. control; ** vs. 0% FBS, at the corresponding time point.

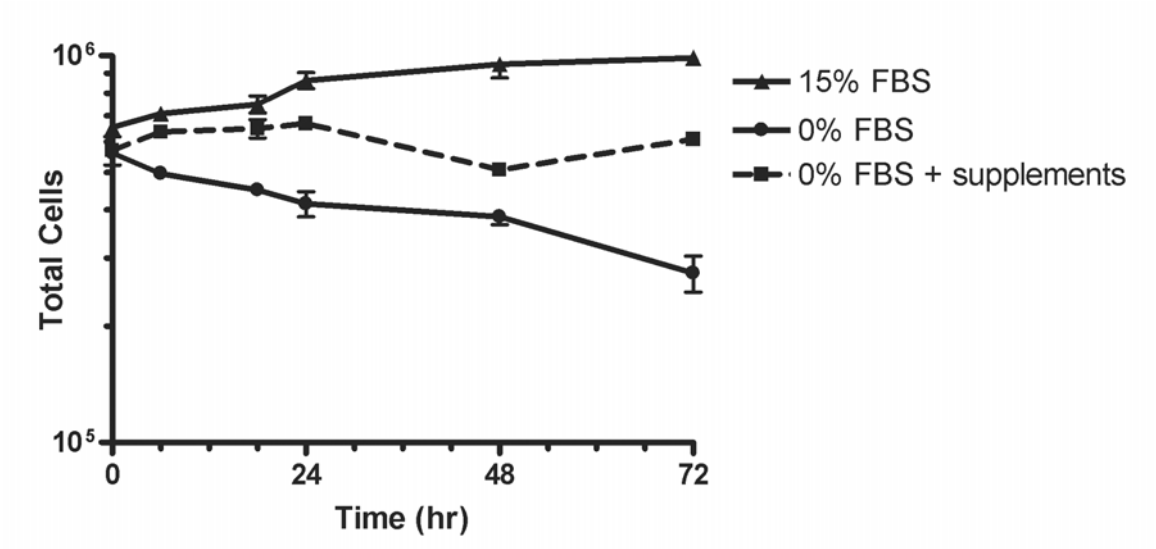
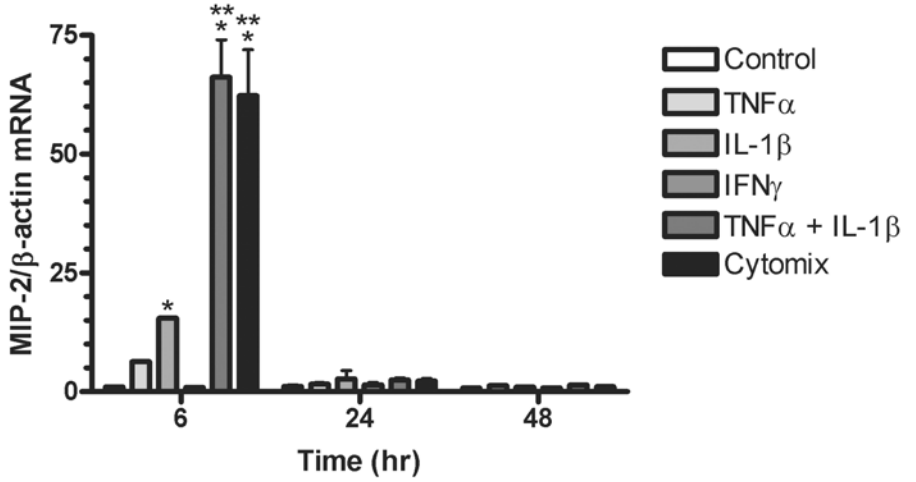


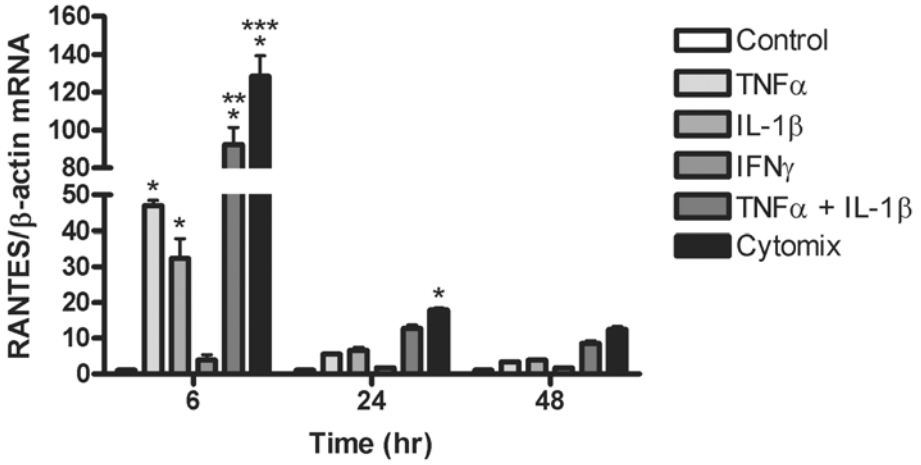
Figure 5. Growth of LA-4 cells in optimized serum-free medium. LA-4 cells were grown to confluence (9-10 days in culture) in normal medium (Ham's F12k) containing 15% FBS. The cells were cultivated for an additional 72 hr either in normal medium (Ham's F12k) containing 15% FBS, medium containing 0% FBS, or medium with 0% FBS supplemented with BSA + insulin + mEGF (0% FBS + supplements). Total live cell counts were analyzed by trypan blue exclusion using a hemocytometer. Data are expressed as mean total number of calculated cells per dish (± SEM).

Figure 6. MIP-2, RANTES, and iNOS mRNA expression in cytokine-treated LA-4 cells. LA-4 cells treated were treated with saline (control), TNFα, IL-1β, IFNγ, TNFα + IL-1β, or cytomix (TNFα + IL-1β + IFNγ) at 0.2 ng/mL for 24 hr. Cells were harvested for mRNA at 6, 24, and 48 hr after the initial cytokine treatments, and the expression of MIP-2 (A), RANTES (B), and iNOS (C) was quantified by real-time RT-PCR, with normalization to β-actin expression. Data are expressed as the mean (± SEM) fold increase over saline-exposed cells. Significance ($p < 0.05$) is indicated by: * vs. control, ** vs. individual cytokine treatments, *** vs. all other treatments, at the corresponding time point.

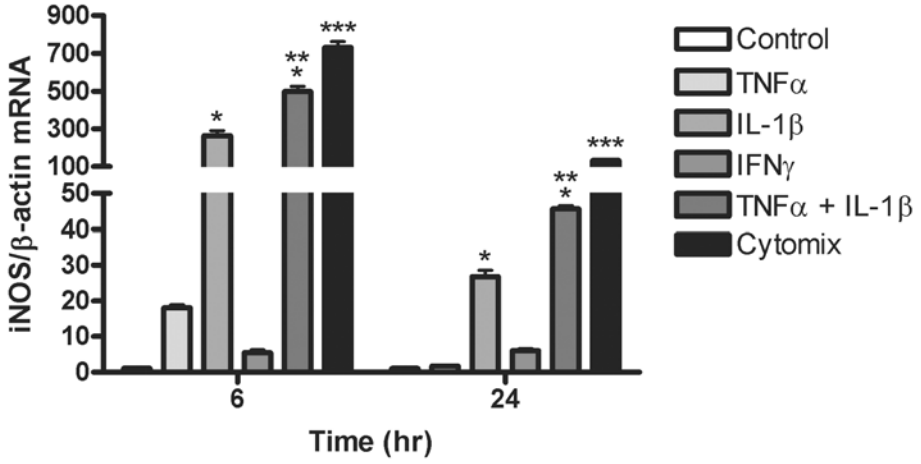
A)


MIP-2/β-actin mRNA
75
50
25
0
6
24
48
Time (hr)
Control
TNFα
IL-1β
IFNγ
TNFα + IL-1β
Cytomix

B)


RANTES/β-actin mRNA
160
140
120
100
80
50
40
30
20
10
0
6
24
48
Time (hr)
Control
TNFα
IL-1β
IFNγ
TNFα + IL-1β
Cytomix

C)


iNOS/β-actin mRNA
900
700
500
300
100
50
40
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0
6
24
Time (hr)
Control
TNFα
IL-1β
IFNγ
TNFα + IL-1β
Cytomix

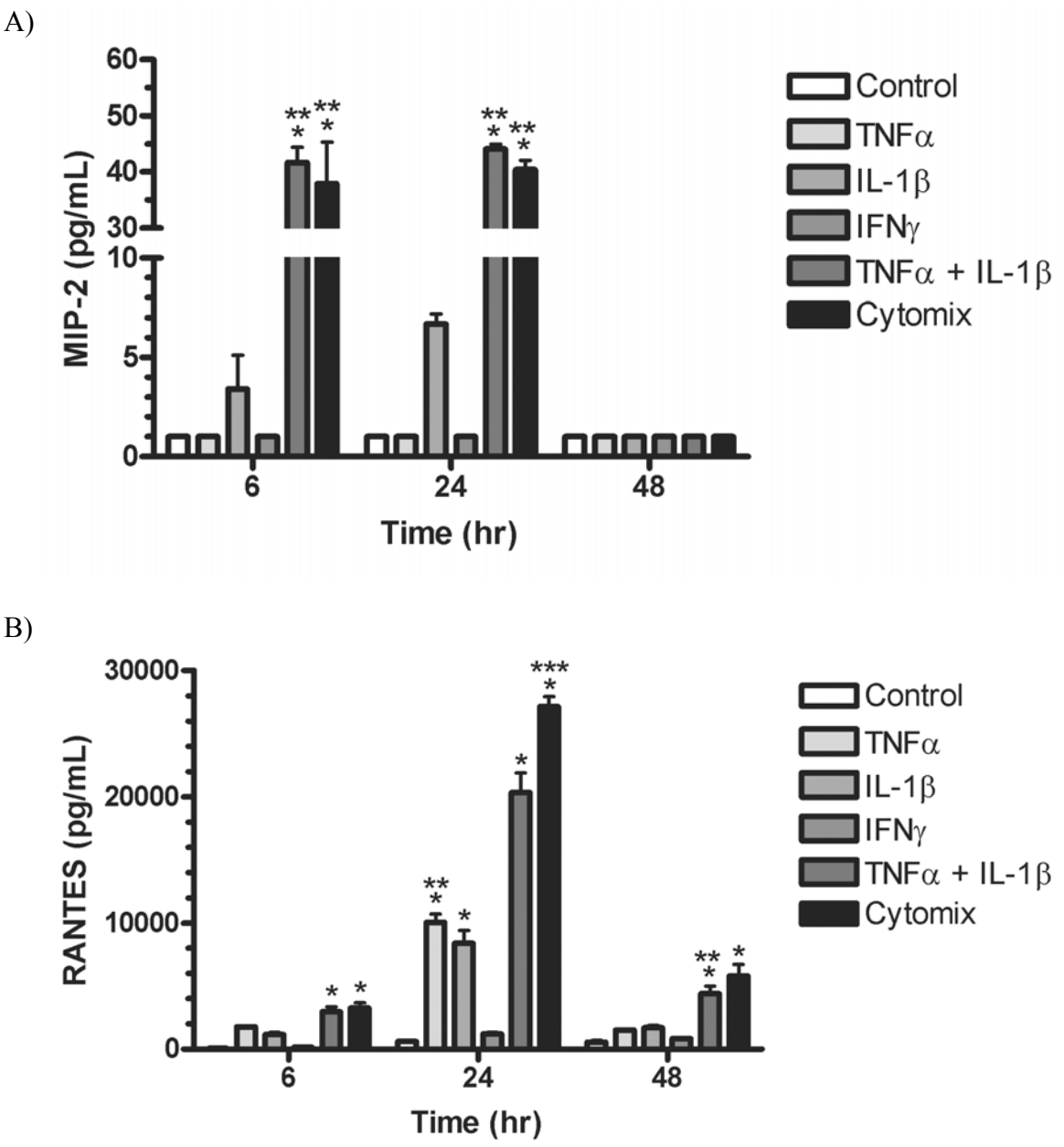
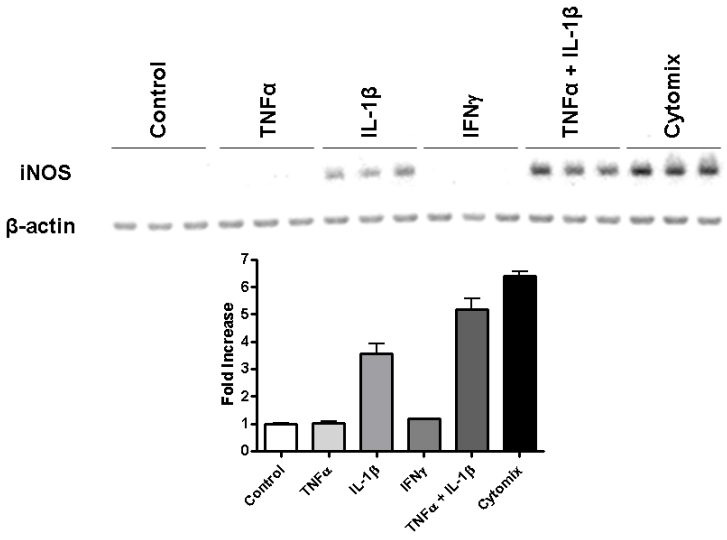


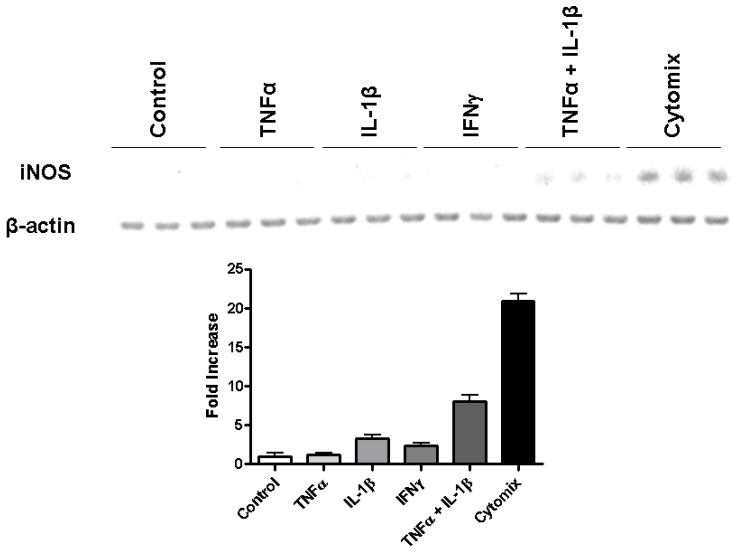
Figure 7. Release of MIP-2 and RANTES in cytokine-treated LA-4 cells. LA-4 cell were treated with (control), TNFα, IL-1β, IFNγ, TNFα + IL-1β, or cytomix (TNFα + IL-1β + IFNγ) at 0.2 ng/mL for 24 hr. Conditioned medium was analyzed for MIP-2 (A) and RANTES (B) concentrations (pg/mL ± SEM) by ELISA at 6, 24, and 48 hr after initial cytokine treatment. Significance ($p < 0.05$) is indicated by: * vs. control, ** vs. individual cytokine treatments, *** vs. all other treatments, at the corresponding time point.

Figure 8. iNOS protein levels and NO production in cytokine-treated LA-4 cells. Cultured LA-4 cells were treated with either saline (control), TNFα, IL-1β, IFNγ, TNFα + IL-1β, or cytomix (TNFα + IL-1β + IFNγ) at 0.2 ng/mL. Protein expression of iNOS at 6 (A) and 24 hr (B) after cytokine treatments were evaluated by western blot. 10 µg of protein extracts were separated on 8% E-PAGE gels, electrophorectially transferred to PVDF and blotted for iNOS and β-actin. The graph below indicates the fold change in densitometry values over control of iNOS protein (normalized to β-actin) in cytokine-treated LA-4 cells. Detection of intracellular NO was accomplished using DAF-FM diacetate, which fluoresces upon reaction with NO (C). LA-4 cells cultured in chamber slides were treated with either saline (control), cytomix (TNFα + IL-1β + IFNγ) 0.2 ng/mL, or cytomix + 1400W (100 µM; Sigma, St. Louis, MO) for 24 hr, after which the cells were washed with HBSS and incubated for 30 min with DAF-FM (10 µM). After a 2 hr exposure, the cells were counterstained with DAPI and imaged using a Nikon Eclipse Ti fluorescent microscope and Nikon Elements Software.

A)


Control
TNFα
IL-1β
IFNγ
TNFα + IL-1β
Cytomix
iNOS
β-actin
Fold Increase
0
1
2
3
4
5
6
7
Control
TNFα
IL-1β
IFNγ
TNFα + IL-1β
Cytomix

B)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TNFα
IL-1β
IFNγ
TNFα + IL-1β
Cytomix
iNOS
β-actin
Fold Increase
0
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10
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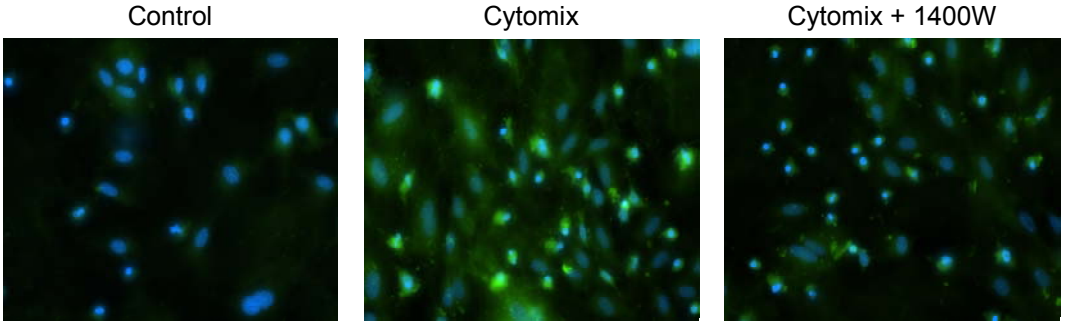
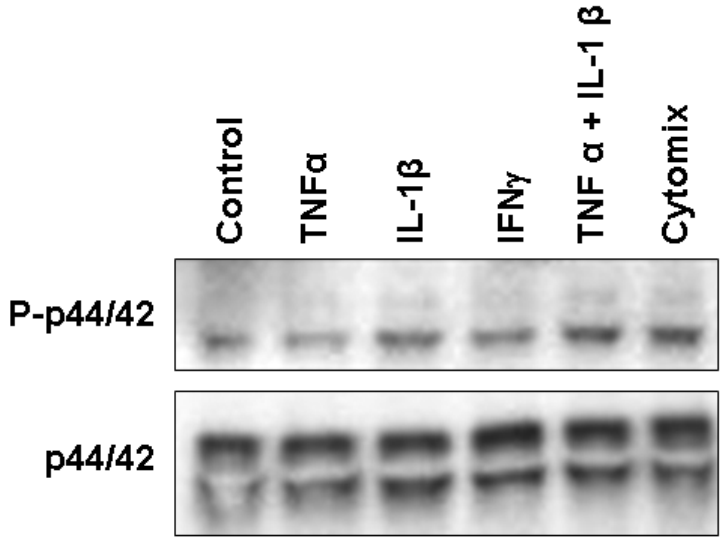
20
25
Control
TNFα
IL-1β
IFNγ
TNFα + IL-1β
Cytomix

C)


Control
Cytomix
Cytomix + 1400W

A)



B)

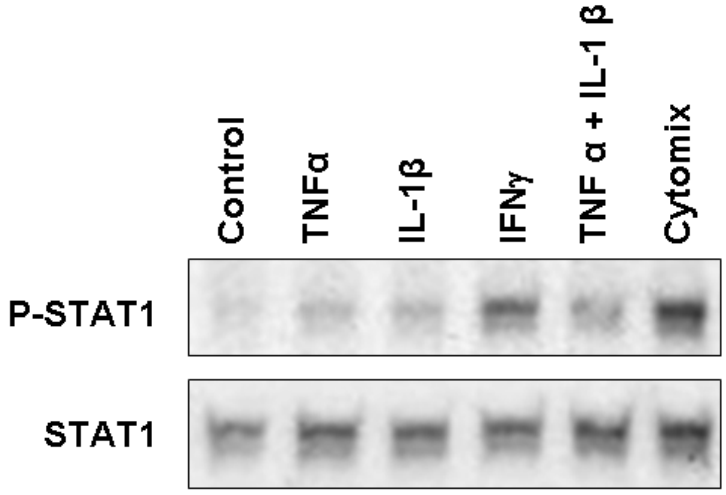


Figure 9. MAPK and STAT protein levels in cytokine-treated LA-4 cells. LA-4 cells were treated with saline (control), TNFα, IL-1β, IFNγ, TNFα + IL-1β, or cytomix (TNFα + IL-1β + IFNγ) at 0.2 ng/mL for 24 hr. Protein expression of phospho-p44/42 (P-p44/42) (A) and phospho-STAT1 (P-SAT1) (B) were evaluated by western blot. 10 μg of protein extracts were separated on 8% E-PAGE gels, electrophorectially transferred to PVDF and blotted for P-p44/42, p44/42, P-STAT1, and STAT1.

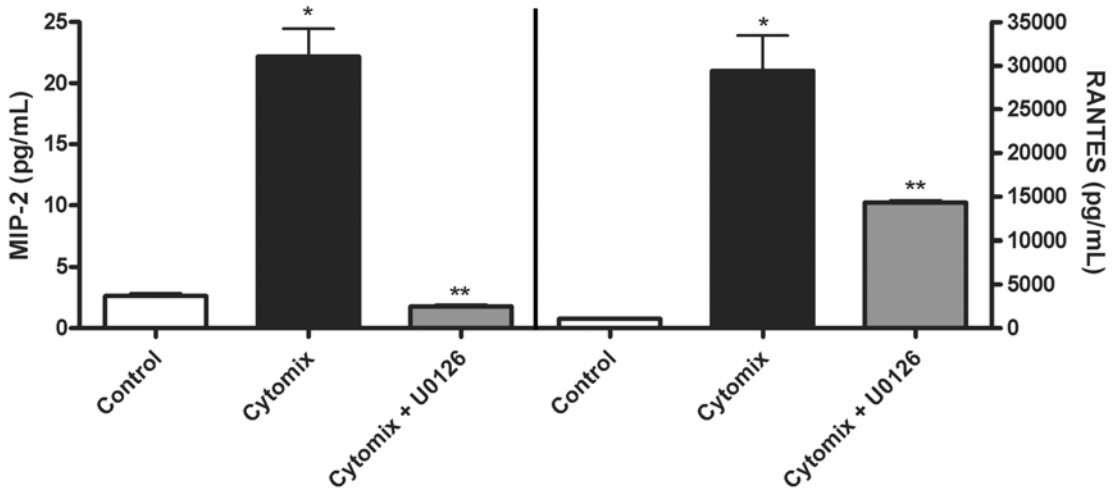


Figure 10. Effects of MAPK inhibition on chemokine release in cytokine-treated LA-4 cells. LA-4 cells were treated with saline (control) or cytomix (TNFα + IL-1β + IFNγ) at 0.2 ng/mL for 24 hr in the presence of U0126 (10 μM). Conditioned medium was analyzed for MIP-2 and RANTES release by ELISA 24 hr after initial cytokine treatment. Data are expressed as the concentration (pg/mL ± SEM) of either MIP-2 or RANTES. Significance ($p < 0.05$) is indicated by: * vs. control, ** vs. cytomix.

CHAPTER 3:

Susceptibility of Cytokine-Treated Alveolar and Airway Epithelial Cells to Injury Induced by Diesel Exhaust Particles of Varying Organic Carbon Content

ABSTRACT

Exposure to traffic-related ambient air pollution, such as diesel exhaust particles (DEP), is associated with adverse health outcomes, especially in individuals with pre-existing inflammatory lung diseases. Using an *in vitro* system to model a pre-existing inflammatory state, the susceptibility of epithelial cells exposed to DEP of varying organic carbon content was studied. Murine LA-4 alveolar type II-like epithelial cells, as well as primary murine tracheal epithelial cells (MTE), were treated with a mixture of cytokines (TNFα + IL-1β + IFNγ) to model a mild inflammatory microenvironment. Epithelial cells were then exposed to DEP of varying organic carbon content, and the resultant cytotoxic, cytoprotective, and antioxidant responses were measured by changes in lactate dehydrogenase (LDH) release, heme oxygenase-1 (HO-1) expression, or glutathione levels, respectively. Data showed that control LA-4 cells exposed to organic carbon-rich DEP (25 μg/cm^2; 24 hr) induced adaptive cytoprotective and antioxidant responses with no apparent cell injury. In contrast, DEP exposure of cytokine-treated LA-4 cells resulted in oxidative stress and cytotoxicity. Likewise, MTE cells exposed to organic carbon-rich DEP (20 μg/cm^2; 24 hr) was seemingly without effect; DEP exposure of cytokine-treated MTE cells resulted in increased epithelial solute permeability. These results suggest that lung epithelial cells stressed by inflammation and then exposed to organic carbon-rich DEP appear unable to respond to the additional oxidative stress, resulting in epithelial barrier dysfunction and cell injury. Adverse health outcomes associated with exposure to traffic-related air pollutants, like DEP, in patients with pre-existing inflammatory respiratory diseases may be due, in part, to similar mechanisms.

KEY WORDS: DEP, *in vitro*, organic carbon, cell injury, HO-1, antioxidants, glutathione

INTRODUCTION

Emissions of traffic-related air pollutants impact air quality for individuals who reside or spend considerable amounts of time near major roadways. Epidemiological data suggests that within these urban populations, individuals with pre-existing lung diseases are at increased risk for experiencing adverse heath effects, including increased hospitalizations and mortality (Halonen et al. 2009; Holguin 2008). In particular, individuals with chronic inflammatory lung diseases, such as asthma (Kim et al. 2008) and chronic obstructive pulmonary disease (COPD) (Sint, Donohue, and Ghio 2008), are uniquely susceptible to the adverse effects of traffic-related particulate matter (PM). Therefore, better characterization of the causative component(s) of traffic-related emissions that render these individuals at greater health risk is needed; as is improved understanding of the cellular and pathophysiologic processes impacted during roadway emission exposure in these susceptible populations.

The lungs of individuals afflicted with asthma or COPD exist in a state of persistent, low-level inflammation (Bousquet et al. 2000; Krewski and Rainham 2007; Elias 2004). This inflammatory state is orchestrated and sustained by a complex network of cells — including resident mononuclear phagocytic cells, various immune surveillance cells, and the airway or alveolar epithelial surface cells (Takizawa 2005). These cells communicate with each other, and in turn, perpetuate the inflammatory processes by releasing mediators and cytokines involved in the recruitment and activation of inflammatory cells. For example, expression and secretion of the pro-inflammatory cytokines tumor necrosis factor-α (TNFα) (Howarth et al. 2005) and interleukin-1β (IL-1β) (Bjornsdottir and Cypcar 1999) are

commonly increased in asthma and COPD. As well, interferon-γ (IFNγ) is frequently increased in bronchoalveolar lavage fluid (BALF) of asthmatics and patients with COPD (Boniface et al. 2003; Elias 2004). Data exist to suggest that IFNγ alleviates (Yoshida et al. 2002) or alternatively, contributes to the disease pathology (Koch et al. 2006). Hence, IFNγ appears to be a key mediator in modulating immune responses amongst these diseases.

In health, the lung epithelial layer acts as a protective barrier, separating the external environment, including traffic-related PM, from penetrating the underlying lung tissue. Exposure to irritants contained in roadway PM may damage this epithelial layer resulting in plasma leakage into the airway lumen or alveolar space (Fernvik et al. 2002). In healthy subjects given sufficient time between exposures, the epithelium is effectively repaired (Patchell and Dorscheid 2006). However, in individuals with inflammatory lung diseases, epithelial repair may be compromised or delayed (Patchell and Dorscheid 2006), thus culminating in progressive lung damage or airway remodeling (Coraux et al. 2008).

For the purpose of this investigation, it was postulated that in pulmonary inflammation, increased levels of key pro-inflammatory cytokines alter or compromise the ability of surface epithelial cells to withstand and adapt to ongoing exposure to traffic-related PM emissions. In many urban areas, diesel exhaust particles (DEP) account for a significant % of traffic-related PM (US EPA 2002; Bedeschi et al. 2007). Owing to their extremely small size, DEP are readily respired, thus depositing in airway and deep lung regions. Composed of an elemental carbonaceous core, DEP possess many organic carbon compounds adsorbed to the particle surface (Singh et al. 2004). Studies suggest that DEP exposure, in particular the organic constituents, might exacerbate allergic lung inflammation

and induce functional lung changes (Matsumoto et al. 2006; Takano et al. 1997; Madden et al. 2003; Steerenberg et al. 2003).

To examine this issue, an *in vitro* epithelial cell culture system was utilized to examine whether traffic-related PM exposure resulted in differential epithelial injury or cytoprotective responses in control or cytokine-treated (TNFα, IL-1β, and IFNγ) cells. Additionally, by using PM samples with relatively high total carbon, but variable organic carbon content, studies further evaluated whether particle-induced responses were generic (i.e., mass-based) or whether they were dependent on the composition of the PM sample. Specifically, it was postulated *a priori* that: (1) DEP exposure of cytokine-treated lung epithelial cells would result in greater cytotoxicity, and (2) the resulting cell injury would be dependent on the relative organic carbon content of the PM sample.

MATERIALS AND METHODS

Cell Culture

LA-4 cells (American Tissue Culture Collection, Manassas, VA), a murine alveolar type II-like epithelial cell line (passages 49 to 55), were grown to 95-100% confluence in Ham's F12K supplemented with 15% fetal bovine serum (FBS), streptomycin (50 μg/mL), penicillin (50 U/mL), and amphotericin B (0.5 μg/mL) in 5% CO_2 at 37°C. Prior to treatment, the medium was changed to FBS-free Ham's F12K supplemented with bovine pituitary extract (BPE; 25 μg/mL), insulin (2 μg/mL,), mouse epidermal growth factor (mEGF; 10 ng/mL), and bovine serum albumin (BSA; 0.5 mg/mL) purchased from Sigma (St. Louis, MO).

Primary mouse tracheal epithelial (MTE) cells were harvested from female BALB/c mice (Charles River Labs, Wilmington, MA), 12–16 weeks of age (19–23 grams), and grown on Transwell membranes in Ham's F12:DMEM as a 50:50 mixture containing 10% FBS, amphotericin-B (250 ng/mL), nystatin (44 IU/mL), gentamicin (100 mg/mL), BPE (104 mg/mL), insulin (5 mg/mL), transferrin (5 mg/mL), mEGF (5 ng/mL), dexamethasone (0.1 μM), cholera toxin (20 ng/mL), and retinol (0.01 μM). The isolation, cultivation, and characterization of MTE cells using this method has been described previously (Lankford et al. 2005). Initially fed from both the apical and basal compartment, once the MTE cells reached 80% confluence they were transferred over to an air–liquid interface (ALI) by removing the apical medium and fed solely basolaterally. Once confluent, approximately 3-4 days post-ALI, the MTE cells were fed basally for 1 day with FBS-free medium supplemented with BSA (0.5 mg/mL) prior to treatment. All protocols were approved by the Institutional Animal Care and Use Committee of the US EPA.

Cytokine Treatment

LA-4 and MTE cell cultures were treated via their respective medium for 24 hr with exogenous cytokines (cytomix: TNFα, IL-1β, and IFNγ; R&D Systems, Minneapolis, MN) each at 0.2 ng/mL for LA-4 cells, and 2 ng/mL for MTE cells (basal exposure only), to induce the *in vitro* equivalent of a state of low-level, acellular, inflammatory microenvironment. These concentrations were based on preliminary studies in which cytokine treatment consistently resulted in increased (at least 5-fold) epithelial cell production of neutrophilic (e.g., MIP-2) or eosinophilic (e.g., RANTES) chemokines, without causing cytotoxicity.

PM Composition

Predominantly carbon-based (e.g., traffic-related) PM samples were evaluated including: the forklift experimentally-generated DEP particle SRM 2975 (National Institute of Standards and Technology, Gaithersburg, MD), fine carbon black particles (CB, Printex 90; a gift from Dr. Vicki Stone, Napier University, Edinburgh, Scotland), and particles from a diesel powered automobile, generated in 1999 (DEP_A; a gift from Dr. Daniel Costa, USEPA, RTP, NC). For comparative chemical composition, we also analyzed an ambient PM sample collected in 1999 near a tunnel in Baltimore, MD (B-PM; a gift from Dr. John Ondov, University of Maryland, Baltimore, MD).

The total elemental carbon (EC), organic carbon (OC), and non-volatile residual fractions of SRM 2975, B-PM, and DEP_A were determined by thermal-optical transmittance (Sunset Laboratories, Forest Grove, OR) as previously described (Birch and Cary 1996). Elemental analysis of deionized water-based (1 hr) extractions of CB, SRM 2975, B-PM, and DEP_A was determined for sulfate (SO_4), transition and heavy metals, and various non-metal elements using ICP-MS (Method 6020a, US EPA 2007) and ICP-AES (Method 200.7 rev 4.4, US EPA 1994). This speciation scheme is useful for comparing the approximate amounts of bioavailable (e.g., water-soluble) elements across heterogeneous PM types for short-term exposures. Finally, lipopolysaccharide levels were measured in 50 μL of PBS-resuspended PM (1.1 mg/mL) using the QCL-1000 Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD).

Cell PM Exposure and Injury Assessment

Just prior to cell exposure, particle stock suspensions (11 mg/mL) were prepared in sterile PBS and sonicated three times for 30 sec on ice using a probe sonicator (Misonix Inc., Farmingdale, NY). LA-4 cells were exposed to PM via spiked cell culture medium to achieve exposures ranging from 2.5 to 250 $\mu g/cm^2$. In MTE cells, PM exposures ranging from 5 to 200 $\mu g/cm^2$ was achieved by apical treatment of 100 µL of PM-saline suspension.

Release of lactate dehydrogenase (LDH) was used as a measure of cell injury in LA-4 and MTE cultures. LDH activity was assayed using a commercially available kit (Thermo Electron Corp., Louisville, CO), modified and adapted for use on the KONELAB 30 clinical chemistry analyzer (Thermo Clinical Labsystems, Espoo, Finland). Cell-free conditioned medium and cell lysates collected from cells treated with cold PBS containing 0.1% Triton-X 100 (Sigma, St. Louis, MO) for 30 min were used in the assay.

Real-Time RT-PCR

Total RNA was isolated using RNeasy (Qiagen, Valencia, CA). cDNA synthesis and real-time PCR using gene-specific primers was then performed using SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Grand Island, NY). Heme oxygenase-1 (HO-1) and β-actin primer/probe sets were purchased from Applied Biosystems (Foster City, CA). After determining that β-actin mRNA expression was not altered by our experimental treatments, the level of HO-1 mRNA expression was normalized to β-actin using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

Evaluation of Cellular Glutathione

Cellular glutathione, reduced (GSH) and disulfide (GSSG) forms, were analyzed by high-performance liquid chromatography (HPLC) using a modification of methods described previously (Gan et al. 2005; Jones et al. 1998). Cells were dislodged with a rubber policeman into PBS, sonicated briefly on ice, and then added to an equal volume of cold 10% perchloric acid containing 0.4 M boric acid (Sigma, St. Louis, MO). After centrifugation (20 min, 4ºC, 20,000 g), the cell-free supernatant was treated with dansyl chloride (Sigma, St. Louis, MO) to label GSH and GSSG fractions, followed by HPLC analysis (Jones et al. 1998).

Epithelial Permeability Changes

As an index of epithelial solute permeability for the confluent MTE cultures established at an ALI (Dye et al. 1997), changes in apical protein levels from the MTE cultures were determined using a Coomassie Plus Protein assay kit (Pierce, Rockford, IL) modified and adapted for use on the KONELAB 30 clinical chemistry analyzer. The apical surface of MTE cells were washed for 5 min with 0.5 mL of PBS and this supernatant collected. After a brief centrifugation (10 min, 4ºC, 20,000 g), the resultant supernatant was analyzed for total protein content.

Statistical Analysis

Data are expressed as means ± SEM. Data were analyzed using a one-way or two-way analysis of variance (ANOVA) and where relevant, followed by Bonferroni post-hoc tests for comparison between groups. A value $p < 0.05$ was considered significant.

RESULTS

PM Sample Composition

To better understand the effects of particle composition on pulmonary epithelial cell responses, relative EC, OC and water-soluble elemental content of representative PM samples were determined. As depicted in Figure 1, the experimentally-generated DEP standard reference material (SRM 2975) consisted mainly of EC; while the diesel auto-mobile-generated sample (DEP_A) and ambient PM sample collected near a tunnel in Baltimore, MD (B-PM) contained approximately 35% and 45% OC, respectively. The CB sample, reportedly of very low-PAH content contains only 1-2 % OC (Stoeger et al. 2006). For comparison, EC and OC content of urban ambient PM collected in the Washington DC area during 1976-1977 (SRM 1649a) and that of a residual oil fly ash sample (ROFA no. 3) are included.

Elemental analysis of these PM samples demonstrated that CB and SRM 2975 contained very little water-soluble sulfate, metals or other elements (Table 1). In contrast, sulfate, metals, and other water-soluble elements extracted from DEP_A closely approximated that of the B-PM tunnel sample; based on previously published data (Vincent et al. 1997), it approximated that of the 1993 bag-house filter collected urban air dust sample, EHC-93 (Environmental Health Directorate; Health Canada, Ottawa, ON). Furthermore, based on previous analyses (McGee et al. 2003), compared to SRM 1649a, DEP_A contained somewhat lower levels sulfate and lead; while relative to ROFA no. 3, it contained approximately one tenth of the sulfate and transition metals characteristic of fly ash samples (Figure 1; Table 1). Lastly, none of the PM samples evaluated (i.e., CB, SRM 2975, DEP_A, and B-PM) contained

excessive levels of endotoxin (i.e., 0.0001, 0.13, 0.53, and 1.1 endotoxin units (EU)/mg PM, respectively). Therefore, because the overall composition of DEP_A most closely resembled that of contemporary urban PM samples, in particular the tunnel traffic-related sample (B-PM), DEP_A was used herein as a PM prototype to evaluate whether cytokine-treated epithelial cells were more prone to roadway particle-induced injury than control epithelial cells.

Cytokine-Treated LA-4 Cell Responses to PM Exposure

First, the relative toxicity of DEP_A on untreated-control LA-4 epithelial cells exposed for 24 hr was established. Results demonstrated that significant cell injury, as determined by LDH release, occurred only in control LA-4 cells exposed to the highest concentration of DEP_A, 250 $\mu g/cm^2$ (Figure 2). Thus, for subsequent exposures, control and cytokine-treated LA-4 epithelial cells were exposed to the intermediate, non-injurious DEP_A concentration, 25 $\mu g/cm^2$. However, as depicted in Figure 3, data revealed that cytomix exposure of LA-4 cells to DEP_A (25 $\mu g/cm^2$) resulted in significantly greater cell injury than occurred during comparable exposure of healthy LA-4 cells.

In a subsequent experiment, control and cytomix-treated LA-4 cells were exposed to particles (25 $\mu g/cm^2$; 24 hr) of varying organic carbon content (Figure 4), including the DEP_A sample, the NIST (SRM 2975) sample with relatively little organic carbon, and carbon black (CB) which contained negligible organic carbon. Again, in control LA-4 cells, at this concentration no particle exposure induced significant LDH leakage. However, as noted above, in the cytomix-treated cell, DEP_A exposure was associated with significant epithelial cell injury. Of note, in this experiment the cytomix treatment itself elicited minor, but sig-

nificant injury. Despite this observation, exposure of the cytokine-treated LA-4 cells to either CB or SRM 2975 failed to induce any additional epithelial cytotoxicity (Figure 4).

Cytoprotective Responses in PM-Exposed LA-4 Cells

To assess if the observed cell injury elicited by DEP_A in cytokine-treated epithelial cells was mediated by oxidative stress, exposed cells were subsequently evaluated for alterations in: (1) gene expression of the oxidant-responsive enzyme, heme oxygenase-1 (HO-1), and (2) changes in the relative amount of reduced (GSH) and disulfide (GSSG) forms of oxidant-scavenging glutathione. As above, LA-4 epithelial cells were exposed to PM samples with varying organic carbon content. As shown in Figure 5, in control LA-4 cells, based on real-time RT-PCR assessment of mRNA expression of HO-1, only exposure to the organic carbon-rich DEP_A resulted in significant (12-fold) induction of this cytoprotective enzyme. Interestingly, cytomix treatment of LA-4 cells was itself associated with significant (8-fold) HO-1 induction. Subsequent exposure of cytokine-treated cells to CB and SRM 2975 failed to induce further increases in HO-1 expression; while DEP_A exposure induced a nearly a 15-fold increase versus control cells and a 25% increase over cytomix-treated cells.

Changes in the redox status of LA-4 cells both the GSH/GSSG ratios (Figure 6a) and molar amount of GSH and GSSG (Figure 6b) were evaluated. The data revealed that control LA-4 cells contained approximately 4-fold greater GSH than GSSG; and that exposure of control LA-4 cells to PM, including DEP_A, failed to significantly alter GSH/GSSG ratios. Despite the influence of cytomix treatment on HO-1 gene expression, there was no significant cytomix-induced effect on intracellular glutathione levels or GSH/GSSG ratios.

Exposure of either control or cytokine-treated cells to CB or to SRM 2975 was without effect on absolute glutathione levels or GSH/GSSG ratios. Of note, DEP_A exposure of control LA-4 cells resulted in minor, statistically insignificant increases in GSH and GSSG, maintaining a constant GSH/GSSG ratio, suggestive of an effective antioxidant cell response. By contrast, in cytokine-treated LA-4 cells, DEP_A exposure was associated with striking increases in GSH and GSSG, with significant reduction in the GSH/GSSG ratio — indicative of failure to maintain normal intracellular *redox* status, and evidence that significant intracellular oxidative stress occurred.

Cytokine-Treated MTE Cell Responses to DEP_A Exposure

To extend our *in vitro* findings beyond that of the immortalized LA-4 cells, primary MTE cells grown at an air-liquid interface (ALI) were also used. Since exposure of LA-4 cells to particles with little (SRM 2975) or no organic carbon content (CB) did not elicit any injurious (LDH) or antioxidant responses (GSH or HO-1), it was decided to only expose MTE cells to DEP_A. Analogous to experiments performed in LA-4 cells, the concentration-response to DEP_A (24 hr) was first established in control, confluent MTE cells. Figure 7 shows that significant cell injury was only observed in cells exposed to the highest DEP_A concentration, 200 $\mu g/cm^2$. Thus, subsequent exposures of the control vs. cytokine-treated MTE cells used an intermediate, non-injurious concentration (20 $\mu g/cm^2$) to determine if cytokine-treated MTE cells were also more susceptible to DEP_A exposure. Results demonstrated that despite a trend towards increased LDH release in cytomix-treated MTE cells, subsequent DEP_A exposure did not result in additional LDH leakage (Figure 8). Consistent with a lack of DEP_A-induced cell injury, there were no significant changes

observed in GSH/GSSG ratios (data not shown) or intracellular GSH and GSSG concentrations in either control or cytokine-treated DEP_A exposed MTE cells (Figure 9). It may also be relevant, however, that saline-exposed control MTE cells contained 8.5- and 10-fold more total gluthatione and reduced glutathione, respectively, than was present in the control LA-4 cells.

Lastly, because control MTE cells established at ALI are able to form tight, impermeable cell layers that preclude the transport of basal medium to the apical surface, the effect of PM exposure on solute permeability was assessed as an indicator of epithelial barrier dysfunction. Results revealed that whereas exposure of control MTE cells to DEP_A for 24 hr did not affect solute permeability, cytomix treatment transiently elevated the amount of protein detected on the apical surface (i.e., due to leakage of basal medium) (Figure 10). Moreover, in cytokine-treated MTE cells, DEP_A exposure resulted in still greater increases in the apical protein; with protein levels remaining significantly elevated for up to 72 hr after initial DEP_A exposure. The changes in apical protein were observed without concurrent decreases in cell lysate protein levels (data not shown).

DISCUSSION

Epidemiological data indicates that human exposure to urban air pollutants in the immediate vicinity of large roadways is associated with a range of health effects. In particular, exposure of individuals with pre-existing inflammatory lung diseases, such as asthma (Kim et al. 2008), COPD (Schikowski et al. 2005), chronic bronchitis (Karr et al. 2007), and allergic rhinitis (Cesaroni et al. 2008) to traffic-related PM has been linked to increased morbidity and mortality. To further understand the mechanisms that render

individuals with pre-existing inflammatory disorders at risk of PM exposure, a cell culture system that models both control and cytokine-treated epithelial cells was developed. Analogous to the adverse health effects observed in the above epidemiological studies, herein data demonstrated that DEP exposure of cytokine-treated alveolar and airway epithelial cells induced significantly greater cytotoxicity and solute permeability changes, respectively, than did comparable exposure of healthy (non-inflamed) cells. Furthermore, data showed that the injury induced was seemingly related to the organic carbon fraction of the particles, in that comparable exposure to particles with little (e.g., SRM 2975) or no organic carbon (e.g., carbon black) failed to elicit significant changes. These data are consistent with a number of *in vitro* and *in vivo* studies that examined the effects of PM exposure, and its carbon composition, in control vs. inflamed experimental settings. For example, when human lung cell lines (A549 and HTB54) were primed by TNFα prior to exposure to a metal-rich PM (residual oil fly ash) (Stringer and Kobzik 1998) or an ambient collected PM (Ning et al. 2004), primed cells produced significantly more IL-8, a potent neutrophil chemokine, as compared to control cells. Further, experimental evidence from *in vivo* murine models of allergic inflammation exposed to concentrated ambient road traffic particles rich in organic carbon (Kleinman et al. 2007) or the extracted organic components from an experimentally-generated DEP (Yanagisawa et al. 2006) also resulted in increased chemokine release and lung inflammation. Together, these results suggest that the adverse effects associated with DEP exposure in the setting of inflammation, are due in large part, to PM subcomponents associated with the organic carbon layer of the insulting particle.

Why would cytokine-treated surface epithelial cells be more prone to injury following exposure to organic carbon-rich particles? Although DEP is composed of an elemental carbon core, owing to differences in engine operating conditions and sample collection methods, varying types and amounts of organic carbon compounds become adsorbed to the surface of the carbon core. DEP composition can vary from nearly all elemental carbon to ≥ 50% organic carbon (Singh et al. 2004). Importantly, many of the adsorbed organic compounds are classified as air toxics by the US EPA, including polycyclic aromatic hydrocarbons (PAH), quinoines, alkanes, aldehydes (Liang et al. 2005) and are capable of generating reactive oxygen species (ROS) within exposed cells (Pan et al. 2004). Furthermore, minor but nonetheless detectable quantities of sulfate, nitrate, metals, and other trace materials may also be adsorbed onto this organic-rich layer (Wichmann 2007). Metals typically represent a minor fraction of DEP mass (1-5%), dependent upon diesel fuel metal content, as well as engine load characteristics during DEP generation. Metals may be present in both ultrafine (< 100 nm) and accumulation-modes of DEP, the result of enhanced metal nucleation or heterogeneous condensation, respectively (Lim, Lim, and Yu 2009). Somewhat paradoxically, therefore, it is possible that within DEP samples, inorganic PM compounds such as metals may be preferentially present within the "organic carbon" layer.

Accordingly, in addition to having greater OC content, PM elemental and endotoxin assessments revealed that compared to CB or SRM 2975, DEP_A also had more water-soluble iron, zinc, and endotoxin. Of note, however, DEP_A contained only minor amounts of the *redox* active, relatively epitheliotoxic metals (i.e., vanadium, copper, and nickel). Moreover, based on our previous investigations in primary rat tracheal epithelial cells exposed to metal-

rich fly ash (Dye et al. 1999) and steel mill PM extracts (Pagan et al. 2003) the amount of iron, zinc, or endotoxin present in DEP_A would likely be insufficient to elicit the cytotoxicity observed in these LA-4 or MTE cells, interactions of metals and/or endotoxin with OC notwithstanding.

Epithelial cells normally have a wide array of enzymatic (HO-1) as well as non-enzymatic (GSH) defensive mechanisms to effectively adapt to inhaled oxidant insults. Our data revealed that exposure of control LA-4 epithelial cells to the organic carbon-rich DEP_A resulted in adaptive increases in HO-1 and a trend in increased total glutathione, consistent with an effective antioxidant cellular response to PM–related oxidative stress. Although its mechanisms of action are still being defined, HO-1 was shown to confer cytoprotection in the setting of lung insults ranging from hyperoxia (Zhang et al. 2006), hypoxia (Zampetaki et al. 2003), and cigarette smoke exposure (Baglole, Sime, and Phipps 2008). As observed in our control LA-4 and MTE cells, under control conditions the ubiquitous intracellular antioxidant, glutathione, is mainly present in its reduced form. Moreover, maintenance of appropriate GSH/GSSG levels is essential for regulating intracellular *redox* homeostasis (Forman, Zhang, and Rinna 2009). As oxidants are encountered, GSH can become transiently oxidized and converted to its disulfide form (GSSG), thus reducing the GSH/GSSG ratio, indicative of ensuing cellular oxidative stress. Under normal circumstances, relative GSH and GSSG levels are re-established via recycling of GSSG by the NADPH-dependent enzyme, glutathione reductase. Our observations in the cytomix-treated cells, a model of pre-existing inflammation, these antioxidant responses may become overwhelmed and thus not able to efficiently respond to additional oxidative stressors.

This study sought therefore to reproduce or "model" the *in vitro* equivalent of a low-grade inflammatory microenvironment within lung epithelial cell cultures via treatment with a combination of exogenous cytokines (cytomix: TNFα + IL-1β + IFNγ) that are often implicated in the pathogenesis and exacerbation of chronic inflammatory lung diseases (Bjornsdottir and Cypcar 1999; Hansbro et al. 2008; Elias 2004). It was postulated that in individuals with inflammatory disorders, surface epithelial cells would be more or less continually exposed to these mediators. As such, antioxidant and cytoprotective defenses may become altered, thus precluding their ability to respond to further oxidant insults such as PM, ultimately leading to diminished cell function. Interestingly, even prior to DEP_A exposure, it was observed that cytokine-treated LA-4 cells had significantly increased HO-1 expression, suggestive of an adaptive antioxidant response. These results are consistent with a previous study demonstrating that cytomix treatment of human primary epithelial cells, at concentrations 25- to 250-fold greater than used herein, was also associated with HO-1 induction (Donnelly and Barnes 2001). Despite apparent adaptive increases in HO-1 (and possibly GSH) during exposure of the control epithelial cells to organic carbon-rich particles (DEP_A), cytokine-treated lung epithelial cells exposed to DEP_A could no longer maintain appropriate GSH/GSSG ratios, developing instead overt oxidative stress and ultimately cell injury.

To further extend our observations with the lung epithelial cell line, primary MTE cells were used, which when established under ALI conditions exhibit cell populations similar to those present in the airways, with polarized cells forming a pseudostratified columnar epithelial barrier (Lankford et al. 2005). Unlike LA-4 cells, increased LDH release

was not observed during exposure of cytokine-treated MTE cells to 20 μg/cm^2 of DEP_A. Upon further evaluation of the intracellular GSH levels, data revealed that DEP_A exposure failed to alter relative GSH or GSSG levels in either control or cytokine-treated MTE cells. Differences in the propensity of airway versus alveolar cell types to develop injury likely reflected differences in their antioxidant capacity. Comparatively, control MTE cells contained nearly 8.5-fold the amount of total glutathione, with GSH:GSSG ratios of nearly 18:1 vs. the 4:1 ratio found in control LA-4 cells. Relatively high GSH levels have also been reported in airway epithelial cells established using ALI conditions. For example, during transition to an ALI, bovine tracheal epithelial cells experience low-level oxidative stress that results in substantially more intercellular GSH and GSSG than is present in comparable cells grown in the traditional submerged setting (Kameyama et al. 2003).

Exposure of cytokine-treated MTE cells to DEP_A did significantly alter the epithelial barrier since solute permeability increases were still evident up to two days after the removal of the initial particle exposure. Similarly, previous reports by our lab as well as others showed that exposure of lung epithelial cells to metal-rich PM (Dye et al. 1997), extracts of ambient PM (Pagan et al. 2003), or to common indoor allergens (Wan et al. 1999) can significantly impair epithelial barrier function. Although not explored in this study changes in the epithelial permeability, even after the removal of DEP, could be the result of residual DEP present either from phagocytosis or surface adherence to the epithelial cells. This normally restrictive barrier serves to prevent foreign proteins, microorganisms, and other inhaled agents or particles from penetrating the underlying submucosal region. Thus, exposure of individuals with chronic respiratory tract inflammation to traffic-derived

pollutants might allow inhaled substances (i.e., aeroallergens) to encounter the antigen presenting cells within the nasal or air passageways. Enhanced atopic disease, either allergic rhinitis or asthma, could result — consistent with the epidemiologic observations of associations between PM exposure and increased morbidity in these susceptible subpopulations.

In summary, the present study demonstrates that exposure to DEP rich in organic carbon induced an oxidant burden that control epithelial cells were able to tolerate through effective cytoprotective and antioxidant mechanisms. In a setting of pre-existing epithelial inflammatory microenvironment, however, exposure of organic-enriched DEP overwhelmed these defenses resulting in impaired epithelial barrier function and epithelial cell injury. These observations are analogous to epidemiological findings that suggest individuals with pre-existing inflammatory lung disorders exposed to traffic-related PM are uniquely susceptible to PM-associated adverse health effects. Of relevance, oxidant – antioxidant imbalance is thought to play a critical role in initiation as well as amplification of inflammatory processes in asthmatic (Nadeem, Masood, and Siddiqui 2008), COPD (Rahman 2008), and rhinitic (Gratziou et al. 2008) patients. Additional *in vivo* studies are needed to determine whether analogous interactions between pre-existing lung inflammatory conditions and traffic-related PM exposure occur. *In vitro* models, such as ours, that model specific disease states may serve to bridge the efforts to understand PM health-effects in susceptible individuals, consistent with EPA's current mandate to incorporate more high-throughput approaches to toxicant evaluation (Kavlock, Austin, and Tice 2009). Using this type of *in vitro* approach, specific effects of PM exposure may be further used to evaluate critical biological pathways

that when perturbed may result in adverse health outcome and disease. Improved understanding of the differential outcomes in susceptible individuals exposed to air pollutants, like PM, may identify important cell responses, stress pathways, or biomarkers that aide hazard and risk identification as well as the NAAQS standard setting.

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Table 1. Relative EC, OC, and water-soluble elemental content of PM samples.

*Elemental and organic carbon content was determined by thermal optical transmittance of particles: carbon black (CB), NIST SRM 2975, diesel powered automobile particles (DEP_A), and ambient PM collected near a tunnel in Baltimore, MD (B-PM).

**Elemental analysis of water-based PM extractions including sulfate (SO_4), transition and heavy metals, and other elements were determined using ICP-MS and ICP-AES.

	CB	SRM 2975	DEP_A	B-PM	EHC-93	SRM 1649a	ROFA no. 3
Carbon							
% EC*	98-99	77.1	33.0	25.0	N/A	2.16	3.43
% OC*	1-2	7.98	34.9	43.0	N/A	15.7	0.27
% Fraction Residual*	–	N/A	N/A	0.033	N/A	58.9	57.6
% Total Carbon (EC+OC)*	~ 99	85.1	67.9	68.0	N/A	17.9	3.70
Water Soluble Elements**							
Sulfate (SO_4) (μg/gm)	770	5,960	50,900	45,870	44,500	86,720	275,500
(%)	**(0.08 %)**	**(0.60 %)**	**(5.1 %)**	**(4.6 %)**	**(4.5 %)**	**(8.7 %)**	**(27.5 %)**
Other Elements**							
Ca	< 1.8	113	5,730	5,350	26,160	12,650	18,600
Mg	< 1.4	153	119	1,060	1,010	1,140	24,900
Na	408	56	93	7,940	16,070	2,150	44,200
P	< 5.9	191	1,670	166	N/A	1,080	"0"
Al	3.0	16	166	141	196	1,050	6,740
As	< 3.1	0.11	0	< 2.2	N/A	13.5	1.7
SiO_2	27	26	19	69	N/A	N/A	N/A
∑Other Elements (μg/gm)	450	555	7,800	14,700	43,400	18,100	94,400
(%)	**(0.05 %)**	**(0.06 %)**	**(0.8 %)**	**(1.5%)**	**(4.3 %)**	**(1.8 %)**	**(9.4 %)**
Transition, Heavy Metals**							
Cd	< 0.3	0.06	0.64	1.73	5.5	19.9	8.9
Co	< 0.6	0.24	5.4	1.37	0.6	2.1	495
Cu	< 0.6	0	130	33	144	83.6	570
Fe	1.0	10.8	971	297	150	407	760
Mn	< 0.1	1.62	17.5	94	101	77.2	365
Ni	< 0.6	0.55	40.2	23	4.9	32.2	17,030
Pb	< 5.9	0.55	10.3	< 5.9	268	1,380	18
V	< 0.2	0.1	0.55	24	-	N/A	1,750
Zn	< 0.8	148	2,790	794	4,780	504	6,560
∑Trans, Heavy Metals (μg/gm)	≤ 10	162	3,970	1,280	5,450	2,510	27,600
(%)	**(≤ 0.001%)**	**(0.016%)**	**(0.40 %)**	**(0.13 %)**	**(0.55 %)**	**(0.25 %)**	**(2.80 %)**

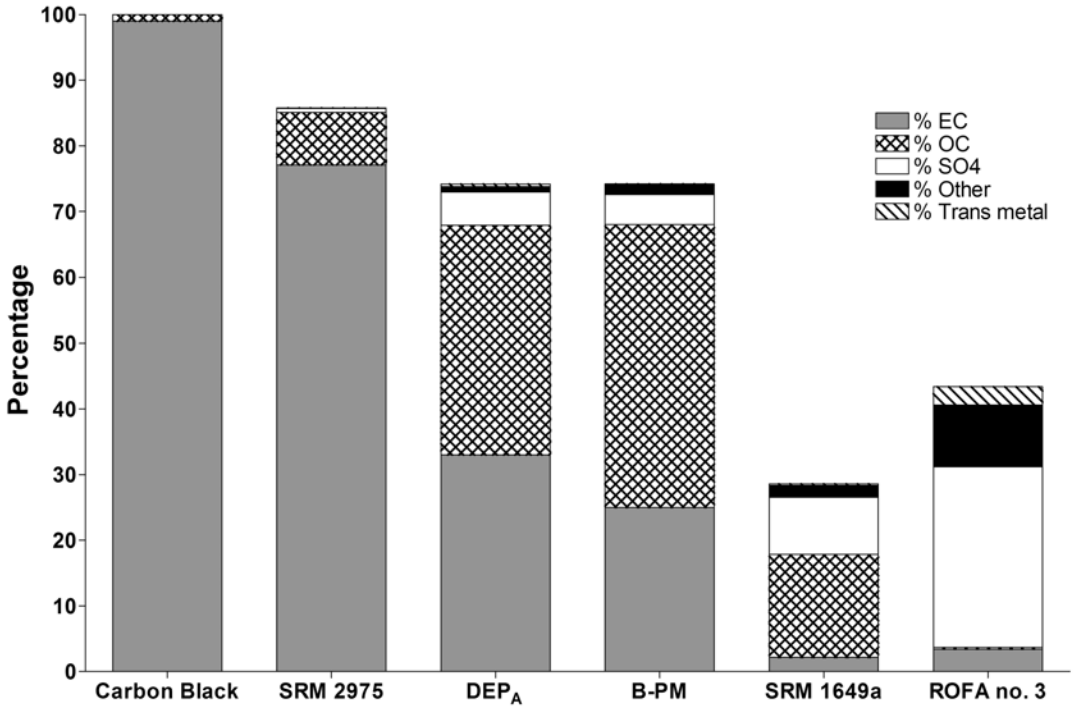


Figure 1. PM composition. Relevant content of elemental carbon (EC), organic carbon (OC), sulfate (SO_4), transition and heavy metals and miscellaneous other elements present in representative PM samples.

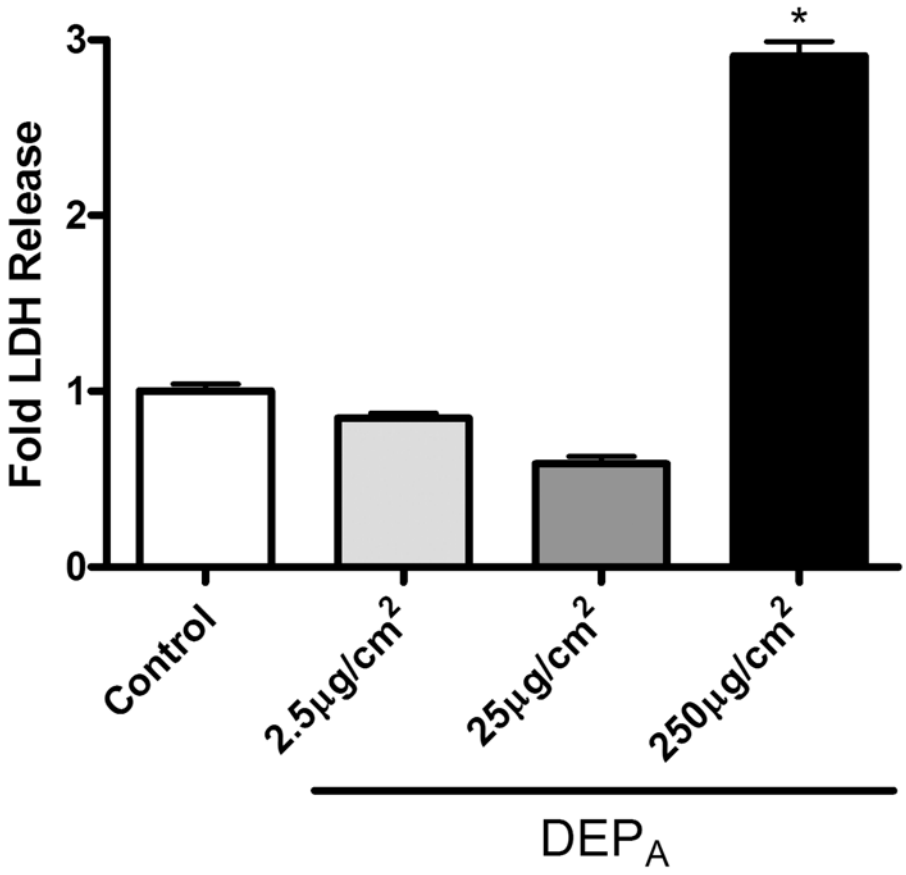


Figure 2. Cell injury of DEP_A exposed LA-4 cells. LA-4 epithelial cells were exposed to saline (Control) or automobile-derived diesel exhaust particles (DEP_A) at 2.5, 25, or 250 $\mu g/cm^2$ for 24 hr. Conditioned medium and cell lysates were analyzed for LDH released. Data are expressed as mean fold increase (± SEM) of LDH release (normalized to total LDH) over saline-exposed control cells (19.4 ± 0.8%). Significantly ($p < 0.05$) greater injury is indicated by: * vs. All other treatments.

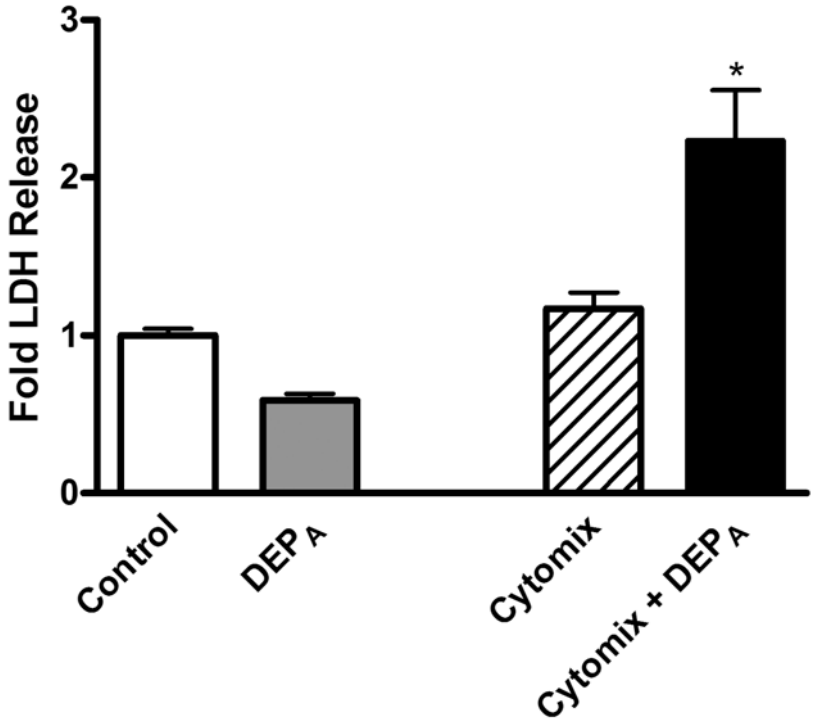


Figure 3. Cell injury of cytokine-treated LA-4 cells exposed to DEP_A. Control and cytokine-treated LA-4 cells were exposed to saline or diesel powered automobile particles (DEP_A 25 μg/cm^2) for 24 hr. Conditioned medium and cell lysates were analyzed for LDH released. Data are expressed as mean fold increase (± SEM) of LDH release (normalized to total LDH) over saline-exposed control cells (20.6 ± 0.8%). Significantly ($p < 0.05$) greater injury is indicated by: * vs. All treatments.

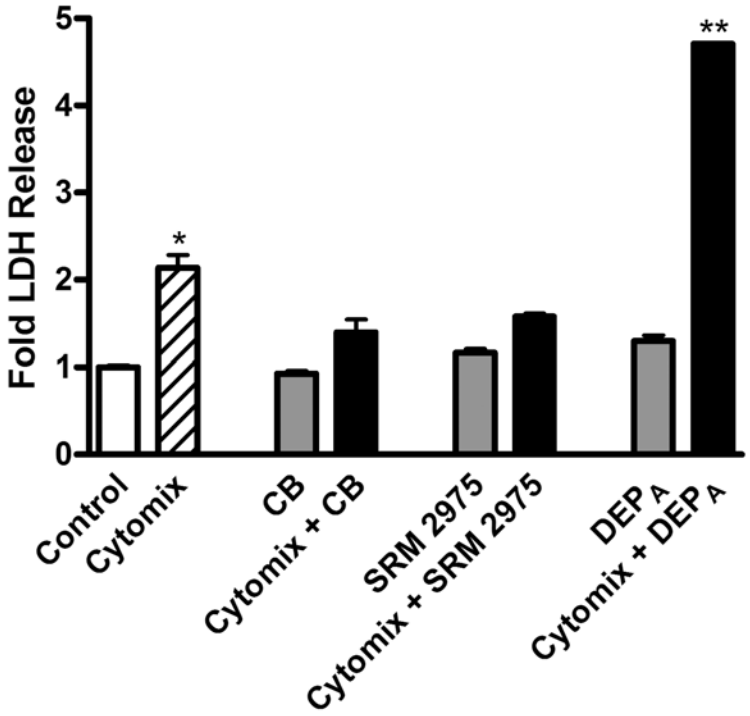


Figure 4. Effect of PM carbon composition on LA-4 cell injury. Control or cytokine-treated LA-4 cells were exposed to either saline (Control), Carbon Black (CB), NIST SRM 2975 (SRM 2975), or diesel powered automobile particles (DEP_A) at 25 μg/cm^2 for 24 hr. Conditioned medium and cell lysates were analyzed for LDH released. Data are expressed as mean fold increase (± SEM) of LDH release (normalized to total LDH) over saline-exposed control cells (19.9 ± 0.5%). Significantly ($p < 0.05$) greater cell injury is indicated by: * vs. Control; ** vs. All Treatments.

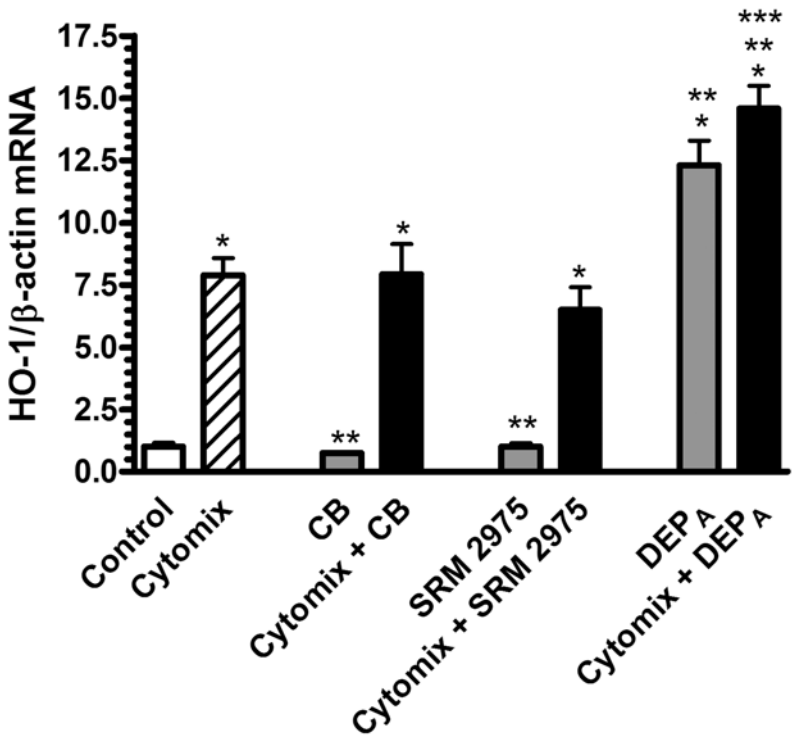
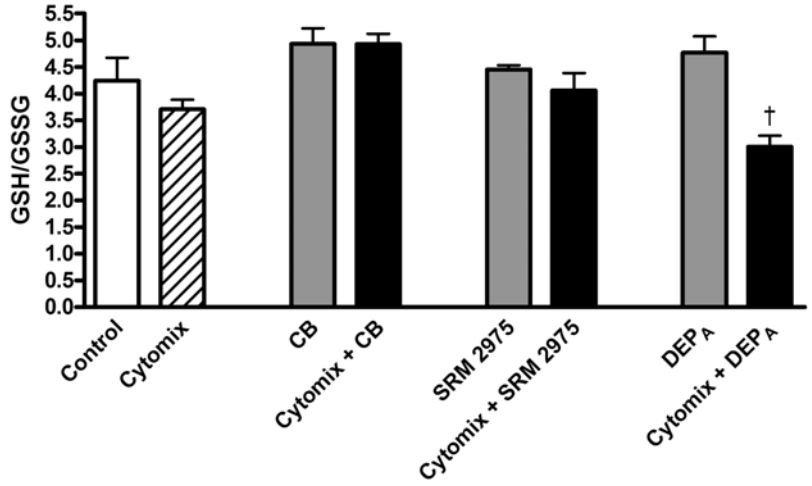


Figure 5. HO-1 expression of cytokine-treated LA-4 cells exposed to DEP_A. Control and cytokine treated LA-4 epithelial cells were exposed to saline (Control), Carbon Black (CB), NIST SRM 2975 (SRM 2975), or diesel powered automobile particles (DEP_A) at 25 μg/cm^2 for 24 hr. Cells were harvested for mRNA, and HO-1 expression was quantified by real-time RT-PCR, with normalization to β-actin expression. Data are expressed as the mean (± SEM) fold increase over saline-exposed control cells. Significance ($p < 0.05$) is indicated by: * vs. Control; ** vs. Cytomix; *** vs. Cytomix + CB and Cytomix + SRM 2975.

A)



B)

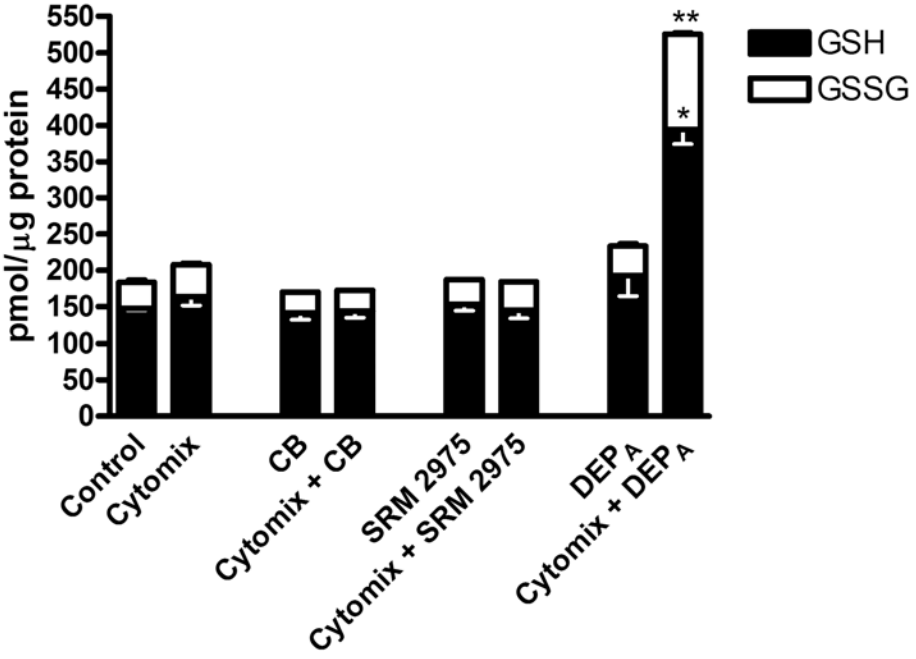


Figure 6. Intracellular glutathione of cytokine-treated LA-4 cells exposed to DEP_A. Control and cytokine-treated LA-4 epithelial cells were exposed to saline (Control), Carbon Black (CB), NIST SRM 2975 (SRM 2975), or diesel powered automobile particles (DEP_A) at 25 $\mu g/cm^2$ for 24 hr. Cell lysates were analyzed for reduced (GSH) and disulfide (GSSG) forms of glutathione by HPLC. Data are expressed as the molar ratios of GSH/GSSG (A) or as pmol/µg of protein in cell lysate (B) ± SEM. Significance ($p < 0.05$) indicated by: * vs. All Treatments (GSH only); ** vs. All Treatments (GSSG only), † vs. DEP_A.

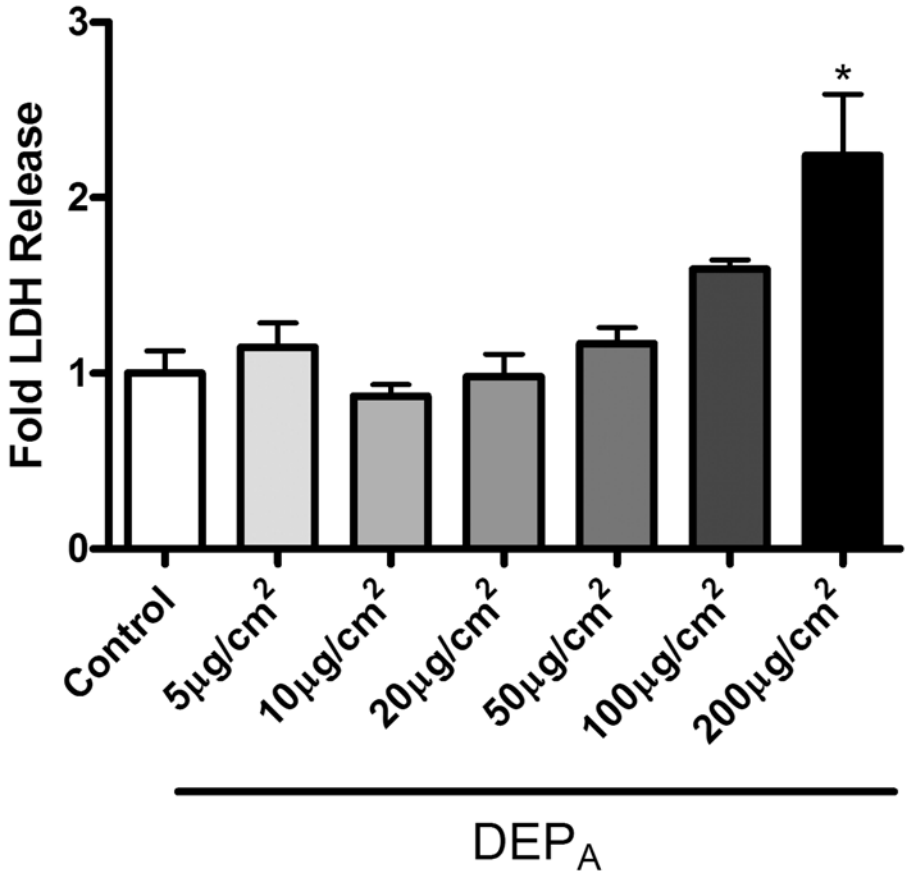


Figure 7. Cell injury of MTE cells exposed to DEP_A. MTE cells were exposed to either saline (Control) or diesel powered automobile particles (DEP_A) at 5, 10, 20, 50, 100, or 200 $\mu g/cm^2$ for 24 hr. Conditioned medium and cell lysates were analyzed for LDH released. Data are expressed as mean fold increase (± SEM) of LDH release (normalized to total LDH) over saline-exposed control cells (4.3 ± 0.5%). Significantly ($p < 0.05$) greater injury is indicated by: * vs. All treatments.

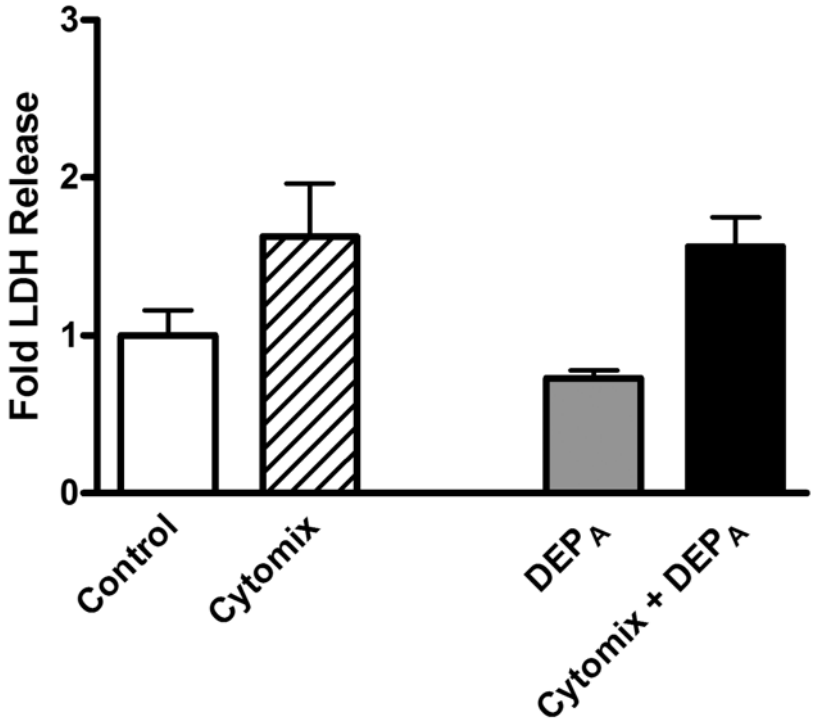


Figure 8. Cell injury of cytokine-treated MTE cells exposed to DEP_A. Control and cytokine-treated MTE cells were exposed to saline or diesel powered automobile particles (DEP_A 20 μg/cm^2) for 24 hr. Conditioned medium and cell lysates were analyzed for LDH released. Data are expressed as mean fold increase (± SEM) of LDH release (normalized to total LDH) over saline-exposed control cells (2.3 ± 0.2%).

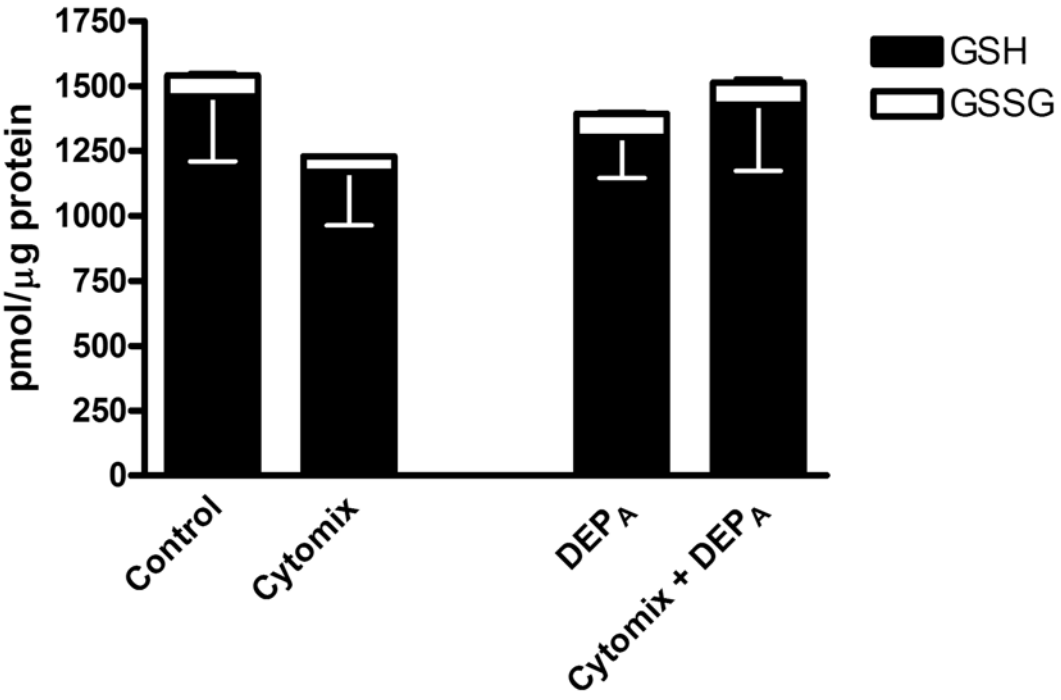


Figure 9. Intracellular glutathione of cytokine-treated MTE cells exposed to DEP_A. Control and cytokine-treated MTE cells were exposed to diesel powered automobile particles (DEP_A) at 20 µg/cm^2 for 24 hr. Cell lysate samples were analyzed for reduced (GSH) and disulfide (GSSG) forms of glutathione by HPLC. Data are expressed as pmol/µg of protein in cell lysate (± SEM).

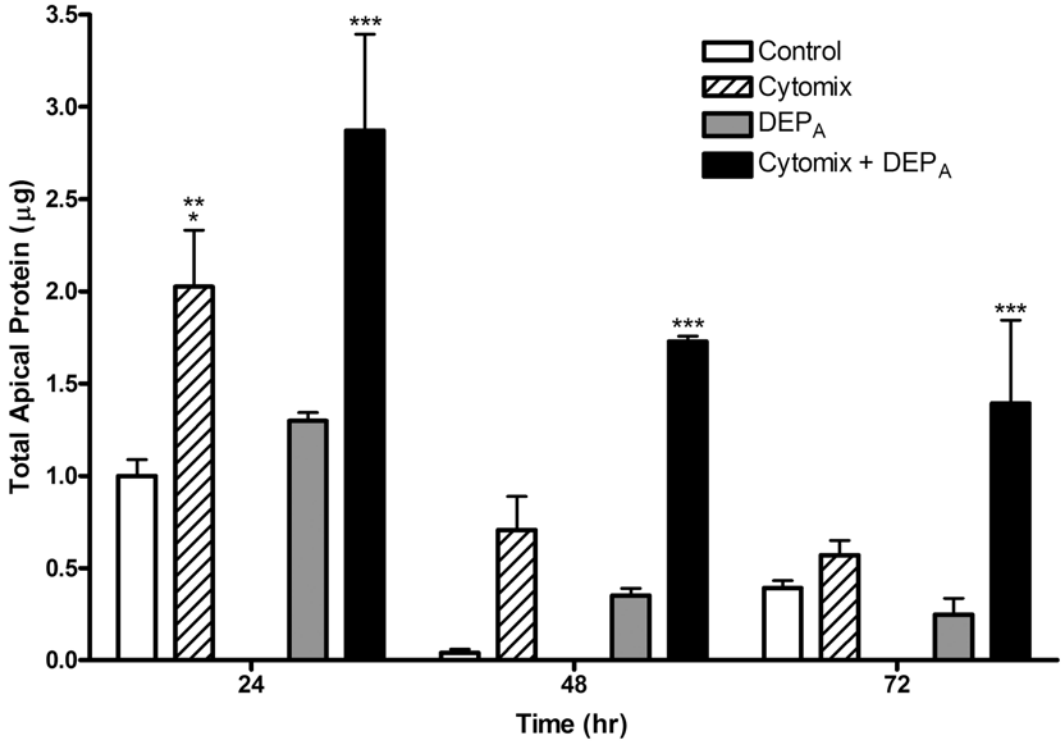


Figure 10. Epithelial solute permeability of cytokine-treated MTE cells exposed to DEP_A. Control and cytokine-treated MTE cells were exposed to either saline (Control) or diesel powered automobile particles (DEP_A 20 µg/cm^2) for 24 hr and subsequently removed. The apical sample was analyzed at 24, 48, or 72 hr after DEP_A treatment to determine apical protein leakage. Data are expressed as mean total apical protein (µg ± SEM). Significance ($p < 0.05$) is indicated by: * vs. Control; ** vs. DEP_A; *** vs. All treatments within time point.

CHAPTER 4:

Interaction of Superoxide and Nitric Oxide in Diesel Exhaust Particles-Induced Cytotoxicity of Cytokine-Treated Epithelial Cells

ABSTRACT

Exposure to diesel exhaust particles (DEP) is associated with adverse health outcomes, in part mediated through the production of reactive oxygen species (ROS), such as superoxide ($O_2^{\cdot -}$). Individuals afflicted with airway inflammation due to disease, have elevated levels of the free radical nitric oxide (NO), and represent a population at greater risk from the effects of DEP exposure. Whereas the cellular toxicity of either NO or $O_2^{\cdot -}$ alone is modest, the two can react together to produce peroxynitrite ($ONOO^-$) a more potent radical with damaging cellular effects. The current *in vitro* study examined the potential cooperation of NO and $O_2^{\cdot -}$ in DEP-induced cell injury in lung epithelial cells established in an inflammatory microenvironment. Murine lung LA-4 epithelial cells pre-treated with TNFα, IL-1β, and IFNγ and exposed to DEP (25 μg/cm^2) for 24 hr had increased ROS production and cell injury. Inhibition of NO with 1400W, a selective iNOS inhibitor, and by catalysis of $O_2^{\cdot -}$, via the addition of superoxide dismutase (SOD), effectively abrogated the resulting ROS. In contrast, when cytokine-treated LA-4 cells were exposed to DEP along with the FeTMPyP, a $ONOO^-$ scavenger, cell injury was prevented. Furthermore, *in vivo* pulmonary administration of these cytokines followed by DEP inhalation resulted in increased ROS production in bronchoalveolar lavage fluid cells, which was effectively abrogated by i.p. administration of FeTMPyP. Together the data show that cytokine-treated epithelial cells are more susceptible to the damaging effects of DEP exposure through the cooperative effects of NO and $O_2^{\cdot -}$, and may represent, at least in part, a contributing mechanism in the adverse health effects of DEP in individuals with chronic inflammatory lung diseases.

KEY WORDS: DEP, nitric oxide, superoxide, cell injury

INTRODUCTION

The air in close proximity of major roadways is polluted by traffic-related emissions, like particulate matter (PM). Whereas epidemiologic data suggests that exposure to PM results in adverse respiratory health outcomes, especially in individuals with pre-existing lung disorders (Dockery et al. 1993), the particle characteristics and biological mechanisms responsible for these effects are still not fully understood. Increasing evidence suggests that many of these adverse effects are in part mediated by reactive oxygen species (ROS) (Li et al. 2003). However, little is known about the interaction, or cooperation, of ROS production under pre-existing pathophysiological conditions such as inflammatory lung diseases.

In many urban areas, diesel exhaust particles (DEP) account for a significant amount of traffic-related PM (Bedeschi et al. 2007; US EPA 2002). Generated by the incomplete combustion of fossil fuel, DEP are composed of a carbonaceous core particle onto which many organic compounds are adsorbed. Many of these compounds, such as polycyclic aromatic hydrocarbons (PAHs) and quinones, are capable of producing ROS either through direct and/or secondary effects during particle exposure (Liang et al. 2005; Pan et al. 2004). Owing to the extremely small size (< 0.2 μm), DEP are easily respired and deposited within the airway and alveoli lung regions. The production of ROS can result in oxidative stress possibly triggering cell injury.

Furthermore, the deleterious actions of particle exposure may be further aggravated in the presence of inflammatory lung diseases. In states of pulmonary inflammation, such as in asthma and chronic obstructive pulmonary disease (COPD), the epithelium lining the lung is continually exposed to inflammatory mediators resulting in their activation and subsequent

production of secondary mediators, such as the free radical nitric oxide (NO) (Jiang et al. 2009; Redington 2006). Constitutive expression of NO synthases (nNOS and eNOS) by the pulmonary epithelium produces NO via the oxidation of L-arginine to L-citrulline, and has been shown to play a beneficial role in regulating airway tone (Redington 2006). However, within an inflammatory microenvironment, NO production is increased (up to 1000-fold) by the activity of inducible NOS (iNOS) and can contribute to tissue injury, especially when in the presence of ROS (Brindicci et al. 2009; Ichinose et al. 2000; Redington 2006). Specifically, NO can rapidly interact with superoxide anion ($O_2^{\cdot -}$), often produced by environmental pollutants like DEP, to form the potent, more reactive oxidant peroxynitrite ($ONOO^-$) (Bai, Suzuki, and Sagai 2001; Heiss et al. 1994; Ito et al. 2000). Excessive production of $ONOO^-$ can result in altered protein and lipid functions, damage DNA, and cell death (Martinez and Andriantsitohaina 2008; Ricciardolo et al. 2006; Ricciardolo, Nijkamp, and Folkerts 2006; Xiao, Nel, and Loo 2005). The interaction of DEP-induced ROS in individuals with already large amounts of NO, due to inflammatory lung disease, may offer a possible contributing mechanism to DEP-mediated pulmonary toxicity.

Our lab has previously shown that DEP exposure of inflamed epithelial cells induced significantly greater oxidative stress and resulting cytotoxicity that was seemingly related the organic carbon fraction of the insulting particles (Manzo et al. in press). Accordingly, in the studies herein, we postulated that the enhanced cytotoxicity elicited in cytokine-treated epithelial cells exposed to DEP_A was in part the result of the interaction $O_2^{\cdot -}$ and NO. To test this hypothesis, both *in vitro* and *in vivo* approaches were employed. In doing so, we assessed differential effects of untreated and cytokine-treated epithelial cells exposed to DEP

in terms of: the production of ROS, like $O_2^{\cdot -}$, from DEP exposure; the production of NO elicited by cytokine pre-treatment; the resulting $ONOO^-$ mediated cell injury by DEP exposure in the microenvironment of inflammation.

MATERIALS AND METHODS

Cell Culture

LA-4 cells (American Tissue Culture Collection, Manassas, VA), a murine alveolar type II-like epithelial cell line (passages 49 to 55), were grown to 95-100% confluence in Ham's F12K supplemented with 15% fetal bovine serum (FBS), streptomycin (50 μg/mL), penicillin (50 U/mL), and amphotericin B (0.5 μg/mL) in 5% CO_2 at 37°C. Prior to cytokine treatment or DEP exposure, the medium was changed to FBS-free Ham's F12K supplemented with bovine pituitary extract (BPE) (25 μg/mL), insulin (2 μg/mL,), mouse epidermal growth factor (mEGF) (10 ng/mL), and bovine serum albumin (BSA) (0.5 mg/mL) purchased from Sigma (St. Louis, MO).

Cytokine Treatment

LA-4 cell cultures were treated via their medium for 24 hr with exogenous cytokines (cytomix: TNFα + IL-1β + IFNγ, R&D Systems, Minneapolis, MN) each at 0.2 ng/mL to induce the *in vitro* equivalent of a state of low-level, acellular, epithelial inflammation. These concentrations were based on prior concentration-response studies in which cytokine treatment consistently resulted in increased (at least 5-fold) epithelial cell production of neutrophilic (e.g., MIP-2) or eosinophilic (e.g., RANTES) chemokines, without causing cytotoxicity. To selectively inhibit iNOS, LA-4 cells were pre-treated with the chemical

inhibitor 1400W dihydrochloride (100 μM, Sigma, St. Louis, MO) for 24 hr prior to cyomix treatment.

PM Exposure

Particles from a diesel powered automobile, generated in 1999 (DEP_A) (a gift from Dr. Daniel Costa, US EPA, RTP, NC) and carbon black (CB) were used. The total elemental carbon (EC), organic carbon (OC), non-volatile residual fractions, elementals analysis of transition and heavy metals, as well as endotoxin content have been described previously (Manzo et al. in press). In short, where as CB contained little OC (1-2%), the diesel automobile-generated sample (DEP_A) closely resembled the OC (35%) and extractable water-soluble metals to that of a contemporary urban tunnel traffic-related sample PM sample.

Just prior to cell exposure, particle stock suspensions (11 mg/mL) were prepared in sterile phosphate-buffer saline (PBS, Sigma, St. Louis, MO) and sonicated three times for 10 sec on ice using a probe sonicator (Misonix Inc., Farmingdale, NY). Particle treatment of LA-4 cells was achieved via the cell culture medium at 25 $\mu g/cm^2$. Inhibition of $O_2^{\cdot -}$ was achieved by the supplemental addition to the cell culture medium of superoxide dismutase (SOD; 200 U/mL, Sigma, St. Louis, MO), a potent $O_2^{\cdot -}$ catalyzing enzyme.

Cell Injury

Release of lactate dehydrogenase (LDH) was used as a measure of cell injury in LA-4 cells. LDH activity was assayed using a commercially available kit (Thermo Electron Corp., Louisville, CO), modified and adapted for use on the KONELAB 30 clinical chemistry analyzer (Thermo Clinical Labsystems, Espoo, Finland). Cell-free conditioned medium and

cell lysates collected from cells treated with cold PBS containing 0.1% Triton-X 100 (Sigma, St. Louis, MO) for 30 min were used in the assay.

Detection of Reactive Oxygen Species (ROS)

Intracellular ROS production was quantified by measuring 7'-dichlorofluorescin diacetate (H_2DCFDA) (Invitrogen, Carlsbad, CA), a cell-permeable probe that fluoresces upon oxidation by ROS. Prior to DEP_A exposure, LA-4 cells cultured in clear bottom black 96-well plates were washed with Hank's Balanced Salt Solution (HBSS, Invitrogen, Grand Island, NY) and aspirated to remove the growth medium. LA-4 cells were then incubated for 30 min with H_2DCFDA (10 μM in HBSS) after which the cells were washed as above, and exposed to DEP_A. After a 2 hr exposure, the fluorescence was read using a multiwell fluorescence plate reader (Packard FluoroCount BF10000, excitation 485nm, emission 530nm). Alternatively, H_2DCFDA was visualized in LA-4 cells grown and treated as above on chamber slides counterstained with DAPI, and imaged using a Nikon Eclipse Ti fluorescent microscope and Nikon Elements Software.

Detection of Superoxide

Dihydroethidium (DHE) (Invitrogen, Carlsbad, CA) is a cell-permeable $O_2^{\cdot-}$ indicator that once oxidized by $O_2^{\cdot-}$ will fluoresce red and intercalate with nucleic acids. Prior to PM exposure, LA-4 cells cultured in chamber slides were washed with HBSS and aspirated to remove the growth medium. LA-4 cells were then incubated for 30 min with DHE (10 μM in HBSS) after which the cells were washed as above, and exposed to PM. After a 2 hr exposure, the cells were counterstained with DAPI and imaged using a Nikon Eclipse Ti fluorescent microscope and Nikon Elements Software.

Activity of Superoxide Dismutase (SOD)

LA-4 cells were collected with the use of a rubber policeman and centrifuged 2,000 x g for 10 min. The pellet was then sonicated three times for 10 sec on ice using a probe sonicator (Misonix Inc., Farmingdale, NY) in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose and centrifuged (1,500 x g, 5 min, 4˚C). The resulting supernatant was assayed for SOD activity with a commercially available kit (RANSOD, RANDOX Laboratories Ltd, Co. Antrim, United Kingdom), modified and adapted for use on the KONELAB 30 clinical chemistry analyzer (Thermo Clinical Labsystems, Espoo, Finland).

Real-Time RT-PCR

Total RNA was isolated using RNeasy (Qiagen, Valencia, CA). cDNA synthesis and real-time PCR using gene-specific primers were then performed using SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA). Inducible nitric oxide synthase (iNOS) and β-actin primer/probe sets were purchased from Applied Biosystems (Foster City, CA). After determining that β-actin mRNA expression was not altered by our experimental treatments, the level of iNOS mRNA expression was normalized to β-actin using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

iNOS Protein Expression

Whole cell lysates were prepared by lysing the cells in RIPA buffer (Tris 50 mM, Deoxycholic acid 0.5%, Sodium Chloride 150 mM) containing 0.1% Sodium Dodecyl Sulfate (SDS), 1% Triton X-100, and protease inhibitors (Leupeptin, Aprotinin and Sodium Orthovanadate). Protein extracts (10 µg) were separated on E-PAGE 8% gels (Invitrogen,

Carlsbad, CA), under reducing conditions, using an E-BASE electrophoresis device (Invitrogen, Carlsbad, CA). Following electrophoresis, the proteins were electrophoretically transferred to PVDF iBlot Transfer stacks (Invitrogen, Carlsbad, CA) using the iBlot dry blotting system (Invitrogen, Carlsbad, CA). The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween 20. After blocking, the membranes were incubated overnight at 4˚C with specific antibodies to either iNOS (1:500 BD Transduction Laboratories, San Jose, CA) or B-actin (1:5000, Sigma, St. Louis, MO). The membranes were then washed with TBS containing 0.1% Tween 20 and incubated for 1 hr at room temperature with the appropriate secondary antibody in 5% nonfat dry milk in TBS containing 0.1% Tween 20. Bands were visualized using chemiluminescence (LumiGlo, Cell Signaling Technology, Danvers, MA) and acquired using an Alpha Innotech 8900 imaging station (San Leandro, CA) and Fluorchem software (Alpha Innotech, San Leandro, CA).

Detection of Nitric Oxide (NO)

Detection of intracellular NO was accomplished using DAF-FM diacetate (Invitrogen, Carlsbad, CA), which fluoresces upon reaction with NO. LA-4 cells were cultured for 24 hr in clear bottom black 96-well plates and exposed to cytomix for an additional 24 hr, after which the cells were washed with HBSS and aspirated to remove the growth medium. Cytomix treated LA-4 cells were then incubated for 30 min with DAF-FM (10 μM) after which the cells were washed as above, and the fluorescence quantified after 2 hr using a multiwell fluorescence plate reader (Packard FluoroCount BF10000, excitation 485nm, emission 530nm).

Additionally, detection of nitrite, formed by the spontaneous oxidation of NO under physiological conditions, was assessed in the conditioned medium of cytomix treated LA-4 cells by a Griess reagent kit (Invitrogen, Carlsbad, CA). Detection of nitrite was quantitated spectrophotometrically by a Thermomax plate reader outfitted with Softmax Pro v2.6.1 software (Molecular Devices, Menlo Park, CA).

Animals

Pathogen-free BALB/c female mice (Charles River Labs, Wilmington, MA), 12-16 weeks of age (19–23 grams) were housed in groups of five in plastic cages with Alpha Dry bedding, maintained on a 12-hr light/dark cycle at approximately 22˚C and 50% relative humidity in our Association for Assessment and Accreditation of Laboratory Animal Care–approved facility. Food (Prolab RMH 3000; PMI Nutrition International, St Louis, MO) and water were provided *ad libitum*. Animals were acclimated for at least 4 days before the study began. All animal studies were approved by the laboratory's Institutional Animal Care and Welfare Committee.

Oropharyngeal Aspiration of Cytomix

Mice were anesthetized in a Plexiglass box using vaporized isofluorane (Webster Veterinary Supply Inc., Sterling, MA). Once anesthetized, the mice were then suspended vertically by their front incisors on a small wire attached to a support, the tongue was gently extended and 50 μL of either sterile PBS (n = 4) or cytomix (TNFα 1.0 ng/g + IL-1β 0.5 ng/g + IFNγ 2.0 ng/g; n = 4) was gently instilled into the oropharynx. The nares were transiently occluded, thus eliciting a deep respiratory effect which subsequently aspirated the instilled material into the airways.

Mouse Exposure to Diesel Exhaust Particles

Diesel exhaust particles were generated in-house at the US EPA laboratories in Research Triangle Park, NC (Cao et al. 2007). The generation and chemical characteristics of these particles have been described previously (Stevens et al. 2008). In brief, DEP were originally generated using a 30 kW (40 hp, 1220 cm^3) 4-cylinder Deutz BF4M1008 diesel engine connected to a 22.3 kW Saylor-Beall air compressor operated to provide a constant ~20% load. Particles were generated and diluted 3:1 with ambient air and collected in a baghouse (35˚C). Similar to the DEP_A particles used in the *in vitro* studies, the baghouse collected DEP used in these *in vivo* studies consisted of 33% OC.

First, normal healthy mice were exposed for 2 days to a either filtered air (n = 4) or DEP (2.0 mg/m^3, n = 4) for 4 hr in two separate 52-port nose-only flow-by inhalation chambers (Lab Products, Seaford, DE) using a previously described exposure system (Farraj et al. 2006). Particles were carried through a charge neutralizer and 2.5 μm cut-point cyclone to remove particles larger than $PM_{2.5}$ before entering the inlet of the nose-only chamber. Prior to exposure, mice were acclimated to the nose-only exposure tubes. Particle concentrations were determined gravimetrically on teflon filters once per hour during the 4 hr exposure period (45 mm diameter with 1 μm pore size; VWR Scientific,West Chester, PA), and real-time PM concentrations were estimated with an aerosol monitor (Dust Track; TSI, Inc., St Paul, MN) connected to the chamber exhaust tubing. Particle size was determined gravimetrically using a Mercer cascade impactor (Intox Products, Albuquerque, NM). The mean concentration for DEP ranged from 2.06 to 2.18 mg/m^3. The MMAD was 1.03 μm and the GSD was 2.57 μm.

Second, mice were treated via oropharyngeal aspiration to either saline or cytomix, as described above, and exposed 48 hr later to either filtered air (n = 16) or DEP (2.0 mg/m^3) (n = 16) for two days, as described above. Twenty four hours prior to oropharyngeal aspiration a subset of mice received i.p. administration of FeTMPyP (10 mg/kg; n = 4) or 1400W (2 mg/kg; n = 4). Administration of FeTMPyP and 1400W was continued for an additional 4 days until the end of the study.

Bronchoalveolar Lavage Fluid (BALF) Collection and Biochemistry Indices

Mice were euthanized by anesthetic overdose (Euthasol, i.p. 150-200 mg/kg), exsanguinated and the tracheas were canulated. The left lobe was isolated, removed and frozen in liquid nitrogen for determination of glutathione. The accessory and right lung lobes were lavaged with three volumes (35 mL/kg) of warmed HBSS. The pooled BALF was centrifuged (800 x g, 1 min, 22˚C) and 150 μL of the resulting supernatant stored at 4˚C for biochemical analysis. The pelleted cells were resuspended in 1 mL of HBSS. Total cell counts were performed on 500 μL of the cell suspension diluted in 10 mL Isoton using a Z1 Coulter counter (Coulter, Hialeah, FL). Additionally, cell differentials were determined by centrifuging 200 μL of the resuspended cell pellet onto slides at 250 rpm for 10 min using a Cytospin (Shandon, Pittsburgh, PA). Slides were subsequently stained with a modified Wright-Giemsa using an automated slide stainer (Hematek 2000, Miles Inc., Elkhart, IN) and at least 200 cells enumerated per animal. Finally, ROS production of BALF cells was determined in 10,000 cells in 300 μL PBS that were seeded on to clear bottom black 96-well plates treated with H_2DCFDA (10 μM in HBSS) and quantitated as stated above.

Total protein, microalbumin (MIA), and N-Acetyl-B-D-glucosaminidase (NAG) in the BALF were analyzed using kits modified and adapted for the Konelab 30 clinical chemistry analyzer (Thermo Clinical Labsystems, Espoo, Finland). Total protein concentrations were determined with the Coomassie plus protein Reagent (Pierce Chemical, Rockford, IL) with a standard curve prepared with bovine serum albumin from Sigma-Aldrich (St. Louis, MO.). Microalbumin concentrations were determined with the MALB SPQ II kit (Diasorin, Stillwater, MN). NAG activity was determined from a commercially available kit from Roche Diagnostics (Pemzberg, Germany).

Evaluation of Cellular Glutathione

Twenty to 30 mg of frozen (-80˚C) lung tissue was homogenized in 1.0 mL ice cold 4% perchloric acid (PCA) containing 0.2 M boric acid, 4 mM diethylenetriaminepentaacetic acid (DTPA), and 20 μM γ-glutamyl glutamate. Homogenates were centrifuged (20,000 x g, 15 min, 4˚C), and the supernatants were stored at -80˚C. A 300 μL sample of each supernatant was labeled with dansyl chloride as previously described (Jones et al. 1998). Briefly, 60 μL of iodoacetic acid was added to each sample and allowed to stand at room temperature for 20 min to stabilize the GSH. The pH of each sample was adjusted to 9.0 by addition of 1 M KOH in saturated potassium tetraborate. Then, 300 μL of a 20 mg/mL solution of dansyl chloride in acetone was added and allowed to stand for 24 hr in the dark at room temperature. The dansyl chloride was extracted with 500 μL of chloroform, and 90 μL of the aqueous layer was used for HPLC analysis of GSH and GSSG. Samples of GSH/GSSG standard solutions (0.625, 1.25, 2.5, 5.0, 10.0 μM each) in 4% PCA/0.2 M boric acid/4 mM DTPA were also dansylated as above for use as a standard curve.

Gradient HPLC was carried out using a Discovery C_{18} 250 mm x 4.6 mm, 5 μm pore column (Sigma Aldrich, St. Louis, MO) using a previously described and modified method (Gan et al. 2005). The initial solvent was 20% acetonitrile in 0.1% (v/v) formic acid, which ran at a flow rate of 1.0 mL/min for 3 min, followed by a linear increase to 60% acetonitrile in 0.1% formic acid over 22 min, then a return to initial conditions in 2 min. This was maintained for 15 min before the next sample was injected. Dansylated GSH and GSSG were monitored by fluorescence (excitation 335nm, emission 515nm) using an Agilent model 1100 fluorescence detector (Agilent Technologies, Santa Clara, CA). The efficiency of the recovery of GSH and GSSG following extraction and dansylation was monitored using the known concentration of the γ-glutamyl glutamate. Data were collected using ChromPerfect Chromatography Data System software (Justice Laboratory Software, Denville, NJ). The 10.0 μM, 5.0 μM and 2.5 μM portion of the standard curve was used to determine the concentration of GSH in the tissue homogenates, and the 2.5, 1.25, and 0.625 μM portion was used for GSSG.

Statistical Analysis

Data are expressed as means ± SEM. Data were analyzed using an analysis of variance (ANOVA) and where relevant, followed by Bonferroni post-hoc tests for comparison between groups. A value $p < 0.05$ was considered significant.

RESULTS

Effects of DEP_A Exposure in Cytokine-Treated LA-4 Cells

Previously we have shown that in the cellular microenvironment of cytokine-induced inflammation, murine lung LA-4 epithelial cells exposed to organic carbon-rich DEP_A

developed significantly greater cell injury than similarly exposed control epithelial cells (Manzo et al. in press). Herein, we first replicated these findings in saline- and cytokine-treated (cytomix: TNFα+IL-1β+IFNγ @ 0.2 ng/mL for 24 hr) LA-4 cells exposed to DEP_A (25 μg/cm^2, 24 hr) and assessed for cell injury by the release of LDH (Figure 1). Whereas cytokine treatment and DEP_A exposure alone resulted in no increase in LDH release, exposure of cytokine-treated LA-4 cells to DEP_A resulted in significantly greater cell injury.

Effects of PM Exposure on ROS Production in LA-4 Cells

To address whether DEP_A exposure was capable of increasing ROS formation in LA-4 cells, we assessed H_2DCFDA oxidation 2 hr after DEP_A exposure (25 μg/cm^2). It has been shown that H_2DCFDA is a useful probe in detecting a wide variety of ROS. Results show that H_2DCFDA oxidation was significantly increased (> 2-fold) in DEP_A exposed cells, whereas saline- and CB-treated cells did not exhibit this response (Figure 2). Furthermore, pre-treatment of LA-4 cells with superoxide dismutase (SOD), which dismutes superoxide anion ($O_2^{\cdot -}$), prior to DEP_A exposure, significantly reduced the amount of ROS production, although it still remained significantly elevated above control and CB treated LA-4 cells.

Next, to more specifically determine which ROS were increased during DEP_A exposure of LA-4 cells, we used the $O_2^{\cdot -}$ specific probe DHE. In the presence of $O_2^{\cdot -}$, DHE becomes oxidized to ethidium, intercalates with nucleic acids, thus emitting red fluorescence. As shown in Figure 3, the DHE probe was added to LA-4 cells prior to being exposed to DEP_A and visualized on a fluorescent microscope. Whereas normal LA-4 cells exhibited little cytoplasmic red fluorescence and no nuclear staining, DEP_A exposed cells had increased overall red fluorescence, and focal nuclear staining.

To further elucidate the source and mechanism of the resulting DEP_A induced ROS, we next evaluated the mRNA expression of the $O_2^{\cdot -}$ generating enzyme xanthine dehydrogenase (XdH) as well as the activity of mitochondrial (Mn-SOD) and cytosolic (CuZn-SOD) SOD. Figure 4 shows that DEP_A exposure (25 μg/cm^2, 24 hr), but not CB exposure, of LA-4 resulted in almost a 2.5-fold increase in XdH, thus further implicating DEP_A in the production of $O_2^{\cdot -}$. As well, DEP_A exposure of LA-4 cells resulted in significantly decreased activity of CuZn-SOD (Control: 0.52 ± 0.09 U/mL vs. DEP_A: 0.24 ± 0.04 U/mL, p = 0.0467), whereas Mn-SOD was unaffected (Control: 0.27 ± 0.03 U/mL vs. DEP_A: 0.28 ± 0.05 U/mL, p = 0.7489).

Effects of Cytomix Treatment on iNOS Expression and Nitric Oxide (NO) Production

To determine the involvement of NO in the cell injury induced by exposure of cells to DEP_A, we assessed the ability of cytomix treatment of LA-4 cells to alter NO production, by assessing for changes in expression of iNOS mRNA and protein levels, as well as NO accumulation using both the NO-specific fluorescence probe DAF-FM diacetate and nitrite production via a griess test.

Twenty four hours after cytomix treatment, LA-4 cells had significantly increased iNOS mRNA expression (Figure 5a) and intracellular protein levels (Figure 5b) as compared to saline-treated control cells. In addition, fluorescence of the NO specific probe DAF-FM diacetate was significantly increased in cytoline-treated LA-4 cells, which returned to control levels when treated with 1400W, a iNOS specific inhibitor (Figure 5c). Comparable effects were also noted using the griess reaction which detects nitrites formed by the spontaneous oxidation of NO (Figure 5d).

ROS Production in Cytokine-Treated LA-4 Cells Exposed to DEP_A

It is known that under conditions of both $O_2^{\cdot -}$ and NO production, these oxidants can react to form the potent, and more reactive, oxidant peroxynitrite ($ONOO^-$). Because the fluorescent probe H_2DCFDA is capable of detecting a broad range of ROS, including $ONOO^-$, we again used this probe to determine if cytokine-treated LA-4 cells exposed to DEP_A would have greater ROS production as compared to saline-treated controls. Following a 24 hr cytomix treatment, LA-4 cells were exposed to DEP_A for an additional 24 hr. ROS production was monitored using H_2DCFDA, both visually on a fluorescent microscope and quantitatively using a multiwall fluorometer. Visually, whereas DEP_A exposure and cytokine-treatment alone of LA-4 cells resulted in minimal fluorescence, cytokine-treated cells exposed to DEP_A had brighter fluorescence (Figure 6a). Furthermore, quantitation of the relative fluorescence revealed that cells exposed to DEP_A had twice the detectable fluorescence of saline-treated cells, whereas DEP_A-exposed cytokine-treated cells had 4-fold greater fluorescence. Cytokine-treatment alone was without effect on ROS production (Figure 6b).

To more specifically implicate the involvement of NO and $O_2^{\cdot -}$ with the increased ROS levels of cytokine-treated cells exposed to DEP_A, we assessed changes in H_2DCFDA oxidation in the presence of SOD and 1400W. In contrast to the robust increase in ROS produced in cytokine-treated cells exposed to DEP_A, supplemental treatment with SOD or 1400W resulted in significantly less ROS production (Figure 6c).

Effects of $ONOO^-$ Scavenging on DEP_A-Induced Cell Injury

To further elucidate if $ONOO^-$ plays a contributing role in the enhanced cell injury observed in cytokine-treated LA-4 cells exposed to DEP_A, experiments were conducted using FeTMPyP, which catalyzes the decomposition of $ONOO^-$. As determined by the release of LDH, cytokine-treated LA-4 cells exposed to DEP_A had significantly greater cell injury compared to saline-treated control cells. However, pre-treatment with FeTMPyP significantly abolished LDH release to near control levels (Figure 7).

Effects of DEP Exposure in Mice

To extend our findings beyond our *in vitro* model, we next created a comparable *in vivo* model in which mice with (and without) cytokine-induced pulmonary inflammation were exposed to DEP. First, to induce a state of low-level pulmonary inflammation, mice were exposed via oropharyngeal aspiration to the same three cytokines, but the relative amounts varied, (cytomix: TNFα 1.0 ng/g + IL-1β 0.5 ng/g + IFNγ 2.0 ng/g). Changes in bronchoalveolar lavage fluid (BALF) 48 hr after cytomix administration was used to assess lung inflammation. Data revealed a significant increase in total cells, consisting largely of neutrophils and lymphocytes (Table 1). Analysis of BALF biochemical indices suggest that a mild non-injurious degree of pulmonary edema was also elicited by cytomix treatment, evident by increases in total protein and microalbumin without any appreciable change in LDH release (Table 1). Together, the results suggest that oropharyngeal aspiration of these inflammatory cytokines (cytomix) results in significant, but minimally injurious, pulmonary inflammation.

Second, the pulmonary effects of nose-only DEP inhalation (2 mg/m^3, x 4 hr/day, 2 days) were assessed in healthy mice. Cellular analysis of BALF after DEP inhalation revealed that while there was no significant increase in total cells, there was a small, but significant, increase in absolute numbers of neutrophils and lymphocytes, although these cell types only consisted of 1-2% of the total cells present (Table 2). Based on biochemical indices in the BALF (LDH, total protein, microalbumin), DEP inhalation did not result in significant pulmonary injury or edema (Table 2). In addition, since DEP inhalation can cause oxidative stress, changes in the relative amount of lung tissue reduced (GSH) and disulfide (GSSG) forms of the oxidant scavenging glutathione were also evaluated. Of note, after DEP inhalation, healthy mice resulted in significant increases in GSH, with only minor, but insignificant increases in GSSG suggestive of an effective antioxidant response (Figure 8).

Finally, 48 hr after saline or cytomix treatment, BALB/c mice were exposed to air or DEP (2 mg/m^3, x 4 hr/day, 2 days), with or without i.p. administration of 1400W or FeTMPyP, and assessed for cellular and biochemical changes in the BALF, ROS production from recovered BALF cells, and total lung tissue glutathione. Analysis of BALF indices of cellularity (total cells and differential) as well as biochemical indicators of cell injury (LDH) and edema (total protein and microalbumin) showed that by 48 hr after the last DEP inhalation, there were no significant changes in any of these indices (Table 3). However, when BALF cells were assessed for ROS production using H_2DCFDA, only the cytokine pre-treated mice exposed to DEP showed significant increases in fluorescence (Figure 9). Interestingly, when these mice were treated with FeTMPyP, the $ONOO^-$ scavenger, the

increase in ROS production was significantly inhibited (Figure 9), and there was a concomitant increase in lung tissue GSH (Figure 10).

DISCUSSION

Epidemiological data indicates that exposure to PM is associated with a range of health effects, especially in individuals with pre-existing respiratory diseases (Halonen et al. 2009; Holguin 2008; Kim et al. 2008; Sint, Donohue, and Ghio 2008). Although still largely unknown, understanding the particle characteristics and biological mechanism(s) responsible for these effects may help to explain these population based studies. Whereas there is increasing evidence implicating the role of PM induced ROS in healthy cells, there are few investigations into the consequence of PM induced ROS production in the setting pre-existing pulmonary inflammation. To further understand the mechanisms that render individuals with pre-existing inflammatory lung disorders, like asthma and COPD, at increased risk of PM exposure, the current study investigated the cooperation of $O_2^{\cdot -}$ and NO in DEP-induced injury. Using a murine lung epithelial cell line, LA-4, we report that the production of ROS, including $O_2^{\cdot -}$, was significantly increased following exposure to DEP_A, an organic carbon rich DEP, but not by exposure to an equivalent mass of carbon black. We further demonstrated that cytokine-treated LA-4 cells produced increased levels of NO. Importantly, when these cytokine-treated cells were exposed to DEP_A, there was a significant increase in the level of ROS generated, with a commensurate degree of cytotoxicity, in part mediated by the potent oxidant $ONOO^-$. Furthermore, inhalation of DEP in mice similarly pre-treated with cytomix, had evidence of increased ROS production in BALF cells, which also appeared to be mediated by $ONOO^-$.

The lung epithelium is continually exposed to PM and in part serves as a physical barrier, preventing particles, like DEP, from penetrating the underlying lung tissue. Both *in vitro* and *in vivo* experimental evidence has showed that exposure to DEP induces the production of ROS, a contributing factor to DEP-induced lung injury and cellular responses (Baulig et al. 2003; Pandya et al. 2002). The present data shows that LA-4 epithelial cells exposed to DEP results in increased ROS production in part due to $O_2^{\cdot -}$. Using the fluorescent probe H_2DCFDA, we show that DEP exposure results in a greater than 2-fold increase in ROS. Furthermore, using specific inhibitors and fluorescent probes, the resulting ROS production is in part the result of increased production of $O_2^{\cdot -}$. Interestingly, we noted that DEP exposure of normal cells resulted in reduced activity of cytosolic SOD, possibly contributing to the $O_2^{\cdot -}$ burden. Similar decrements in SOD have been noted in mice intratrachealy exposed to DEP, which could be rescued by the exogenous administration of SOD (Kumagai, Taira, and Sagai 1995; Sagai et al. 1993; Sagai, Furuyama, and Ichinose 1996). In accordance with the data presented above, similar DEP *in vitro* exposures of both bronchial and alveolar epithelial cells resulted in significant ROS production, as measured by H_2DCFDA, which was effectively inhibited with the nonspecific antioxidant NAC (Amara et al. 2007; Baulig et al. 2003). In addition, DEP exposure of both bronchial and alveolar epithelial cells has been shown to produce $O_2^{\cdot -}$ (Li et al. 2002; Sugimoto et al. 2005). Production of DEP-induced ROS can arise either directly from the particles or through its interaction with the targeted cell. Although DEP is composed of an elemental carbon core, differences in engine operation and fuel/oil types can result in varying types and amounts of organic carbon compounds becoming adsorbed onto the surface of the carbon core. Impor-

tantly, many of the adsorbed organic compounds are classified as air toxics by the US EPA and are capable of redox cycling to produce ROS. Specifically, adsorbed to the surface of the particles there can exist polycyclic aromatic hydrocarbons (PAH) and quinones (Liang et al. 2005; Pan et al. 2004) which can result in direct production of ROS via extracellular redox cycling. Alternatively, intracellular ROS can be produced after DEP exposure through a variety of sources such as membrane bound enzymes, such as NADPH oxidases (NOX) (Amara et al. 2007; Mo et al. 2009), metabolic activation of compounds adsorbed to the particle surface via pulmonary P_{450} monooxygenase systems, and mitochondria dysfunction (Zhao et al. 2006; Zhao et al. 2009). Although we did not specifically investigate the involvement of the mitochriondrial dysfunction, or NOX and P450 enzymes in our DEP induced ROS production, our data did show that expression of xanthine dehydrogenase (XdH) was significantly elevated after DEP exposure. Normally involved in the metabolism of purines, XdH can be converted to xanthine oxidase (XO) and lead to additional $O_2^{\cdot -}$ production. Other *in vitro* studies have also shown the expression and activity of XO to be significantly increased in pulmonary endothelial cells upon exposure to cigarette smoke condensates (Kayyali et al. 2003). An *in vivo* model investigating the cooperation of endotoxin and DEP exposure in enhanced pulmonary toxicity showed XO as a major producer of ROS (Arimoto et al. 2005). While there is increasing evidence that suggests that many of the DEP-induced adverse effects are at least in part mediated by ROS, little is known about the interaction of ROS with other free radicals present in states of inflammation.

Asthma and COPD represent two diseases in which many of the clinical manifestations are orchestrated by, or the result of, pulmonary inflammation. This inflammatory state is maintained by a complex network of cells through the continual release of inflammatory cytokines, such as TNFα (Howarth et al. 2005), IL-1β (Bjornsdottir and Cypcar 1999), and IFNγ (Boniface et al. 2003). As well as actively participating in the inflammatory process, the pulmonary epithelium is continually exposed to these mediators and activated to release additional potent mediators, like NO. In our studies, murine LA-4 epithelial cells were treated with exogenous cytokines (cytomix: TNFα + IL-1β + IFNγ) found in the BALF of asthmatics and patients with COPD, to induced an inflammatory microenvironment. As a result, LA-4 cells showed the concomitant increase of both iNOS mRNA and protein as well as the production of NO. Whereas we did not investigate the contribution of other NOS isoforms, we expect that the iNOS accounts for the majority of increased NO since treatment with 1400W, an iNOS specific inhibitor, was able to prevent NO production from cytokine-treated LA-4 cells. Supporting evidence of increased NO production from iNOS has been shown in patients afflicted with asthma and COPD, as well as both animal and *in vitro* models (Brindicci et al. 2009; Ichinose et al. 2000; Robbins, Springall et al. 1994). In healthy subjects, NO is present in exhaled breath in low concentrations due to constituently expressed NOS isoforms (nNOS, eNOS) (Redington 2006; Rodway et al. 2009). However, several studies looking at asthmatics, and to lesser extent patients with COPD, have shown that the exhaled NO is significantly increased (Beg et al. 2009; Rodway et al. 2009). Furthermore, *in situ* hybridization and immunohistochemistry of human airways and deep lung from asthmatics and patients with

COPD have shown iNOS to be present and localized to the epithelium (Redington et al. 2001; Ricciardolo et al. 2005). *In vitro* studies of both primary and transformed pulmonary epithelial cells (human and murine) have shown that iNOS expression is regulated by exposure pro-inflammatory cytokines. Specifically, treatment of A549, BEAS-2B, and LA-4 cells with a similar cytokine profile (TNFα + IL-1β + IFNγ) was shown to increase NO production that was the result of transcriptional activation of iNOS mRNA and resulting iNOS protein (Asano et al. 1994; Hamid et al. 1993; Robbins, Barnes et al. 1994; Watkins et al. 1997). Whereas low concentrations of NO, produced by constitutively expressed NOS isoforms, air important in regulating airway tone, high concentrations of NO, the result of iNOS activation, can react with other free radicals and result in further inflammation (acellular mediator release and cell infiltration), vascular permeability resulting in edema, and cytotoxicity (Ho et al. 2002; Que et al. 2009; Tadie et al. 2008).

The cooperative action of $O_2^{\cdot -}$ and NO to form reactive nitrogen species (RNS) is important in the innate immune response to invading microorganisms. However, in the lung, improper production of these free radicals by disease or environmental pollutants can result in adverse effects. When produced simultaneously and in excessive quantities, NO and $O_2^{\cdot -}$ rapidly react to form the more potent oxidant $ONOO^-$ that can result in cytotoxicity (Castillo et al. 2007; Martinez and Andriantsitohaina 2008; Redington 2006; Ricciardolo et al. 2006). Common targets of RNS include modification of lipids, nucleic acids, and proteins such as proteins involved in antioxidant defense (Martinez and Andriantsitohaina 2008). The current study demonstrates that exposure of cytokine-treated lung epithelial cells to a normally benign concentration of DEP, resulted in enhanced ROS production and subsequent

cytotoxicity that was seemingly the result of $ONOO^-$, via the interaction of $O_2^{\cdot -}$ and NO. Control cells exposed to DEP resulted in a 2-fold increase in ROS, as measured by H_2DCFDA fluorescence, without any change in cell injury. Consequently, when maintained in an inflammatory microenvironment, DEP-exposed LA-4 cells had greater ROS production (4-fold), with subsequent cell injury (2.5-fold). Whereas the increased ROS production of cytokine-treated cells exposed to DEP could be significantly reduced by the addition of SOD or inhibition of iNOS, the resulting cell injury was only abrogated by treatment with FeTMPyP, an $ONOO^-$ scavenger. Furthermore, we demonstrated that DEP exposure of mice with cytokine-induced pulmonary inflammation had greater ROS production in BALF cells that was inhibited by FeTMPyP.

In summary, the present study demonstrates that pulmonary epithelial cells exposed to DEP results in the production of ROS, including $O_2^{\cdot -}$, which presumably through efficient antioxidant defenses does not result in cell injury. However, pulmonary epithelial cells established in an inflammatory microenvironment, increased NO production which cooperated with DEP-induced ROS resulting in $ONOO^-$ production and overt cell injury. Moreover, production of $ONOO^-$ in the BALF cells of the analogously cytokine-treated and DEP exposed mice suggest that such interactions may occur in multiple lung cell types.

Together the data show that cytokine-treated epithelial cells are more susceptible to the damaging effects of DEP exposure through the cooperative effects of NO and $O_2^{\cdot -}$, and thus may represent, a contributing mechanism in the adverse health effects elicited by DEP in individuals with chronic inflammatory lung diseases. These results provide a possible mechanism that may further explain the adverse associations observed in numerous

epidemiological studies between individuals with pre-existing inflammatory lung disorders and PM exposure. Furthermore, whereas there exists several studies that have investigated the role of DEP induced NO and $O_2^{\cdot -}$ in healthy cell cultures and animal models, to our knowledge the present study is unique in its investigation of the involvement of $O_2^{\cdot -}$ and NO using both an *in vitro* and *in vivo* studies that model a pre-existing diseased setting.

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Table 1. Cellular and biochemical profile in BALF of cytomix-treated mice.
Mice were treated with 50 μL of either saline or cytomix (TNFα 1.0 ng/g + IL-1β 0.5 ng/g + IFNγ 2.0 ng/g) via oropharyngeal aspiration. Total cell counts, differential cell counts, and biochemical indices of injury were determined on BALF 48 hr post-cytomix instillation. Results are presented as mean (± SEM). Asterisk (*) indicates significantly different from saline-exposed mice.

	Saline	Cytomix
BALF Counts ($x10^3$)		
Total Cells	88.7 ± 4.8	263.9 ± 25.5*
Macrophages	72.5 ± 9.5	100.9 ± 7.1
Neutrophils	14.7 ± 6.7	119.4 ± 13.1*
Lymphocytes	1.4 ± 0.7	57.1 ±16.7*
BALF Biochemistries		
LDH (U/mL)	37.5 ± 9.1	55.3 ± 13.7
Total Protein (μg/mL)	67.6 ± 5.2	126.6 ± 21.4
Microalbumin (μg/mL)	15.2 ± 1.9	24.6 ± 2.5*

Table 2. Cellular and biochemical profile in BALF of DEP-exposed mice.
Mice were exposed to either air or DEP (2 mg/m^3) 4 hr/day x 2 days via nose only inhalation. Total cell counts, differential cell counts, and biochemical indices of injury were determined on BALF. Results are presented as mean (± SEM). Asterisk (*) indicates significantly different from air-exposed mice.

	Air	DEP
BALF Cell Counts (x10^3)		
Total Cells	85.3 ± 7.4	110.6 ± 12.3
Macrophages	84.9 ± 7.3	107.5 ± 11.1
Neutrophils	0.1 ± 0.1	1.4 ± 0.40*
Lymphocytes	0.2 ± 0.1	1.7 ± 0.7*
BALF Biochemistries		
LDH (U/mL)	18.78 ± 3.1	12.6 ± 1.8
Total Protein (μg/mL)	62.9 ± 5.6	49.6 ± 11.1
Microalbumin (μg/mL)	15.1 ± 0.9	10.9 ± 3.8

Table 3. Cellular and biochemical profile in BALF of saline- or cytomix-treated mice exposed to DEP.

Fourty eight hours after oropharyngeal aspiration of 50 μL saline or cytomix (TNFα 1.0 ng/g + IL-1β 0.5 ng/g + IFNγ 2.0 ng/g), with or without daily i.p. treatment of 1400W (2.0 mg/kg), a iNOS inhibitor, or FeTMPyP (10 mg/kg), a peroxynitrite scavenger, mice were exposed to air or DEP (2 mg/m^3) 4 hr/day x 2 days via nose only inhalation. Total cell counts, differential cell counts, and biochemical indices of injury were determined on BALF. Results are presented as mean (± SEM).

Asterisk (*) indicates significantly different from air-exposed mice.

	Air				DEP			
	Saline	Cytomix	1400W: Cytomix	FeTMPyP: Cytomix	Saline	Cytomix	1400W: Cytomix	FeTMPyP: Cytomix
BALF Cell Counts (x10^3)								
Total Cells	109.8 ± 52.43	96.4 ± 22.2	82.9 ± 21.6	109.2 ± 10.7	84.8 ± 20.7	106.7 ± 35.2	130.4 ± 11.9	84.3 ± 10.2
Macrophages	100.7 ± 44.1	89.3 ± 20.0	78.2 ± 19.9	100.8 ± 10.5	83.2 ± 21.21	96.43 ± 32.7	112.5 ± 12.1	78.0 ± 9.1
Neutrophils	8.2 ± 8.1	4.1 ± 1.8	3.1 ± 1.2	5.5 ± 1.5	0.4 ± 0.1	7.5 ± 2.6	11.0 ± 3.6	4.7 ± 0.8
Lymphocytes	0.9 ± 0.6	3.0 ± 0.7	1.6 ± 0.4	3.0 ± 1.2	1.2 ± 0.5	2.8 ± 0.7	4.4 ± 0.3	1.6 ± 0.7
BALF Biochemistries								
LDH (U/mL)	37.0 ± 9.8	38.8 ± 8.6	38.9 ± 3.5	38.1 ± 1.6	23.5 ± 1.3	43.7 ± 6.7	37.9 ± 2.5	36.4 ± 2.6
Total Protein (μg/mL)	66.8 ± 17.8	82.0 ± 8.4	68.9 ± 8.5	74.2 ± 1.7	68.1 ± 2.4	88.1 ± 16.9	76..1 ± 1.0	75.9 ± 2.3
Microalbumin (μg/mL)	15.9 ± 3.1	16.6 ± .05	15.9 ± 1.4	14.1 ± .08	16.9 ± 0.4	14.7 ± 1.7	15.9 ± 0.4	14.7 ± 0.6

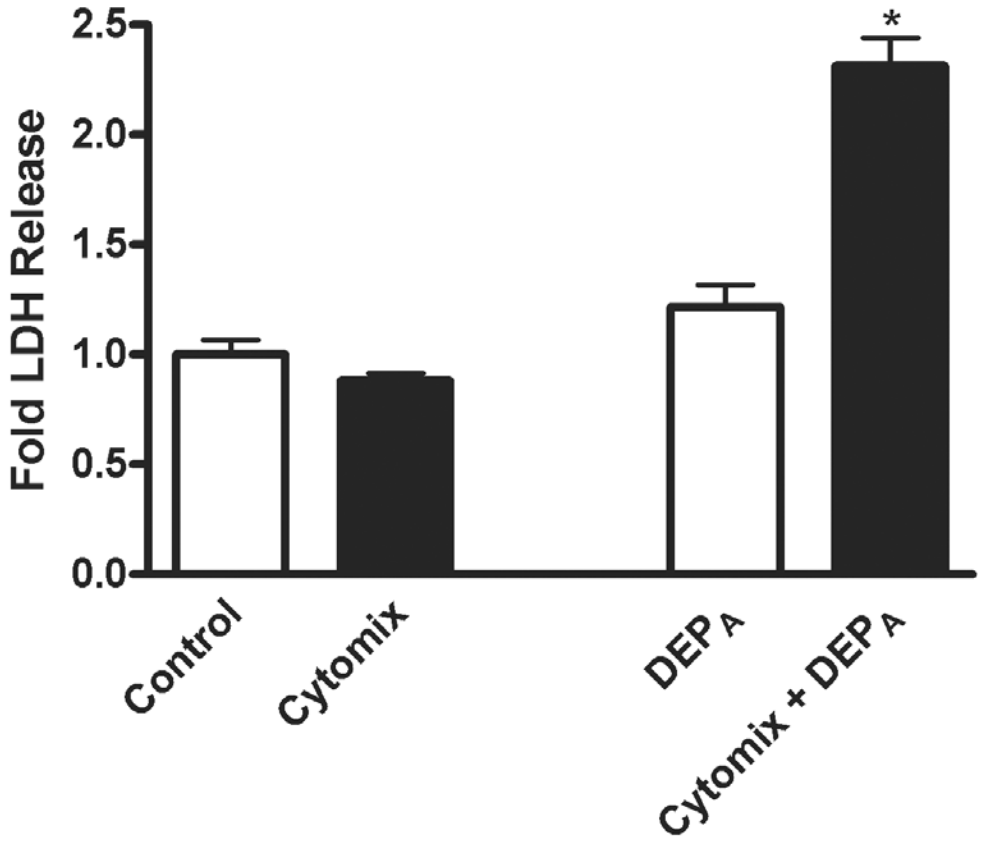


Figure 1. Effects of DEP_A exposure in cytokine-treated cells. Cell injury in LA-4 epithelial cells pre-treated with saline (control) or cytomix for 24 hr and subsequently exposed to diesel exhaust particles (DEP_A) at 25 μg/cm^2 for 24 hr. Conditioned medium and cell lysates were analyzed for LDH released. Data are expressed as mean fold increase (± SEM) of LDH release (normalized to total LDH) over saline-exposed control cells. Significantly ($p < 0.05$) greater injury is indicated by: * vs. All other treatments.

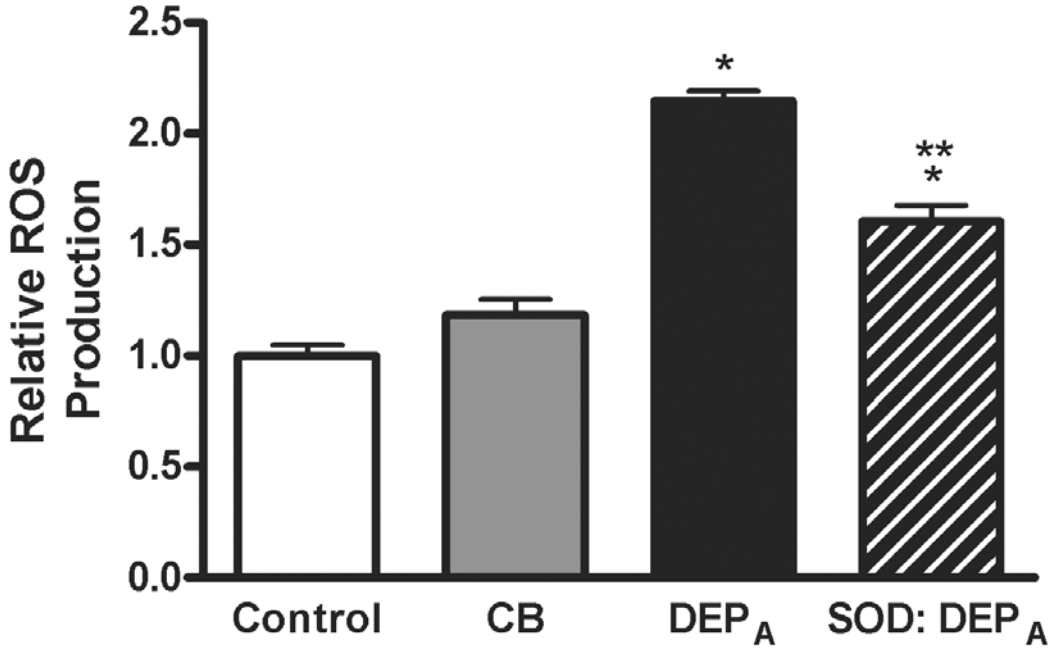


Figure 2. Effects of PM exposure on ROS production. LA-4 epithelial cells were exposed to saline (control), carbon black (CB), or diesel exhaust particles (DEP_A 25 μg/cm^2), with or without SOD (200 U/L), in the presence of H_2DCFDA. Oxidation of H_2DCFDA was measured fluorometrically after 2 hr. Data are expressed as mean fold increase (± SEM) over saline-exposed control cells. Significance ($p < 0.05$) indicated by: * vs. Control and CB; ** vs. DEP_A.

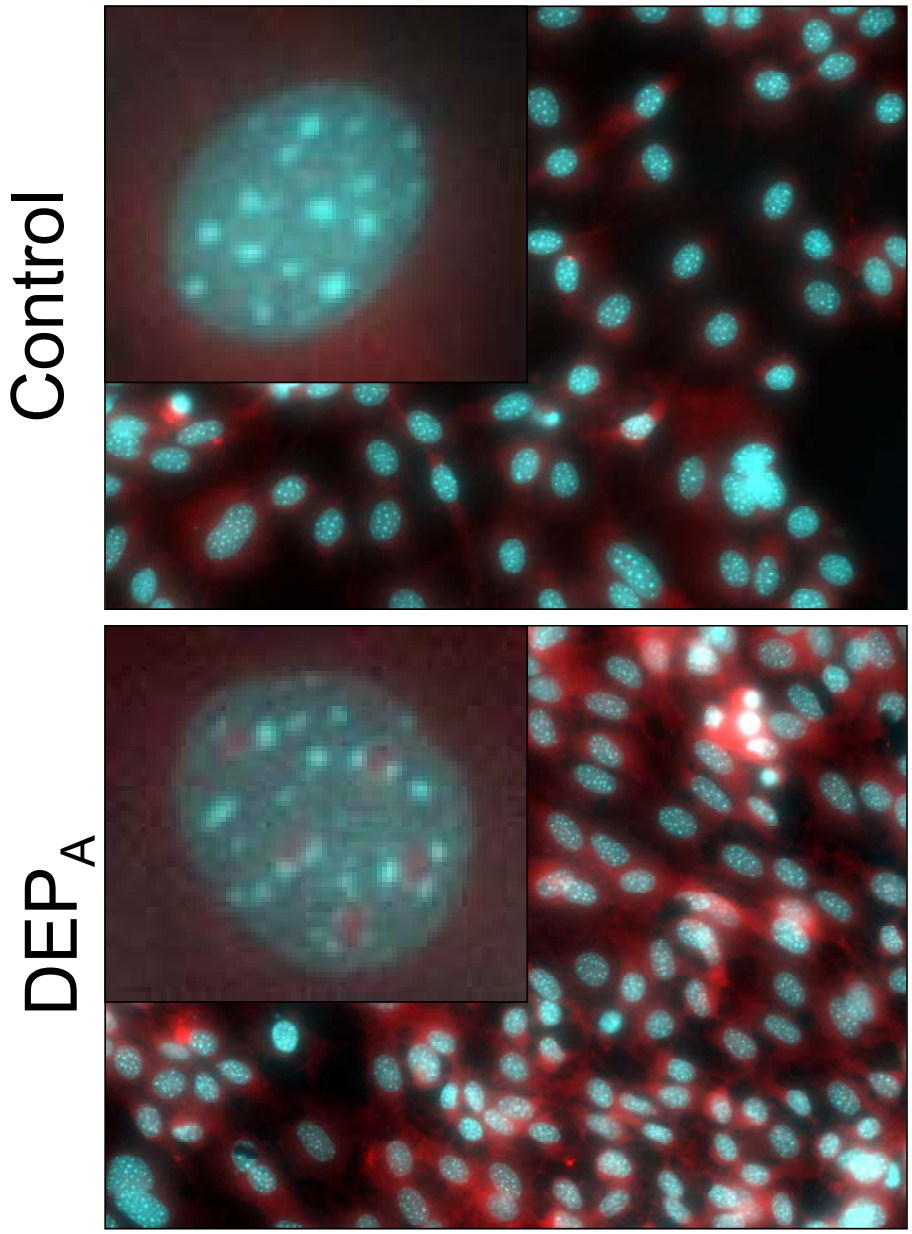


Figure 3. $O_2^{\cdot -}$ production from DEP_A exposed cells. LA-4 epithelial cells were grown on chamber slides and exposed to saline (control) or diesel exhaust particles (DEP_A) at 25 $\mu g/cm^2$ for 2 hr in the presence of DHE. Cells were counterstained with DAPI and visualized using a fluorescent microscope.

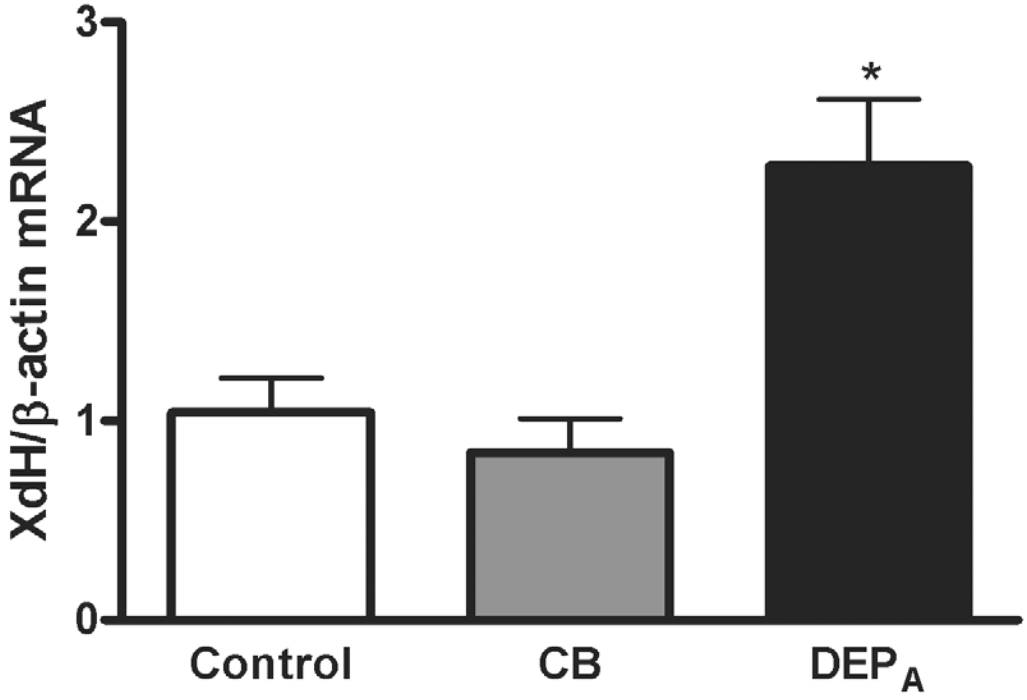
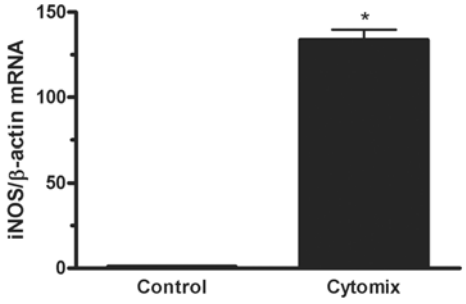


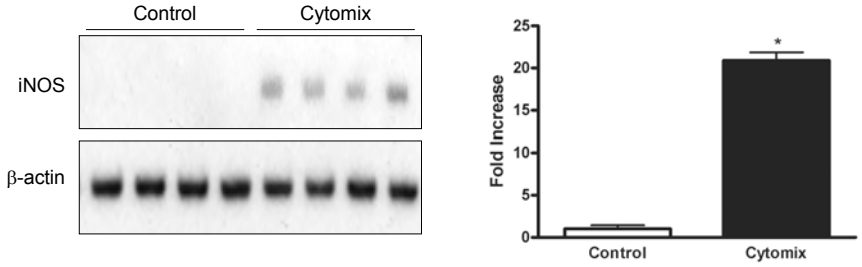
Figure 4. XdH expression in DEP_A exposed cells. LA-4 epithelial cells exposed to saline (control), carbon black (CB), or diesel exhaust particles (DEP_A) at 25 μg/cm^2 for 24 hr. Cells were harvested for mRNA, and XdH expression was quantified by real-time RT-PCR, with normalization to β-actin expression. Data are expressed as the mean (± SEM) fold increase over saline-exposed control cells. Significance ($p < 0.05$) is indicated by: * vs. All Treatment.

Figure 5. iNOS expression and NO production in cytokine-treated cells. LA-4 cells were treated for 24 hr with either saline (control) or cytomix and the resulting mRNA (A) and protein (B) expression of iNOS, as well the production of NO by DAF-FM diacetate oxidation (C) and nitrite release (D) was evaluated. (A) 10-20 μg of protein extracts separated on 8% E-PAGE gels, electrophorectially transferred to PVDF and blotted for iNOS and β-actin. The graph below indicates the fold change in densitometry values, per control of iNOS protein (normalized to β-actin). (B) Cells were harvested for mRNA, and iNOS expression was quantified by real-time RT-PCR, with normalization to β-actin expression. Data are expressed as the mean (± SEM) fold increase over saline-exposed cells. (C) Intracellular oxidation of DAF-FM diacetate by NO was measured fluorometrically after 24 hr of cytomix exposure in the presence of 1400W (100 μM). Data are expressed as mean fold increase (± SEM) over saline-exposed control cells. (D) Nitrite levels from spontaneous oxidation of NO were evaluated in condition cell culture medium after 24 hr of cytomix treatment in LA-4 cells, with 1400W (100 μM). Data are expressed as the mean (± SEM) μM amounts of nitrite. Significance ($p < 0.05$) is indicated by: * vs. Control; ** vs. Cytomix.

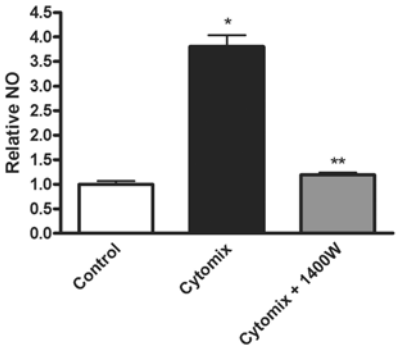
A)


iNOS/β-actin mRNA
150
100
50
0
*
Control
Cytomix

B)


Control
Cytomix
iNOS
β-actin
Fold Increase
25
20
15
10
5
0
*
Control
Cytomix

C)


Relative NO
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Control
Cytomix
Cytomix + 1400W

D)

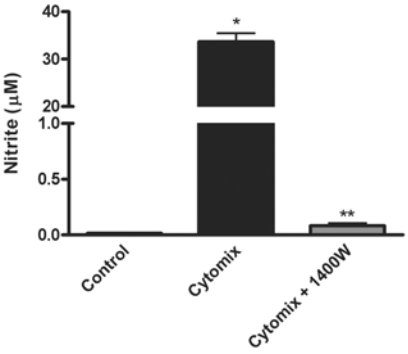
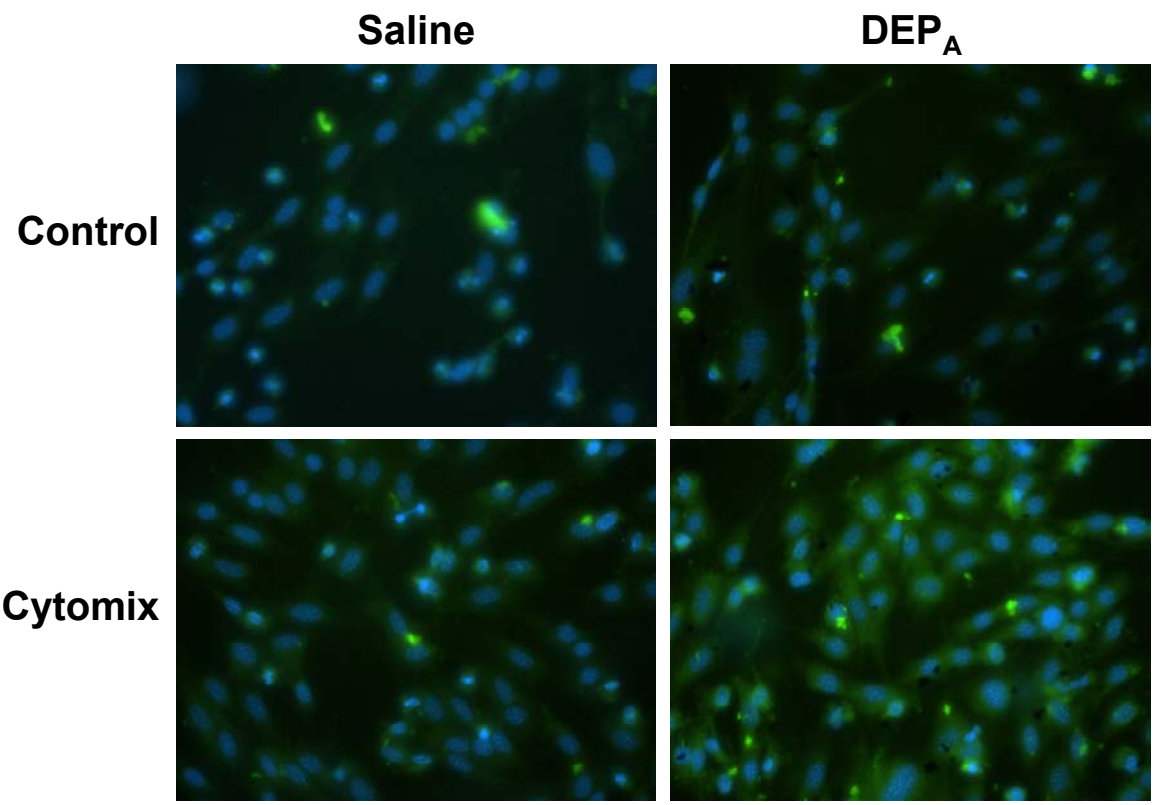
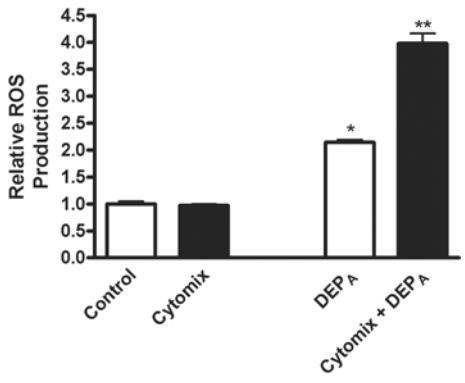

Nitrite (μM)
40
30
20
1.0
0.5
0.0
*
**
Control
Cytomix
Cytomix + 1400W

Figure 6. ROS production in cytokine-treated LA-4 cells exposed to DEP_A. Control or cytomix-treated LA-4 epithelial cells were exposed to saline or diesel exhaust particles (DEP_A) at 25 μg/cm^2 in the presence of H_2DCFDA and the oxidation of H_2DCFDA was visualized on a fluorescent microscope (A) and measured fluorometrically (B) after 2 hr. Data are expressed as mean fold increase (± SEM) over saline-exposed control cells. (C) Cytomix-treated LA-4 epithelial cells were exposed to saline (control) or diesel exhaust particles (DEP_A) at 25 μg/cm^2 in the presence of SOD (200 U/L) or 1400W (100 μM), in the presence of H_2DCFDA and the oxidation of H_2DCFDA was measured fluorometrically after 2 hr. Data are expressed as mean fold increase (± SEM) over saline-exposed control cells. Significance ($p < 0.05$) indicated by: * vs. Control; ** vs. All other treatments; † vs. Cytomix + DEP_A.

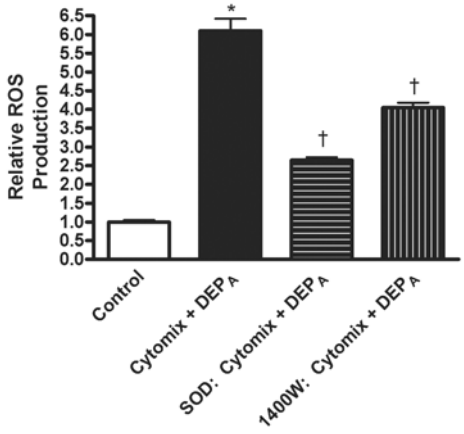
A)


Saline
DEP A
Control
Cytomix

B)


Relative ROS Production
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Control
Cytomix
DEP A
Cytomix + DEP A

C)


Relative ROS Production
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
†
†
Control
Cytomix + DEP A
SOD: Cytomix + DEP A
1400W: Cytomix + DEP A

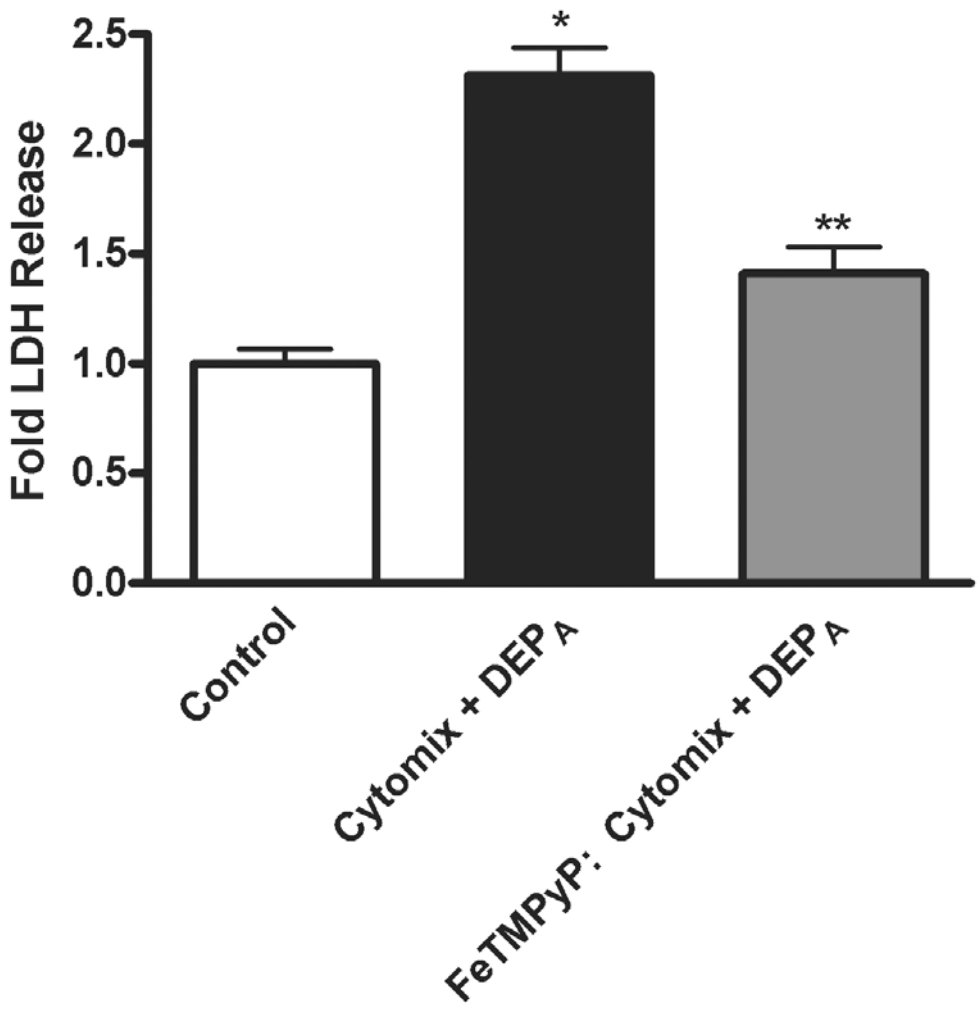


Figure 7. Effects of $ONOO^-$ scavenging on DEP_A-induced cell injury. Control or cytomix-treated LA-4 cells were exposed to saline or diesel exhaust particles (DEP_A) at 25 μg/cm^2 for 24 hr in the presence of FeTMPyP (10 μM). Conditioned medium and cell lysates were analyzed for LDH released. Data are expressed as mean fold increase (± SEM) of LDH release (normalized to total LDH) over saline-exposed control cells. Significantly ($p < 0.05$) greater injury is indicated by: * vs. Control; ** vs. Cytomix + DEP_A.

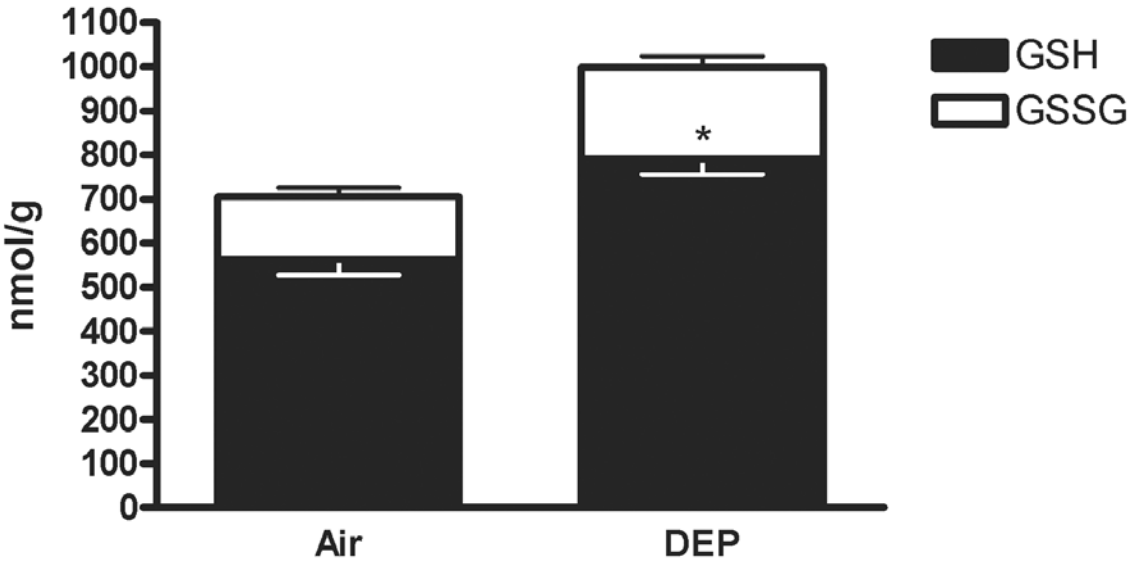


Figure 8. Changes in intracellular glutathione in DEP exposed mice. Female BALB/c mice were exposed to DEP (2 mg/m^3, 4 hr/day, 2 days) via the nose. Lung homegantes were analyzed for reduced (GSH) and disulfide (GSSG) forms of glutathione by HPLC. Data are expressed as the mean nmol/µg of protein (± SEM) of GSH or GSSG. Significance ($p < 0.05$) indicated by: * vs. Air.

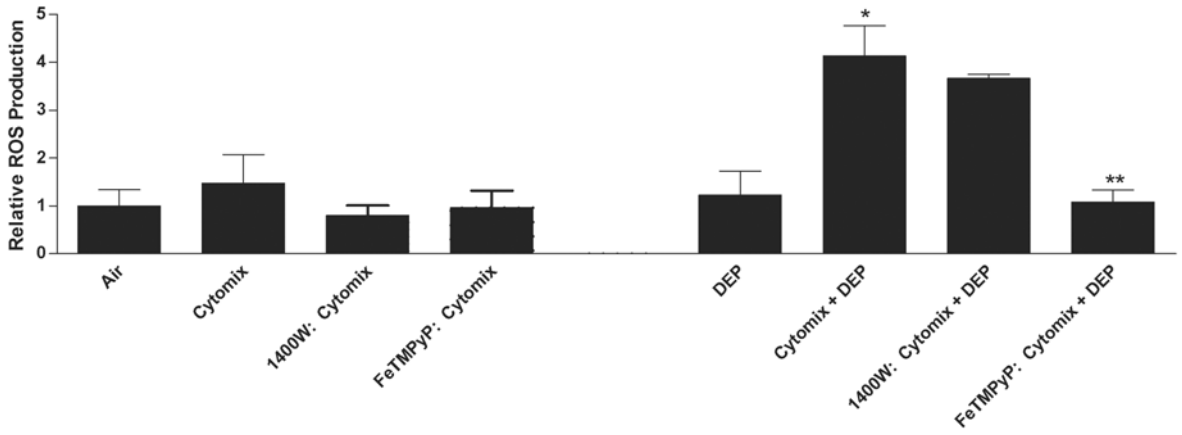


Figure 9. Effects of DEP exposure on ROS production in cytomix-treated mice. Female BALB/c mice were treated once via oropharyngeal aspiration to PBS or cytomix (TNFα 1.0 ng/g + IL-1β 0.5 ng/g + IFNγ 2.0 ng/g) in the presence of 1400W (100 μM) or FeTMPyP (10 μM). Forty eight hours later, the mice were exposed to either air or DEP (2 mg/m^3, 4 hr/day, 2 days). The resulting ROS production from BALF cells was analyzed by H_2DCFDA oxidation. Data are expressed as mean (± SEM) fold increase over air exposed mice. Significance ($p < 0.05$) indicated by: * vs. Air, Cytomix, DEP; ** vs. Cytomix + DEP.

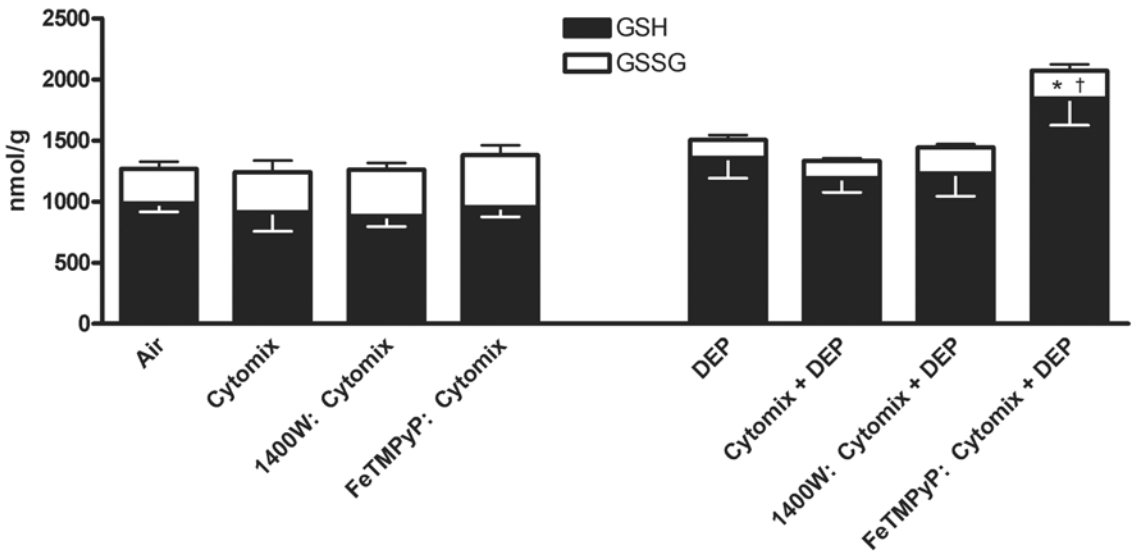


Figure 10. Changes in intracellular glutathione in healthy or cytomix-treated mice exposed to DEP. Female BALB/c mice were treated once via oropharyngeal aspiration to PBS or cytomix (TNFα 1.0 ng/g + IL-1β 0.5 ng/g + IFNγ 2.0 ng/g) in the presence of 1400W (100 μM) or FeTMPyP (10 μM). Forty eight hours later, the mice were exposed to either air or DEP (2 mg/m^3, 4 hr/day, 2 days). Lung homogenates were analyzed for reduced (GSH) and disulfide (GSSG) forms of glutathione by HPLC. Data are expressed as the mean nmol/μg of protein (± SEM) of GSH or GSSG. Significance ($p < 0.05$) indicated by: * vs. Air; † vs. Cytomix and FeTMPyP: Cytomix.

CHAPTER 5:

Overall Discussion

Epidemiological data indicates that exposure of individuals with pre-existing inflammatory lung diseases to traffic-related particulate matter (PM) are susceptible to increased morbidity and mortality. This dissertation set out to further understand what makes individuals with pre-existing inflammatory lung diseases more susceptible to particulate air pollutant exposure. The data presented here describes (1) the development of an *in vitro* model that mimics the epithelial inflammatory microenvironment found in individuals with pre-existing inflammatory lung diseases, (2) the differential cellular effects of normal and "inflamed" lung epithelial cells exposed to a specific traffic-related PM, and (3) the contributing mechanism(s) involved in these differential cellular effects.

Since the pulmonary epithelium is exposed to numerous pro-inflammatory stimuli in the setting of inflammatory lung diseases, and it is a primary target to inhaled insults like PM, it represents an appropriate cell to investigate the effects of PM exposure. Within this dissertation we have described an *in vitro* epithelial cell culture model that mimics the generic inflammatory microenvironment via treatment with exogenous pro-inflammatory cytokines found to be elevated in inflammatory lung diseases like asthma and chronic obstructive pulmonary disease (COPD). More specifically, cytokine-treated (cytomix: TNFα + IL-1β + IFNγ) airway and alveolar epithelial cells resulted in the activation and release of secondary mediators involved in leukocyte chemotaxis (RANTES, MIP-2), and free radical production (iNOS, NO) involved in inflammation.

Using this *in vitro* model the differential effects of PM exposure were evaluated in the context of normal and cytokine-treated cells. This dissertation shows that cytokine-treated alveolar and airway epithelial cells exposed to diesel exhaust particles (DEP), a traffic-

related particle representative of an urban contemporary air shed, induced oxidative stress, impaired epithelial barrier function, and cell injury. Furthermore, in agreement with numerous other investigators, the data presented here showed that the resulting oxidative stress and injury induced by DEP exposure was related to the organic carbon fraction of the particles.

The evidence collected to date, including that presented here, suggests that the deleterious actions of DEP exposure cause oxidative stress, in part mediated by reactive oxygen species (ROS). Inflammation itself can result in ROS production from several different sources (inflammatory cells and lung epithelial cells) which can combine with other available ROS to create more potent radicals. Specifically, nitric oxide (NO) can rapidly interact with superoxide anion ($O_2^{\cdot -}$) to form the radical peroxynitrite ($ONOO^-$) resulting in altered proteins and lipids, damage DNA, and cell death. Herein we showed that pulmonary epithelial cells that produce increased amounts of NO, the result of cytokine-treatment, interact with DEP-induced ROS, including $O_2^{\cdot -}$, resulting in $ONOO^-$ mediated cell injury. Furthermore, as a proof of concept, in a comparable *in vivo* model, mice with pulmonary inflammation, elicited by exogenous aspiration of inflammatory cytokines, and exposed to DEP inhalation produced increased amounts of ROS. Similarly, the *in vivo* results suggeste that in the context of pre-existing pulmonary inflammation, subsequent DEP exposure results in enhanced ROS production, in part a consequence of $ONOO^-$. The interaction of DEP-induced ROS in individuals with already large amounts of NO, due to inflammatory lung disease, may offer a possible contributing mechanism to DEP-mediated pulmonary toxicity.

The production of ROS is known to play a major role in several inflammatory lung diseases, including asthma and COPD (Kirkham and Rahman 2006). Under normal physiological conditions, low level ROS production is produced by a variety of processes, including cellular respiration and enzymatic reactions, and serve important roles as secondary messengers that regulate the function of signaling molecules, transcription factors, and gene expression (Park, Kim, and Lee 2009; Rahman 2006; Rahman and Adcock 2006; Rahman, Biswas, and Kirkham 2006; Rahman, Biswas, and Kode 2006; Rahman and Kilty 2006). During pulmonary inflammation, ROS production is further increased the result of increased cytokine levels as well inflammatory cells (neutrophils, eosinophils, macrophages). The result of increased ROS is the aberrant oxidation of proteins, DNA, and lipids that not only perpetuate the inflammatory process, but can also cause cell injury to surrounding tissue (Park, Kim, and Lee 2009; Rahman 2006; Rahman and Adcock 2006; Rahman, Biswas, and Kirkham 2006; Rahman, Biswas, and Kode 2006; Rahman and Kilty 2006).

Since the lung exists in a high oxygen environment it has a well developed antioxidant system, consisting of enzymatic (SOD, catalase, HO-1) and non-enzymatic (GSH, vitamins C & E, cartenoids, flavanoids) systems that help to neutralize excess oxidants. Inflammatory lung diseases not only have exaggerated ROS production, but also depressed antioxidant mechanisms, thus creating an oxidant - antioxidant imbalance, further putting them at increased risk of the adverse health effects of PM. Deficiencies in both non-enzymatic and enzymatic antioxidants have been reported in both asthmatics and patients with COPD (Nadeem, Masood, and Siddiqui 2008; Rahman 2006; Rahman and Adcock 2006; Rahman, Biswas, and Kirkham 2006; Rahman, Biswas, and Kode 2006; Rahman and

Kilty 2006). The reduced form of glutathione (GSH) represents the most abundant non-enzymatic antioxidant in the lung and it is normally found in very high levels not only within lung epithelial cells, but also in the epithelial lining fluid. However, decreased GSH levels have been documented in bronchoalveolar lavage fluid (BALF) of individuals afflicted with asthma and COPD (Biswas and Rahman 2009; Nadeem, Masood, and Siddiqui 2008; Sackesen et al. 2008). Similarly, decreased levels of several enzymatic antioxidants have been observed in inflammatory lung diseases (Nadeem, Masood, and Siddiqui 2008; Rahman, Biswas, and Kode 2006). Superoxide dismutase (SOD), which exits in three isoforms (CuZnSOD, MnSOD, ECSOD), are important for dismutating $O_2^{\cdot -}$ to the less toxic H_2O_2. However, like GSH, both the expression and activity of CuZnSOD and MnSOD are decreased in asthmatics (Comhair and Erzurum 2005; Comhair, Ricci et al. 2005; Comhair, Xu et al. 2005; Jarjour, Busse, and Calhoun 1992; Kinnula, Crapo, and Raivio 1995). Likewise, catalase activity, which is responsible for decomposing H_2O_2 to water, is decreased in asthmatics (Varshavskii et al. 2003). To combat such deficiencies, several therapeutic approaches based on GSH augmentation have been explored, but have not yielded promising results for several reason ranging from poor half-life, poor cellular uptake, and adverse effects (airway hyper-reactivity) (Nadeem, Masood, and Siddiqui 2008; Park, Kim, and Lee 2009).

Taken together, the adverse health effects of individuals with pre-existing inflammatory lung diseases exposed to PM, like DEP, may be in part due to already heightened ROS production and reduced antioxidant systems, which in the context of additional oxidative stress (PM) is unable to effectively adapt resulting in further

inflammation. The studies discussed within this dissertation demonstrate how residential exposure to a common pollutant present in many urban environments can have adverse effects on individuals with pre-existing pulmonary disease. Undoubtedly, many mechanisms contribute to this result, but from this dissertation it is believed that the cooperation of NO and $O_2^{\cdot -}$ plays a contributing role to the PM-induced adverse health effects observed in individuals with pre-existing inflammatory pulmonary disease. Considering that individuals living in urban environments are chronically exposed to PM, further understanding of its effects of PM is needed as is a greater understanding of the effects on lung antioxidants not only in health but also disease. Hopefully, such advances in our understanding will translate into possible therapeutic treatments that can be implemented in addition to ongoing efforts to reduce vehicular emmisions.

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