EXAMINATION OF DIESEL AND BIODIESEL EXHAUST EXPOSURE INDUCED DISRUPTION OF LIPID ARACHIDONIC ACID METABOLISM.

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ABSTRACT

Laya R. Bhavaraju: Examination of diesel and biodiesel exhaust exposure induced disruption of arachidonic acid metabolism.
(Under the direction of Drs. Michael C. Madden and Urmila P. Kodavanti)

Diesel combustion emissions contributes a large amount of particulate matter found in air pollution collected in high-traffic urban areas. Current epidemiology studies indicate a strong association between traffic and increased susceptibility to adverse respiratory and cardiovascular events. Current air quality initiatives have introduced biodiesel combustion as an alternative for diesel fuel to reduce particulate emissions. Incomplete biodiesel (BD) combustion emissions analysis has detected the presence of unique emissions components, such as fatty acid methyl esters. However, the biological activity of BD exhaust emissions have yet to be studied unlike diesel exhaust. Particle extraction based cellular-response studies have challenges with collection bias and sample loss. In this research, an improved extraction method was established with both diesel and BD exhaust particle for use in cell culture exposure assessments. Critical comparisons of BD and diesel particle extractions allowed for assessment of composition based response alterations of arachidonic acid (AA) metabolism. Multiple cellular functions are regulated by AA metabolites including inflammation and vascular tone. Endothelial cells regulate vascular tone by releasing lipid signaling molecules (PGI_2 and $PGF_{2\alpha}$) to signal relaxation of smooth muscle cells. Alveolar macrophages (AM) release prostaglandins (PGE_2) indicating cellular inflammation response. The work in this dissertation compared *in vitro* responses of diesel and BD exposure with alterations to AA metabolism. We initially observed the AA metabolism changes with response to BD and diesel emission particles in AM. The significant increase in prostaglandin production and release from BD relative to diesel led to further assessment of AA metabolite production alterations in endothelial cells. The mechanistic response of BD extract was addressed with human umbilical cord endothelial cells (HUVECs) and the findings revealed a reduction in prostaglandins and prostacyclin

relative to diesel exposure. The increased incorporation of AA back into the phospholipid membranes by acyltransferase was associated with BD exposure resulting in reduction of prostacyclin and prostaglandins. Cumulatively, these studies increase the depth of knowledge of diesel and BD induced disruption of AA metabolism. Thus, the composition of exhaust can induce differential cellular responses of lipid mediators affecting AM inflammation signaling and the HUVECs lipid remodeling pathways.

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PREFACE

The experiments detailed in chapter two compare differences of prostaglandin release in macrophages from exposure to diesel and biodiesel exhaust particles. The entirety of this chapter has been published in Chemosphere 2014. The alveolar macrophages were harvested fresh from Wistar Kyoto rats and a sample of spontaneously hypertensive rats. Bronchiolar lavage was used to collect the cells from the lung environment prior to culturing the cells in media, as preparation for in vitro particle exposure. The animal handling, injections of anesthesia and lung lavages were conducted by the author, after initial assistance from Urmila Kodavanti and Jonathan Shannahan. Post exposure extraction of mRNA and conversion into cDNA followed with RT-PCR quantification was conducted by the author. The particle calculations of metals which were generated from ICP-OES, in adherence to EPA protocol, was conducted by John McGee. The data conducted on particle sequestration of lipid mediator PGE_2 was performed by Michael Madden. The data generated from *in vitro* exposures of macrophages to diesel particles and the resulting analysis of prostaglandins in media with ELISA and HPLC detection, were done by the author. Additional experiments involving AM exposure with pre-treated particles and post exposure challenges were also conducted by the author. Microscopy of trypan blue and the H& E stained images were generated by the author.

Chapter three characterization experiments utilized multiple methods and new uses of existing equipment. The endotoxin particle test was conducted by CapeCod Inc. The calculations of elemental and organic carbon content using a thermo-optical based method of sequential pyrolytic vaporization, was conducted at Sunset Labs. The initial adaptation of fatty acids detection from particles was done by Dr. Sheila Flack and slightly modified by the author. Assistance to run Malvern DLS zetasizer for quantification of homogenous suspensions was given by Dr. Lenibel Santiago.

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LIST OF ABBREVIATIONS

A549 Human Lung Adenocarcinoma Epithelial Cell Line

ANOVA Analysis of Variance

AP-1 Activating Protein-1

AA arachidonic acid

AHR acryl hydrocarbon receptor

AM Alveolar macrophages

B100 biodiesel exhaust particle

B20 biodiesel blend 20%

β-actin Beta-Actin

BSA Bovine Serum Albumin

cDNA Complementary Deoxyribonucleic Acid

CRE calcium response element

CV Cardiovascular

DEP Diesel Exhaust Extract

DMEM Dulbecco's Modified Eagle's Medium

DMSO Dimethyl Sulfoxide

DNA Deoxyribonucleic Acid

DPI Diphenyleneiodonium

E-selectin Endothelial Adhesion Molecule 1

EA.hy926 Hybrid Epithelial and Endothelial Cell Line

ECL Enhanced Chemiluminescence

EDTA Ethylene Diamine Tetraacetic Acid

EGM-2 Endothelial Growth Medium

ELISA Enzyme linked immuno staining assay

EPA Environmental Protection Agency

EC/OC elemental carbon /organic carbon ratio

GC-MS gas chromatography and mass spectrometry

HPLC High pressure liquid chromatography

HUVECs Human Umbilical vein endothelial cells

FBS Fetal Bovine Serum

HCAEC Human Coronary Artery Endothelial Cells

HCL Hydrochloric Acid

HO-1 Heme Oxygenase-1

H_2O_2 Hydrogen Peroxide

HRP Horseradish Peroxidase

HUVECs Human Umbilical Vein Endothelial Cells

ICAM-1 Intercellular Adhesion Molecule-1

IgG Immunoglobulin G

IL-1β Interleukin-1 Beta

IL-6 Interleukin-6

IL-8 Interleukin-8

LD 50 lethal dose fifty percent

LDH Lactate Dehydrogenase

LPCAT Lysophosphatidylcholine acyltransferase

mRNA Messenger Ribonucleic Acid

NaCl Sodium Cloride

Nrf2 Nuclear Factor (Erythroid-Derived 2)-Like 2

NF-κB Nuclear Factor-Kappa B

NOx Nitric Oxide species

PAH polycyclic aromatic hydrocarbon

PBS Phosphate Buffer Saline

PCR Polymerase Chain Reaction

PGH_2 Endoperoxide prostaglandin h2

PLA_2 Phospholipase A2

PLC phospholipase C

PM Particulate Matter

PM10 Coarse Particulate Matter

PM2.5 Fine Particulate Matter

RNA Ribonucleic Acid

ROFA – residual oil fly ash

ROS Reactive Oxygen Species

RT-PCR Reverse Transcriptase Polymerase Chain Reaction

SDS Sodium Dodecyl Sulfate

SHR Spontaneously hypertensive rats

SOD Superoxide Dismutase

TF Tissue Factor

TNF-α Tumor Necrosis Factor-Alpha

TM Thrombomodulin

UF Ultrafine Particle

UPLC Ultra high performance liquid chromatography

VCAM-1 Vascular Adhesion Molecule-1

VEGF Vascular Endothelial Growth Factor

Chapter 1.

Introduction

1.0. Air pollution and human health effects.

Re-examining historical air pollution events illustrates the evolving impact of air pollution on human health. Adverse human health effects due to air pollution were recognized as early as the dawn of industrialization, around the 1860s. However, only after several catastrophic historical events during the post-industrial revolution in the 1950s was air pollution finally measured and stringently assessed. Increased urban air pollution, combined with stagnant-winter air, led to the "Great London Smog" in December 1952. For five days, an impenetrable smog settled on the city of London, during which the death toll dramatically increased, which established a recognizable pattern of air pollution inhalation-induced mortality. The smog accumulation was a mixture of sulfur dioxide and other persistent chemical air pollutants produced by burning coal and wood. The five-day period of smog resulted not only in fatalities, but also increased hospitalizations associated with fatal pulmonary infections [1]. This was one of many global historical events that was used as a documented case study to illustrate the human health outcomes of unregulated air pollutants in urban industrial settings.

An example of a national historical event, occurred in 1948 in Donora, Pennsylvania, where air pollutants from a zinc smelting factory were linked to around 6000 hospitalizations and hundreds of local deaths [2]. This was a national case study showing clear association of the health dangers with air pollution from industry and manufacturing. Although the zinc factory was shut down due directly to the health impact of poor air quality, no immediate national action was undertaken regarding air pollutants. Decades later, in the 1960s, the federal government decided to address air quality by setting limits on air pollutants. The Clean Air Act of 1963 was the first federal legislation to regulate air pollution in the United States [3]. The Act was expanded in 1967 to include the right to monitor and control pollution levels. Finally in 1970, the Clean Air act was amended to establish the National Ambient Air Quality Standards

(NAAQS), which set emission levels for six "criteria" air pollutants. Criteria air pollutants were defined as air pollutants found in high concentrations in smog. These are ozone, lead, sulfur dioxide, nitrogen oxides, carbon monoxide and particulate matter (PM). The NAAQS regulate the levels of these six criteria pollutants, but there is a larger collection of pollutants listed as Hazardous Air Pollutants (HAPs) which have established federal guidelines and standards. Together, the HAPs and NAAQS, nationally regulate air pollutants with careful consideration of new toxicology evidence to minimize harmful health effects.

Air Pollution PM Classifications by size.

The six criteria air pollutants have been evaluated regarding acceptable levels of exposure. Particulate matter (PM) specifically, was further regulated based on size. There are three size classifications, of which two are individually regulated. The first is PM_{10}, or coarse particulate matter, less than or equal to 10μm in aerodynamic diameter. The current NAAQS standard is 150 $\mu g/m^3$ over a 24-hour period [4]. The second regulated class of PM, is $PM_{2.5}$, or fine particulate matter, less than or equal to 2.5 μm in aerodynamic diameter. Its current NAAQS standard recently changed from 15$\mu g/m^3$ to 12$\mu g/m^3$ because new evidence indicated acute exposure was found to be strongly associated with adverse cardiovascular events (NAAQS US EPA 2013). The third is ultrafine particles (UFPs), defined as particles less than or equal to 0.1 μm in diameter, which makes them susceptible to agglomeration [5]. Currently, there is no ambient air standard-regulated level for UFPs. The standards set by the NAAQS are federal limits, but some states have their own standards that are lower than the federal limits. In addition to size, PM is also classified as primary or secondary. Primary PM is defined as the fraction of PM that is emitted directly into the atmosphere; secondary PM forms from photo oxidation in the atmosphere when PM reacts with pre-existing gases such as SOx and NOx. Ambient air pollution is also classified by the source because PM measurements have previously found that anthropogenic sources generate smaller PM ($<PM_{2.5}$) than natural sources, such as lightning [4]. Densely populated urban regions are considered to emit the largest volume of anthropogenic PM [4]. PM classification allows for increased accuracy when measuring ambient PM and can help identify regions where there is increased need for air monitoring.

Epidemiological studies establishing ambient PM induced adverse health effects including cardiovascular events

An extensive air pollution epidemiology study called the "Harvard Six Cities Study," which began in 1974, was critical in establishing an association with ambient PM and adverse health effects. The study correlated air pollution effects with mortality in approximately 8,000 adults in six U.S. cities classified as either polluted or less polluted. The most polluted urban cities had 89.0μg/m^3 PM for a 24hr sampling period and the least polluted had one third the PM concentration of the most polluted. The study lead by Dockery et al [6] found that cities with a higher concentration of ambient PM, such as Steubenville, Ohio, had an increased rate of mortality compared to cities with lower ambient PM levels (e.g., Portage, Wisconsin) [7-9]. Data from this pioneering study along with several hundred additional epidemiology studies, since the early 1990s, indicate that increased concentrations of PM are clearly linked to a large number of PM-associated deaths [4].

The direct association between air pollution PM exposure and cardiovascular disease is critical to understand, since heart disease is now the number-one cause of death worldwide [10]. Air pollution epidemiology research has long established a strong correlation between increasing PM exposure and adverse cardiovascular health effects. Field epidemiology studies indicated a positive correlation between increasing ambient PM (i.e., PM_{10}) and an increase in hospital admissions associated with respiratory and cardiovascular injury [2, 11]. This study noted not only the increase in hospital admissions but also found 1.4% increase in cardiovascular-related deaths (e.g. myocardial infractions or stroke) with 10μg/m^3 mean daily average of PM_{10} suggesting that there are links between increased PM and adverse cardiovascular events [7]. Study participants involved in the Multi-Ethnic Study of Atherosclerosis (MESA) cohort, exhibited changes in blood pressure from exposure to an average of 10μg/m^3 of $PM_{2.5}$ daily for 30 days [12]. In this study, the significant increase in systolic blood pressure (SBP) was strongly associated with subjects who lived within 300m of a highway, yet subjects who lived more than 400m did not show significant blood pressure changes [12]. This study suggests that highway generated PM is crucial to altering cardiac biomarkers. Similarly, another cardiac biomarker was identified in a study conducted with

PM collected in close proximity to Los Angeles highways. This study of Los Angeles residents showed significantly increased carotid intima-media thickness (CIMT), an atherosclerosis marker, in healthy subjects with prolonged daily exposure to elevated PM concentrations (e.g. $PM_{2.5}$ approximately 10μg/m^3 collected near a highway) [13]. Based on these studies, epidemiology evidence supports PM exposure induced cardiovascular injury. These include both physiological changes (e.g., blood pressure and heart rate) and pathological changes (e.g., intima-media thickness). In addition, these studies find that the source of PM (e.g., highway PM) can impact the intensity of cardiac biomarkers.

Study of PM near roadways and highways drew focus to the traffic–generated PM effects originating from cars and trucks. Epidemiology studies focused on cardiovascular events associated with traffic-generated PM identified increased cardiac injury associated with pathological (e.g., plaque buildup) and physiological (e.g. heart rate variability) alterations. A field study conducted in the Netherlands [14], indicated residents living within 50m from a major roadway have increased risk of cardiopulmonary linked mortality. In this study the close proximity to a roadway correlated with 95% greater risk of mortality after normalizing for factors like age, smoking habits, diet and regional poverty [14]. Another study, with subjects in occupations requiring large amounts of time in or near highways (e.g., taxi drivers), found increased intima-media thickness of the carotid artery [15]. This study further supports the role of PM emissions from vehicles as a source of adverse cardiovascular effects.

In addition to measuring the intima-media thickness, one study measured coronary artery calcification (CAC). This was assessed by electron beam-computed tomography, which measured the plaque size. The study found of 4,814 subjects living near a major roadway (i.e., <200m from roadway) were associated with increased CAC after normalization for extraneous factors (e.g., income, age) [16]. Based on occupational exposures and field studies, traffic PM significantly increased plaque buildup which can potentiate atherosclerosis or disrupt vascular tone. These studies support the hypothesis that traffic-generated PM can contribute significantly to mortality and cardiac injury markers.

The traffic-generated PM exposures mediated both pathological and physiological responses. The composition of traffic-generated PM may play a critical role in certain biological responses. Traffic-generated PM is typically composed of exhaust emissions from diesel and gasoline powered vehicles, but

it also includes non-combustion -derived compounds (e.g., dust gathered by wearing down of components from automobile brake linings and tires) and environmental bio aerosols entrained from the ground (e.g., mold, pollen or bacteria) [17]. Air pollution researchers now have data associating PM with critical mechanistic factors involved in potential adverse cardiovascular health outcomes.

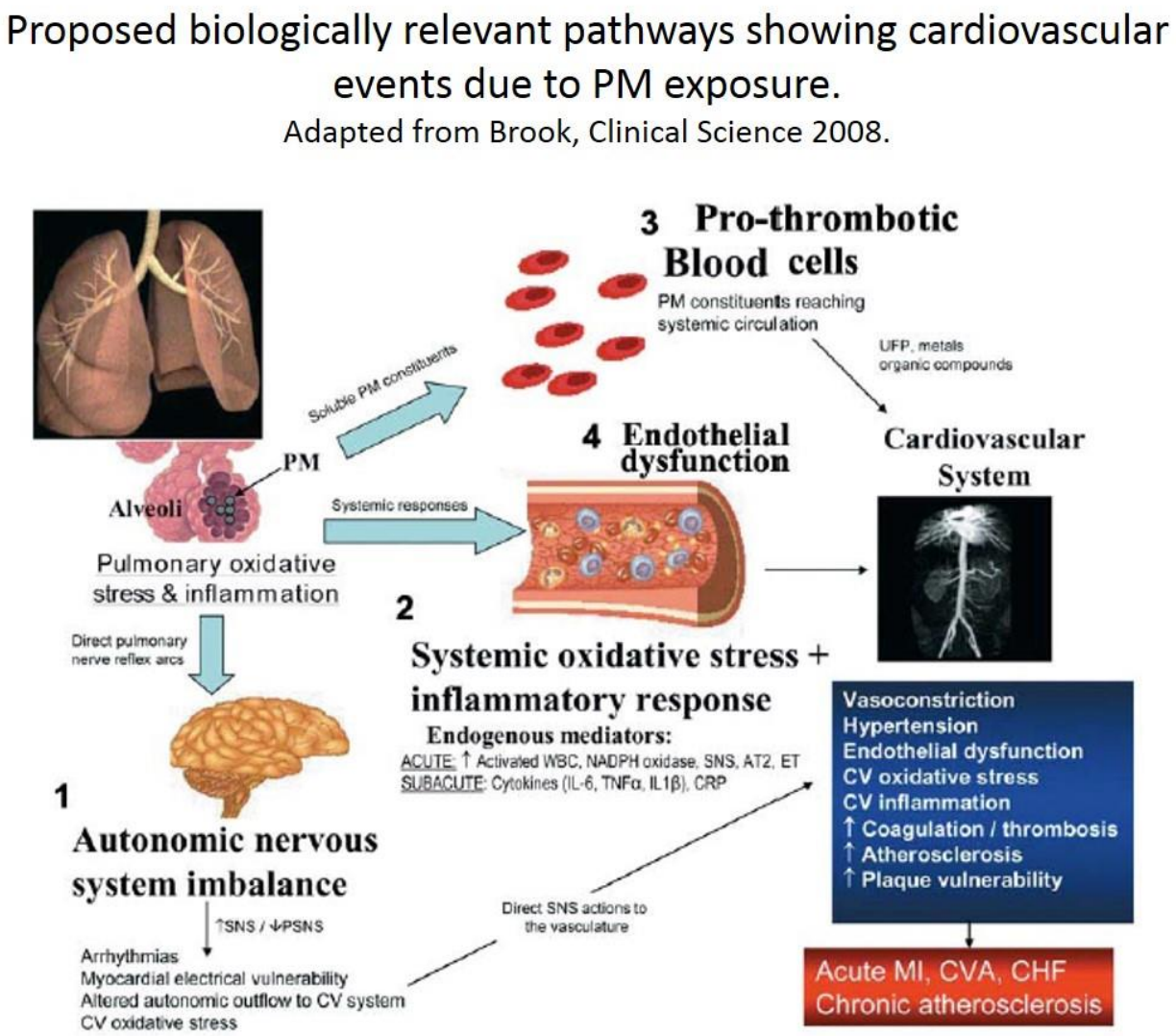

Figure 1.1. Proposed mechanism of cardiovascular injury with PM exposure [5].

2.0. Proposed particle exposure induced mechanisms of cardiovascular injury.

PM exposure may induce biological responses by a combination of several modes of action. Inhalation of PM may induce adverse cardiovascular injury from particle and also from soluble compounds on the surface of particle, as illustrated in Figure 1.1[5]. Particle inhalation results in deposition of particles within the lung alveolar regions. Deposited particles and soluble compounds can generate adverse cardiovascular events via three critical routes (Figure 1.1), which are not mutually exclusive and can act concurrently. The first proposed PM-induced route of injury generates autonomic nervous system imbalance, that results in adverse cardiovascular events including arrhythmias and sympathetic nervous system mediated disruption of vascular tone. A second proposed PM-induced route is initiated with respiratory and peripheral cellular responses resulting in transport of inflammation mediator into circulation, that results in endothelial cell disruption and potentiating cardiovascular injury. The last proposed route relies on soluble compounds from PM dissolution, thus translocating into circulation to initiate endothelial cell injury potentiating into adverse cardiovascular events. All three routes initiate a cascade of precursory events resulting in disruption of vascular tone, endothelial dysfunction, and plaque buildup. These precursory events, along with PM-induced systemic inflammation, lead to a complex multi-layer cardiovascular response. PM-induced cardiac injury is mediated by several critical steps and exploration by air pollution researchers has established PM- soluble compounds induced both physiological and pathological responses.

PM deposition and translocation.

Inhaled-PM deposition in the lung is a critical initiating factor for PM translocation and component solubilization. PM deposition into the lung is primarily determined by a particle's aerodynamic diameter. Empirical data, along with predictive modeling data, can help determine the location of deposition of PM with variable diameters. Volunteers inhaling, 5μm diameter particles had the largest deposition fraction (40%) in the alveolar region [18]. The smaller 3μm particles had less (i.e. 50%) total lung deposition, but with similar total percent (42%) deposition in the alveolar region, suggesting particle sizes resulted in similar total deposition of the alveolar region. However with even smaller diameter particles, in a separate study, there was a larger percent total deposition in the same region [19]. This study was also conducted in healthy human volunteers, but with particles ranging from 0.04μm - 0.1μm and it identified up to 66%

alveolar deposition of particles from inhalation exposure [19]. These studies found that total deposition at the alveolar region varies with particle diameters, suggesting that there is a higher concentration of 0.04μm PM retained near the alveolar region of lung, in humans.

Recent predictive modeling programs have generated reliable accurate data regarding PM deposition and translocation. The modeling data indicated particles of larger diameter measured between 2 to 10μm deposit primarily in the nose and upper airways [20] and smaller particles (e.g., fine, ultrafine PM) deposit deeper in the alveolar regions [20]. The combined experimental and predictive modeling data found PM total and regional deposition is based in part on size, but the translocation from the site of deposition might be influenced by other factors such as PM solubility, size, phagocytosis of PM by alveolar macrophages and clearance.

PM translocation is a topic of great debate since it is unclear if the whole particle, fragments of particle or soluble compounds from particle diffuse into peripheral tissue, such as the vasculature, after initial inhalation exposure. PM potential translocation of ultrafine particles was addressed in an *in vivo* rodent study. In this study, inert particles, TiO2 nanoparticles (~20nm), were initially intratracheally instilled and recovered after 1hr exposure from broncholalveolar lavage fluid (BALF). A little less than 20% of the total deposited PM amount was estimated to be recovered, based on measurement of amounts in broncholalveolar lavage fluid (BALF), suggesting that majority translocated into pulmonary tissue or extrapulmonary tissue or remained in AM after phagocytosis [21]. This study also found TiO2 present predominantly in the alveoli indicating that nanoparticles can likely translocate into the periphery or they would be recovered in BALF. A second study with both inhalation and instillation of plain carbon particles into animal lungs demonstrated carbon particles (20-29nm in diameter) were also taken up by the epithelial cells and were re-distributed into lipophilic layers [22]. In addition, dog autopsies found inhaled aerosolized ultrafine particles may translocate into circulation through the pulmonary capillaries and lymphatics to deposit in the lung and peripheral organs [23]. Several studies observed particle inhalation resulted in detection of PM, not only in lungs, but within the vascular lining, liver and olfactory bulb possibly by crossing the blood-brain barrier [24, 25]. These studies suggest inhalation of ultrafine

PM may result in translocation of whole particle into the peripheral tissues and organs, thus possibly validating the proposed mechanism of action of PM in Figure 1.1.

Experimental evidence of soluble PM constituent – induced responses.

As previously stated in the Figure 1.1, cardiac injury could result from soluble constituents of inhaled PM. Evidence that soluble constituents from PM induce cellular effects was identified in an *in vivo* study comparing pure carbon particles with diesel exhaust particles, which contain several soluble compounds. This study identified significant response differences from pure carbon particles with diesel exhaust particles. The 4 hr rodent inhalation exposure to diesel exhaust particle and carbon black particles at a relatively high ambient concentration (500μg/m^3) resulted in elevated levels of endothelin-1 (ET-1) and endothelin -3 (ET-3) in plasma that is typically associated with constriction of the vascular tone response [26]. Diesel particles, but not carbon black particles, altered hemodynamics. Also evaluated in this study were water washed ambient Ottawa particles that had polar organic compounds removed but other compounds remained [26]. These treated particles also caused initial ET-1 and ET-3 increase in plasma after exposure, but changes in blood pressure or heart rate were not found. This study indicated that both particles and the non-water soluble compounds adsorbed to the particle are likely responsible for hemodynamic plasma signal changes in rodent exposures [26]. Similarly, in a study using rodent models of hypertension, particles with and without metals were evaluated for an inflammation response. In this study, hypertensive rats had exposure to two different PM-with and without metals, and also polycyclic aromatic hydrocarbons (PAHs). Exposure via intra-tracheal instillation resulted in increased inflammation response that was significantly correlated only with particles containing metals [27]. These findings highlight the importance of the soluble PM bound chemicals in generating robust cellular responses.

Evidence from inhalation exposure of systemic responses and release of mediators.

Also shown in Figure 1.1, Brook et al hypothesized that soluble mediators that result from local inflammation spread to induce a systemic response which subsequently leads to adverse cardiovascular events [5]. The detection of soluble mediators in plasma after exposure may not be statistically

significant however the collection of soluble mediators can be evaluated for biological significance by exposing the plasma to cell cultures *in vitro*. Plasma was collected from a human exposure study to dilute diesel exhaust. The dilute plasma contained markers that were capable of activating an inflammatory response in culture (i.e., human coronary artery endothelial cells). Incubation with the dilute plasma resulted in increased cellular adhesion molecules and interleukin -8 (IL-8) [28]. Evaluation of results from this study possibly suggest there are mediators of inflammation that were released into the periphery post-diesel exposure, resulting in a systemic inflammatory response. There is a possibility that compounds from the diesel exhaust translocated into plasma and the response is linked to the compounds, but no measurements were made of metals or other organic substances in plasma. Additionally, the probability is relatively low for soluble mediators, considering that the inhalation exposure was 106μg/m^3, which is three-fold lower than common diesel exhaust exposure concentrations that previously measured inflammation responses. Measurement of PAHs in urine and exhaled breath condensate from these diesel exhaust-exposed subjects showed that the diesel exposure did not result in PAH increase relative to the air control exposure suggesting a low inhaled dose [29]. There is greater probability that the increased adhesion molecules and IL-8 are a result of inflammatory mediators released from the lung or peripheral vascular tissue, validating a hypothesis of systemic response. For further examination of the theory presented in Figure 1.1, air pollution researchers are focused on characterizing the PM-associated compounds and finding specific cellular responses that may lead to cardiovascular injury.

3.0. Diesel and Biodiesel exhaust composition and response comparisons.

Anthropogenic $PM_{2.5}$ concentrations across the U.S, in a 24hr period, were concentrated in high-traffic urban settings [4] and calculated to originate from diesel emissions. In a 2012 study conducted in Hong Kong, 43% of total $PM_{2.5}$ were traced back to idling diesel-powered naval vessels in the shipyard [30], demonstrating that globally diesel-combustion generates the most $PM_{2.5}$. Recently reduced PM emission standards for mobile diesel engines (i.e., 0.01 g per brake horsepower-hour [g/bhp-h]) forced introduction of new emission-control technologies such as diesel particle filters and improved fuel injection systems. These specifications may also reduce harmful emissions such as sulfur, CO and NOx

species [4]. Historically urban traffic-generated pollution was composed of exhaust emissions from diesel and gasoline [4]. However in 2012, the U.S government passed the Energy policy Act expanding the renewable fuels initiative that increased production of biodiesel (BD). As of 2014 the national biodiesel production reached a record high of 1.8 billion gallons, exceeding the proposed levels stated in the 2014 Renewable Fuel Standard Rule. The global volume of all feedstock biodiesel produced and consumed is steadily increasing in billions of liters every year [31]. The most common commercially available biodiesel is however a blend - B20, which is 20% soy biodiesel and 80% petroleum diesel [32], which has been successfully utilized in the U.S transportation industry. Biodiesel powered trucks are increasing in popularity yet exhaust composition analysis remains as complicated as diesel and requires further research regarding emissions chemistry and potential health effects. Diesel combustion exhaust is composed of particles and gases consisting of hundreds of different chemical components. In general, the gas-phase volatiles in diesel exhaust include olefins (1, 3-butadiene), aromatics (benzene, ethylbenzene, toluene, and xylenes), PAHs (including nitro-PAHs and oxy-PAHs), alkanes, oxides of nitrogen, carbon, and sulfur and carbonyls. Common PAHs found in diesel exhaust are phenanthrene, fluorenes, naphthalenes, pyrenes, fluoranthrenes [33, 34]. The particles emitted from diesel combustion typically consist of a carbon-rich core with adsorbed chemicals on the surface as illustrated in Figure 1.2 [35].

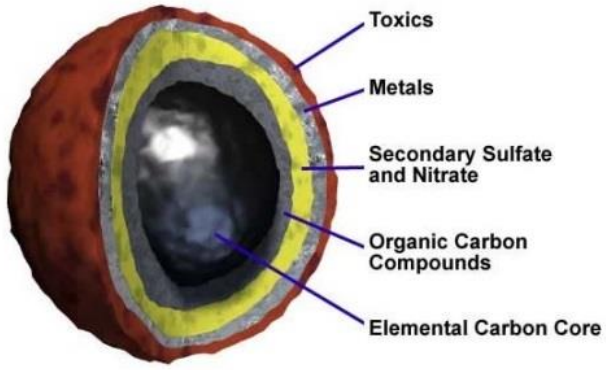

Figure 1.2. Diesel particle schematic. Elemental carbon core and soluble compounds adsorbed to surface [35].

Common surface compounds include metals, ions, organics and incomplete-combustion species [36]. Transition metals such as Fe and Zn are abundant in both diesel and biodiesel exhaust particles [37]. PAH are more abundant in diesel exhaust compared to biodiesel exhaust emissions [38]. However, unique to biodiesel combustion are whole (C18) and fragmented (C3- C4) fatty acid methyl esters (FAMEs). Biodiesel emissions release an abundant amount of FAMEs even though biodiesel emits fewer PM total mass per gallon of fuel relative to diesel [39].

GC-MS analyses of biodiesel combustion exhaust identified several FAME species [34, 40]. These species are likely incomplete combustion products from the original fuel. Biodiesels are typically created by a chemical reaction between plant oils or animal fat and an alcohol, typically methanol, in the presence of catalyst, which generates FAMEs. An alternative for methanol is to use enzymes to generate FAMEs [41]. Inefficiencies in the biodiesel-refining processes after transesterification result in a fuel with impurities consisting of plant oil triglycerides, glycerol and intermediate-reaction products (e.g., fatty acids) that can be subsequently emitted in exhaust. The fuel is the source of the unique emission compounds found with biodiesel PM exhaust. There are hundreds of chemicals that are also released in the exhaust with known biological or health effects that are not linked directly to the unburned fuel. In a U.S. Environmental Protection Agency (EPA) summary report, comparison of biodiesel and diesel exhaust emissions shows that hydrocarbons (HC), PM and CO decreased over 10% compared to diesel neat, as displayed in Table 1.1 [4]. The differences in the exhaust emissions between diesel and biodiesel are important to understand because health effects can be directly associated with exhaust composition. Current research on PM-induced health effects generally has examined singular chemical compounds and cellular responses to assess potential risks with human inhalation exposure, and the responses to some inorganic (e.g., transition metals) and organic (e.g., PAHs) chemicals are discussed below.

Emission impacts of 20 vol% biodiesel
for soybean-based biodiesel added to an average base fuel

	Percent change in emissions
NOx	+ 2.0 %
PM	- 10.1 %
HC	- 21.1 %
CO	-11.0 %

Table1.1. Emissions changes from diesel to biodiesel (20%) [32].

Metals in emissions linked to oxidative stress response.

Specific cellular responses have previously been associated with individual chemical species emitted in exhaust, such as oxidative stress with metals in the emissions. Metals are typically emitted from the combustion of petroleum diesel and biodiesel, and have been predicted to originate from both the fuel and engine lubricants [42]. Several studies have detected from particle analysis both petroleum diesel and biodiesel exhaust contain metals [27, 43]. The most common metals found include Zn, Fe, Mn and Cr [44] from both diesel and biodiesel, however, Zn was noticeably elevated in biodiesel. Respirable Zn particles were previously identified to deposit within the lung and disperse into the cardiac tissue. A rodent exposure study, found stable Zn isotopes in murine extrapulmonary tissue (i.e., cardiac tissue) after intratracheally instillation [45], suggesting PM-associated metals can be inhaled and translocate from the lung into the cardiac tissue. Like Zn, other transition metals can induce intracellular oxidative stress from production of reactive oxygen species (ROS) that have unpaired electron(s) in their outer valance shell. ROS products consist of free radicals and non-radicals but the non-radicals include species with high oxidizing potential that modify proteins and lipids and generating cellular toxicity. ROS generation and induction of lipid peroxidation species has been observed with *in vitro* exposures of residual oil fly ash (ROFA), a metal-rich PM. In this exposure study with lung epithelial cells, ROS products were significantly increased as were inflammatory mediators from arachidonic acid metabolism such as prostaglandin E2 (PGE_2) and 15-eicosatetraenoic acid [46]. Another ROFA exposure study with alveolar macrophages (AMs) found increased ROS products and pro-inflammation markers [47-49], suggesting lung epithelial and AMs are sensitive to oxidative stress induced by metals. The metal-rich ROFA also increased expression of pro-inflammatory markers, IL-8, IL-6, and tumor necrosis factor-α (TNF-α) in bronchial epithelial cells [50, 51]. In a whole animal exposure study comparing PM that is metal rich compared to PM without metals, results indicate greater inflammation response in the BALF, with metal rich PM exposure [27]. Further evidence of metal-rich PM-induced oxidative stress comes from studies in which the metals were removed. Metal chelation of ambient PM with deferoxamine resulted in significant reduction of oxidative stress responses and reduced antioxidant levels [52, 53] indicating that removal or reduction of metals from particles can decrease the potency of particle-induced cellular

responses [54]. PM from biodiesel and diesel combustion include soluble compounds such as metals that may translocate to the periphery, where they initiate pro-inflammation and oxidative stress responses, potentially leading to adverse cardiovascular events. Particle-bound metals are potent soluble mediators of oxidative stress and pro-inflammatory responses that are commonly found in both diesel and biodiesel exhaust exposure.

PAH emissions and cellular responses.

PAH are generated and emitted from diesel and biodiesel combustion [34, 55] and induce variable cellular responses. A recent review of studies involving subjects with prolonged occupational exposure to PM rich in PAHs, concluded that there is increased risk of lung cancer and significantly increased levels of chromosome aberrations in populations with greatest exposure [56]. Interestingly, most of the studies were conducted in Europe where diesel fuel use is more common than in the U.S and diesel emits more PAHs relative to gasoline and biodiesel [57]. *In vitro* mechanistic studies with diesel exhaust particles (DEP) identified that PAHs bind to the acyl hydrocarbon receptor (AHR), initiating nuclear translocation and dimerization with ARNT. The dimerization binds and activates target sequences such as hydrocarbon response elements or antioxidant response elements. Thus, initiating downstream inflammation and oxidative stress-signaling in cells. PAHs commonly react with other species in the cellular environment. For example, quinones generated from PAH components undergo cyclic-reduction reactions with oxygen in the cells, followed by oxidative coupling with NADPH, resulting in the formation of extremely unstable semiquinone radicals that can also initiate oxidative stress. PAH mode of action is complex and can activate multiple pathways within cells. An *in vitro* study examined the methanol extractable DEP compounds that include hydroxy-PAHs, carboxy-PAHs, nitro-amino-PAHs and found inflammogenic responses [58]. In this study bronchial epithelial cells released elevated levels IL-6 after exposure to the most polar extract collected from DEP, suggesting PAHs derivatives potentially induce inflammatory responses and have increased cytotoxicity relative to the parent PAHs [58]. Recent toxicology efforts to establish a mode of action for diesel found similar pathways activated with diesel and individual PAH compounds suggesting the PAH content of diesel is substantial enough to induce similar responses.

FAME induced cellular responses.

Biodiesel exhaust emissions have elevated amounts of fatty acids methyl esters (FAMES) and fragmented fatty acids methyl esters, relative to diesel based on GC-MS analysis [34]. There is limited data associated with cellular responses and health effects due to FAME exposure and therefore biodiesel exhaust induced health effects remains unclear. An *in vitro* study comparing saturated fatty acids with methylated fatty acids (18:1) and (18:3) found that methylated fatty acids were unable to translocate iron (i.e., 59Fe isotope) into pulmonary artery endothelial cells, while saturated fatty acids (18:1 and 18:3) were able to increase intracellular iron significantly [59]. The exposure of the cells to the C18:1 and C18:3 fatty acids induced cytotoxicity likely due to increased iron transport into cells, suggesting exposure to saturated fatty acids but not methylated analogues generated adverse effects. However, in a recent study, methyl palmitate has been discovered in rat retinal tissue and was identified to function as a vasoactive dilator, based on the findings with rat aortic ring exposures [60]. Other than a few studies, most research with FAMEs did not result in significant cellular alterations. But since FAMEs are one of the most common PM-associated exhaust emissions from biodiesel, further research should be considered to better address health effects. Exhaust emissions based cellular response research is vital in directing areas of engine advancements to reduce harmful emissions species.

4.0. Engine conditions and fuel composition alter exhaust emissions composition potentially changing biological *responses.*

Reduction of PM can be achieved with modifications to combustion technology resulting in fewer PM and thereby lessening adverse health effects from inhalation exposure. The physiochemical characteristics of PM from petroleum diesel engine exhaust vary with fuel, engine conditions, fuel additives and after-treatments fitted to an engine. Emission analysis of biodiesel indicate that biodiesel blend emissions vary from the combustion of biodiesel neat and blends. Studies generally indicated that 20% blends have different emissions than 30% and 50% biodiesel regarding the increasing volume of organic soot (PM), volatile organic material (e.g., PAH) [39, 61] and CO emissions [62, 63]. The changes in the emissions such as with mass, black carbon (BC), metals, PAH, volatile organic carbon (VOC) and

CO are not necessarily directly proportional (i.e., linear) with the amount of soy-derived biodiesel added to diesel, as shown in Figure 1.3 [64].

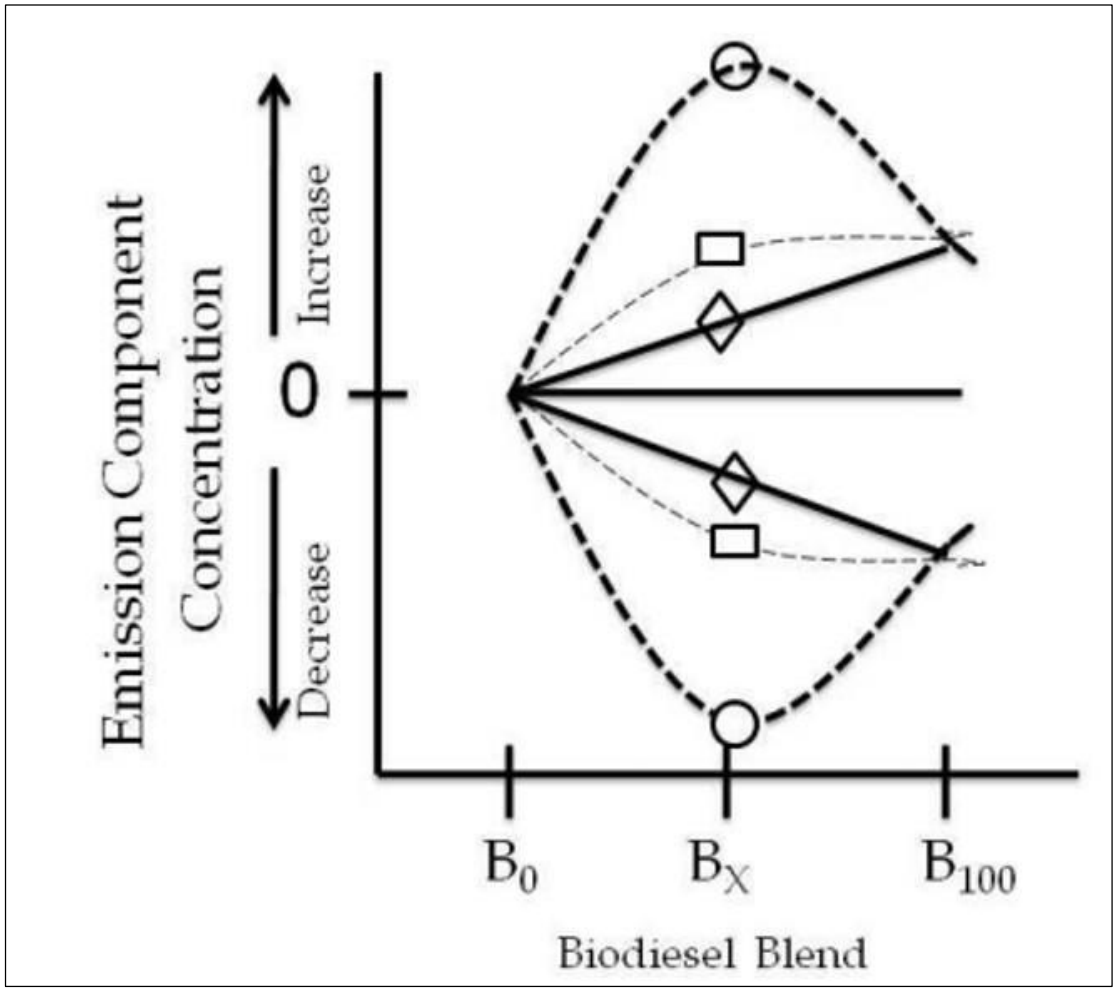

Figure 1.3. Percent change of typical combustion emissions of Diesel, biodiesel blend and B100 [65] .

Physiochemical characterization of emissions species has found that different biodiesel feedstock (type) fuels can generate unique emissions suggesting some biodiesel feedstock may be more toxic than others. For example, the type of metals and PAH differ from combustion of rapeseed methyl esters (biodiesel) blends [66], pure waste cooking oil [67, 68] and kanjar oil [42]. All three fuel types were combusted under similar European driving-cycle standards before exhaust was analyzed using ICP-OES for metals. The results indicated increased metals in plant-based biofuel and fewer metals with waste cooking oils, but the opposite is true for PAH emissions. A general lack of consistency regarding run conditions may prevent the uniformity in cellular responses from combustion emissions. One common engine-run condition that can alter the emissions is the engine load. Compared to a low- load engine, a

high- load engine raises the temperature, which causes emission of PM containing more elemental carbon than organic carbon. A rodent study comparing toxicity from emissions of high-load and low-load engines found significantly more oxidative stress, inflammation and increased sensitivity to viral infections in mice exposed to PM from high- load engine run [55]. In a separate comparative *in vitro* study with normal human bronchial epithelial cells (NHBE), exposure to high and low load combustion emissions resulted in increased expression of IL-8, a pro-inflammatory marker. Though, inflammation doubled with particles emitted from high-load emissions compared to low-load emissions [69]. These studies indicated increased inflammation response from exhaust emissions generated with high-load engine conditions. The high-load exhaust is typically characterized with enriched elemental carbon relative to low-load PM, suggesting combustion conditions affect exhaust chemicals that can interfere with cellular responeses.

Lastly, the addition of fuel additives resulted in exhaust that reduced inflammatory responses. Incorporation of fuel additives, such as iron based Satacen 3, improved the cetane value of the fuel and, reduced incomplete combustion emissions and PM. Human airway epithelium-derived cells exposed to emissions from fuel with and without Satacen 3 produced significant decreases of cytokines IL-8 and TNFα with fuel additives [70]. Not only were the exhaust emissions different, but some changes in exhaust also correlated with different cellular responses.

5.0. Clinical and Toxicological studies involving Diesel and Biodiesel exhaust emissions.

Hundreds of epidemiology studies with both ambient and occupational exposures, and validated findings from controlled human-exposure studies to diesel exhaust whole (DE) and DEP alone, convincingly indicated an association with adverse respiratory and cardiovascular outcomes. Exposure-induced responses ranged from increased neutrophils, alveolar macrophages and pro-inflammatory markers in the lung to vascular-tone alterations mediated by endothelial cells. Controlled human-exposure studies with healthy human volunteers and DEP found increased lung neutrophils in sputum and elevated inflammatory markers in bronchial washes [71, 72]. In one study with healthy young volunteers exposed to 300 $\mu g/m^3$ of DEP (for 1 hour), neutrophils increased significantly in both the bronchial epithelium and peripheral blood, suggesting an inflammatory response expanded from the site of PM deposition. Other notable inflammatory responses include a significant increase of markers: ICAM-

1, VCAM-1, and platelets in blood from diesel-exposed but not air-exposed subjects [73, 74]. Pro-inflammatory gene expression levels of IL-8, IL-18, IL-13 and IL-10 increased more than two-fold in the bronchial epithelium after 6 hr exposure to diesel, further supporting signaling of neutrophil recruitment [73, 74]. Even 1-hour DEP exposure with 300 μg/m^3 resulted in signaling increase of pro-inflammatory cytokines IL-6 and TNFα in plasma [75], possibly indicating inflammatory markers can migrate into the vasculature or may be induced by extrapulmonary tissues due to downstream responses to DEP exposure.

Human exposure studies found diesel PM not only impact lung inflammatory response, but also can lead to changes in blood flow. One controlled human-exposure study with diluted diesel exhaust containing only 100-200 μg/m3 PM found changes to vascular resistance, as measured by brachial artery diameter [76]. In this study, the results showed a 1-13% luminal diameter decrease, which was statistically significant with increasing diesel exposure concentrations. In a similar study of healthy male subjects with increased DE exposure (300μg/m^3) for 1 hour, venous occlusion plethysmography was used to measure blood flow. Unlike the previous study, these subjects were given infusions of a vasodilation agonist (e.g., endothelium dependent bradykinin and acetylcholine; endothelium independent sodium nitroprusside and verapamil) and then analyzed for blood flow changes [77]. The results indicated that DE exposure reduced the vasodilation response to bradykinin and acetylcholine but did not alter the vasodilation to the endothelial independent agonists [77]. These results suggested endothelium-dependent agonists like bradykinin are not as active or potent as endothelium-independent agonists of vasodilation after DEP exposure. This controlled human-exposure study suggests a disruption of endothelial cell-mediated vasodilation response after diesel exposure.

The vascular effects of diesel are typically linked with PM and not as strongly with gas-phase components. For instance, filtering out PM from whole exhaust resulted in attenuated vasoconstriction and ex-vivo thrombus formation in humans [75]. This study suggested that a larger contribution of particle fraction, as opposed to gas phase emissions, prompted differential vascular changes. In a rodent-exposure study, evidence supporting particle- mediated vascular effects were evaluated using diesel and ambient particles [26]. Results from this nose-only inhalation exposure identified increased endothelin (ET-3) levels in rats with diesel PM (5mg/m^3 re-suspended particles) at 36 hours after exposure without

introducing any gas phase components [26]. This study validated that particles emitted from diesel combustion contain compounds not found in gas phase or in carbon black particles that can potentiate endothelin release. Additional support for diesel particle potency was previously linked to an inflammatory response in lung neutrophil recruitment. In a study by Nightingale et al, diesel exhaust particle was collected and the PM alone was suspended in a controlled chamber at 200 $\mu g/m^3$ [78]. The subjects were exposed to the diesel particle alone for 2hrs and the results indicated increased neutrophils in sputum, suggesting that the particles alone induced inflammatory responses without including volatile gas- phase emissions from diesel combustion [78]. The compositional differences from biodiesel and diesel may help identify the individual chemical species involved in potential cardiovascular risk. Although there are several inflammation and vascular tone changes previously examined with diesel exposure, the exact pathways activated and the events leading up to the cardiovascular events are still unclear.

Recently new data was obtained from controlled human exposures to biodiesel-blend and biodiesel neat exhaust regarding exposure- induced alterations of vascular tone [79]. Biodiesel, like petroleum diesel, can also impair vasodilation in healthy human volunteers, based on measurements of brachial artery diameter changes from ultrasound detection [77]. A clinical study involving healthy volunteers with exposure to biodiesel-blend (B30) exhaust at 300$\mu g/m^3$ and biodiesel neat at 106$\mu g/m^3$ found impaired vasodilation response from significant number of subjects [79]. Additional results indicated that the blood flow into the forearm remained constricted after infusion of endothelial-dependent vasodilators such as bradykinin. In contrast, infusion of endothelial-independent vasodilator, such as vasopressin, was capable of detectable changes in diameter in Biodiesel exposed subjects, suggesting that the impairment was localized to the endothelial cell mediator production or release [79]. Endothelial cell mediated vascular tone is regulated by multiple pathways and signaling molecules, but the exact mechanism by which biodiesel exposure impaired vasodilation is not yet clearly understood. Exhaust (i.e. both particle and gas phase emission) composition interference with the endothelial cells mediated regulation of vasodilation signaling was a proposed mechanisms for biodiesel and diesel exhaust induced impairment of vasodilation.

6.0. Pathway specific diesel and biodiesel exhaust responses.

Diesel and biodiesel PM-induced cardiovascular dysfunction was mediated by a few pathway specific responses. Some of the known activated pathways include oxidative stress, pro-inflammation signaling and alterations of endothelial cell-mediated vascular tone. Within each pathway exist several different types of signaling markers that include peptides, proteins and lipids. These signaling markers can be measured to assess the potency of each combustion PM. There are there multiple markers for each pathway that can function independently or in concert with other markers to potentiate a singular pathway response. Similarly, diesel and biodiesel PM exposure can initiate multiple pathways that may act independently or in concert with other pathways to generate adverse cardiovascular events.

Oxidative stress response detection from exposure to biodiesel and diesel PM.

Oxidative stress pathway is one of several pathways activated with diesel and biodiesel exposure and it is measured by changes in free radicals, antioxidants or lipid peroxidation species. Exposure studies conducted *in vivo* and *in vitro*, measured DEP exposure induced cellular increases of free radicals and depletion of antioxidants. *In vitro* AM exposure studies with diesel extract generated excessive oxidative stress products such as H2O2 and $O2^-$ [80]. Simultaneously, some exposure studies show depleted antioxidants and consequently increased gene expression levels. Common antioxidant enzymes that neutralize the reactive oxygen species (ROS) are: hemeoxygenase (HO-1), catalase, peroxidase or superoxide dismutase (SOD). Several studies with *in vitro* exposure to diesel and biodiesel (e.g., B99) found elevated antioxidant mRNA and protein expression of SOD [81, 82]. Additionally studies have found depletion of intracellular reduced glutathione as an indicator of oxidative stress. Mice pulmonary exposure to DEP resulted in both decreased GSH and SOD in lung [81]. Markers of oxidative stress are not limited to detection of free radicals and antioxidants but include lipid peroxidation species. Studies with rodent exposures to DEP and gasoline emissions, resulted in elevated lipid peroxidation species discovered in lung tissue [55, 83]. Lipid peroxidation products were also detected at high concentrations in exhaled breath [84, 85] and urine post exposure to concentrated ambient fine and ultrafine PM [85] and with wood smoke exposure [84]. These studies show strong correlation of reactive oxygen species

(ROS) with exposure to both combustion PM from diesel and biodiesel suggesting there are some shared pathways but verification requires observation of other markers for oxidative stress.

Isoprostanes are lipids, though not directly derived from AA, that affect the vascular system and are frequently used as markers of oxidative stress. Specifically, 15-F2t-isoprostane (15-F2t-IsoP), has been found to be activated within oxidative stress responses from exposure to diesel and biodiesel exhaust [86]. Rodent exposure with 200μg/m^3 of diesel $PM_{2.5}$ for seven weeks resulted in significantly elevated 15-F2t-IsoP levels in urine [87]. This study indicated elevated oxidized lipid changes due to diesel exposure which can remain above baseline for over seven weeks. Thus, the data emphasizes the continued production and robust longevity of lipids as markers of oxidative stress. ROS products if not neutralized may initiate systemic inflammation signaling.

Pro-inflammation markers found with diesel and biodiesel exhaust exposure.

Diesel and biodiesel exhaust exposures have been identified to induce pro-inflammatory responses *in vivo* and *in vitro*. DEP exposure induced significantly elevated expression levels of inflammatory markers, IL-6 and TNFα, in human cell culture models as well as in human plasma [75]. In one study, *in vitro* exposure of DEP particles resulted in significantly elevated mRNA levels of IL-1, after only 2hrs of exposure, which indicated the cytokine production initiated post-phagocytosis by alveolar macrophages (AM) [88]. A significant increase of cytokines (e.g., IL-6 and IL-8) resulted from biodiesel-blend exposure to epithelial and endothelial cells [43, 89, 90]. Post endothelial cell exposure to biodiesel, IL-8 mRNA levels were slightly increased [90], but not significantly suggesting the inflammation response was not as robust in vascular cell culture models as it was in AM and human plasma. However, most biodiesel and diesel exposures to date have indicated a consistent increase of cytokine mRNA and protein [43, 90].

Potent de novo inflammation-signaling markers belong to a family of lipids called eicosanoids (i.e., oxygenated AA products) which function primarily in signaling and recruitment of additional inflammation mediators. One subfamily of lipid inflammation mediators, called prostaglandins, is dependent on a canonical pathway involving arachidonic acid (AA) metabolism. AA is the precursor for prostaglandins, which are produced by enzymes COX-1 and COX-2. The isoform COX-1 is constitutively

expressed in cells, and COX-2 formation is generally induced in response to inflammation. The COX-2 gene is co-activated by transcription factor activation of cAMP response element (CRE). Diesel exhaust exposure-induced stress has been found to activate and increase COX-2 gene expression and protein. It has been shown that DEP exposure of lung epithelial cells resulted in dose dependent increase of COX-2 protein and mRNA [91]. COX-1 and COX-2 are critical for enzymatic production of the lipid inflammation and immune system mediator prostaglandin E2 (PGE_2) from the precursor AA. Another study with diesel extracts fraction exposure to bronchial epithelial cells found a combination of COX-2 protein and PGE_2 production was significantly increased relative to control [69], suggesting that the COX-2 increase is directly responsible for the increased PGE_2 production and release. However, a separate contrasting *in vitro* study with human alveolar macrophages found diesel PM exposure resulted in decreased PGE_2 levels [92]. The conflicting results were proposed to be related to particle binding of PGE_2 which prevents accurate detection. A complementary challenge with endotoxins resulted in a return of PGE_2 to control levels which indicated the sequestering by particle was reversible and no permanent damage to the PGE_2 production occurred [92]. This study also signified the role of lipid mediators in inflammation responses. Additionally, a study of diesel exposure to monocyte- derived macrophages that were co-simulated with LPS, found significant increases of PGE_2 release compared to cells exposed to only LPS or only DEP. Thus, suggesting that there was synergy with LPS and DEP response resulting in significantly increased PGE_2 levels [93]. These studies suggested that prostaglandins are detectable inflammation markers with DEP extract and detection can be complicated with whole particle sequestering. Furthermore, there are other lipid inflammatory markers derived from AA metabolism. Other AA metabolites are cell type specific, unlike prostaglandins. Leukotriene B4 (LTB4) largely functions in lung cells. Its primary function is to induce the adhesion and activation of leukocytes to the endothelium. One in vivo study found LTB4 was altered with diesel exhaust exposure. In this two-day study, with mice, whole DE exposure resulted in 3-fold greater LTB4 levels in BALF relative to air [94]. Based on DE exposure induced changes to production of these AA derived lipids signals (e.g., LTB4, PGE_2, 12(S)-HETE), there was increased interest to evaluate biodiesel exposure induced response of lipid inflammation signals.

Vascular tone alterations mediated with NO, ET-1 and PGI_2

Controlled human exposures to biodiesel blend (RME30), biodiesel neat and diesel indicated impaired vasodilation response however the exact mechanism(s) remains unclear. Vasodilation is regulated by the parasympathetic system, as well as by signaling molecules released from the kidney, heart and endothelial cells (i.e., artery and vein). Researchers in this field are actively pursuing proof of a mechanism of action related to the parasympathetic system and signaling molecules generated from the kidney or heart. Additionally, the smooth muscle cells can respond to variable signaling molecules to initiate calcium influx which results in calcium based relaxation response. As previously mentioned, studies have recently identified that controlled human exposures to DE, B30 and B100 resulted in impaired vasodilation and the mechanism behind this may be associated with vascular mediators produced by endothelial cells [77, 79, 95]. The following studies identified that there are a number of signaling molecules associated with endothelial cell mediated vasodilation which includes NO, ET-1 and prostaglandins (PGI_2) which maybe altered by diesel or biodiesel exhaust exposure.

Vascular dilation was regulated via NO, which was produced when O_2 couples with an endothelial NO generating enzyme (eNOS) to generate NO. Diffusion of NO into the nearby vascular smooth muscle activates cyclic guanosine monophosphate (cGMP) and soluble guanylate cyclase (sGC) to generate smooth-muscle relaxation. A diesel study with rodent exposure to 300μg/m^3 for 5 hrs, resulted in NO reduction. Additionally, post-exposure coronary arteries (CA) were isolated and resistance was measured. The results found DE-exposed CA had significantly decreased dilation relative to air [96], suggesting that DE exposure may impair relaxation response. In the same study, DE-exposed CA generated an increase in superoxide anion ($O2^-$), which decreased the ability of eNOS to uncouple thereby offering a potential mechanism for NO reduction. This assay, along with the previous resistance measures, strongly suggests vascular tone impairment was caused by exposure to whole diesel exhaust and involved NO-mediated dilation impairment.

In a second study, aortic rings were isolated from Wistar Kyoto rats and exposed to DE extract (100μg PM equivalent /mL concentration) [97]. The NO concentrations initially doubled from control samples compared to diesel and shortly after the diesel exposed aortic rings were significantly decreased

[97]. These results indicated reduced NO released from rat aortic endothelial cells post diesel exposure, which suggested that NO reduction may explain impairment of vascular relaxation post DE exposure.

Endothelin-1 (ET-1) is a peptide which is known to induce vasoconstriction and is released primarily from endothelial cells. ET-1 is the most researched endothelin isoform that acts on neighboring smooth muscle to initiate constriction. The ET-1 isoform is the substrate released from endothelial cells that binds to either ET A /B receptors (ET_AR / ET_BR) which are G-protein coupled receptors located on smooth muscle cells. The ET_AR is located on smooth muscle cells and when bound by ET-1 will initiate the vasoconstriction process in smooth muscle cells. The ET_BR is located on both on endothelial cells and smooth muscle cells. The ET-1 activated ET_BR receptor located on endothelial cells can initiate release of other vasodilators (i.e., NO and PGI_2), contrastingly ET_BR on smooth muscle cells binds ET-1 and activates vasoconstriction. The same receptor may induce opposite responses if ET-1 binds to receptor A or B. Currently endothelin antagonists have therapeutic uses and function in reducing pulmonary hypertension and arterial hypertension in patients [98, 99]. PM -induced changes in ET-1 release were found in studies of rodent exposures to both urban dust (SRM1649) [100] and mixed-vehicle emissions (e.g., gasoline and DE) [83, 101]. Increased circulating ET-1 levels in plasma suggested that an endothelial cell response to PM exposure was to release ET-1.

An *in vivo* study conducted with rodent pretreatment with receptor antagonists BQ-788 or BQ-123, ET_BR and ET_AR antagonists respectively, prior to DE inhalation exposures found that only ET_BR antagonism resulted in diminished ET-1 mediated vascoconstriction. After 300µg/m^3 DE exposure for 5 hrs resulted in lack of ET_BR receptor activation in coronary arteries, suggesting that DE only inhibited the activation via ET_BR receptor [102]. The study indicated diesel exposure did not alter vasoconstrictive responses, but receptor induced response (i.e., vasodilation) was inhibited. The results of these studies suggest that the diesel exposure induced vasoconstriction response can be explained with mechanisms involving ET_BR and the impaired endothelial cell response to release NO and prostaglandin I2 (PGI_2).

The endothelial cell-mediated potent vasodilation signal not yet discussed is the AA metabolism derived lipid mediator called prostacyclin, PGI_2, which is a member of the prostaglandin family. As shown in Figure 1.4, prostacyclin is a product of AA and COX-1 and 2. Another lipid metabolite derived from AA

metabolism is prostaglandin F2α, $PGF_{2\alpha}$, which inversely functions to signal vasoconstriction of smooth muscle cells. PGI_2 binds the IP receptor located on smooth muscle cells to activate calcium-mediated cAMP activation that is required to initiate calcium-dependent relaxation of veins and arteries. The stable metabolite of PGI_2, 6-keto-$PGF_{1\alpha}$, does not bind the IP receptor but it is synonymous with the amount of PGI_2 produced in cells. To date PM exposure studies have no direct vascular tone measures regarding PGI_2 or $PGF_{2\alpha}$ levels with endothelial cell exposures to diesel exhaust in either rodents or humans. However, in one study of DEP-exposed ApoE-/- mice, the $PGF_{2\alpha}$ plasma concentrations were reduced from control [103], suggesting alterations occur with lipid AA metabolites after diesel exposure. The ApoE-/- mice were treated for 1 hour with intraperitoneal injection of DEP suspended in saline and then immediately blood was collected to measure the amount of $PGF_{2\alpha}$ in plasma [103] and though the data is not significant it indicated levels were decreased relative to control.

Though diesel and biodiesel combustion PM studies have not been examined for PGI_2 changes, other combustion sources were previously analyzed. Cigarette smoke exposure has resulted in changes to PGI_2 levels [104]. In a exposure study with cigarette smokers, a reduction of urinary PGI_2 was found in chronic smokers compared with to non-smokers [104]. Another study with HUVECs exposure to cigarette smoke condensate found a dose-dependent decrease in 6-keto-$PGF_{1\alpha}$, confirming similar *in vivo* and *in vitro* responses. There were critical soluble compounds found in both that disrupted the endothelial cell mediated lipid vasodilation response [105]. This study, combined with the previous evidence of lipid changes with PM exposure suggests that further research can bring awareness of lipids as detectable mediators of exposure induced damage. AA metabolism can generate other eicosanoid subfamilies such as hydroxyeicosatetraenoic acids (HETEs) which indirectly activated inflammation and vasoactive regulation functions. HETEs are created by specific lipoxygenase enzymes that have elevated mRNA expression under pro-inflammatory conditions [106]. Free AA in endothelial cells is taken up by 12-lipoxygenase to generate 12(S)-HETE, which functions as a vasodilator and is the major metabolite in mouse mesenteric arteries [107]. An additional reason to suspect diesel exposure induced changes to lipid mediators of vasodilation is due to lipid regulation via the inflammation response. Studies suggests that an inflammation response specifically from cytokine production and COX-2 protein increases can

alter PGI_2 and PGF_{2a} homeostasis. Independent studies conducted both *in vivo* and *in vitro* linked elevated TNFα and IL-1β levels in endothelial cells with increased production of PGI_2 [108]; [109]. TNFα and IL-1β bind to the endothelial cell receptors to initiate phosphorylation cascades, which led to the activation of cytosolic phospholipase A2 (cPLA2), which can release esterified AA from the phospholipid membrane for conversion into PGI_2 via COX-1 & 2 and PGI_2 synthase. The signaling cascade response from TNFα and IL-1β stimulation effectively released AA which has been shown as the limiting agent in PGI_2 and $PGF_{2α}$ production in most cell types [108]. The release of prostaglandins, which have roles in vascular tone and functions in pro-inflammatory responses suggest that lipids are good markers to study biodiesel or diesel exhaust exposure induced cardiovascular injury.

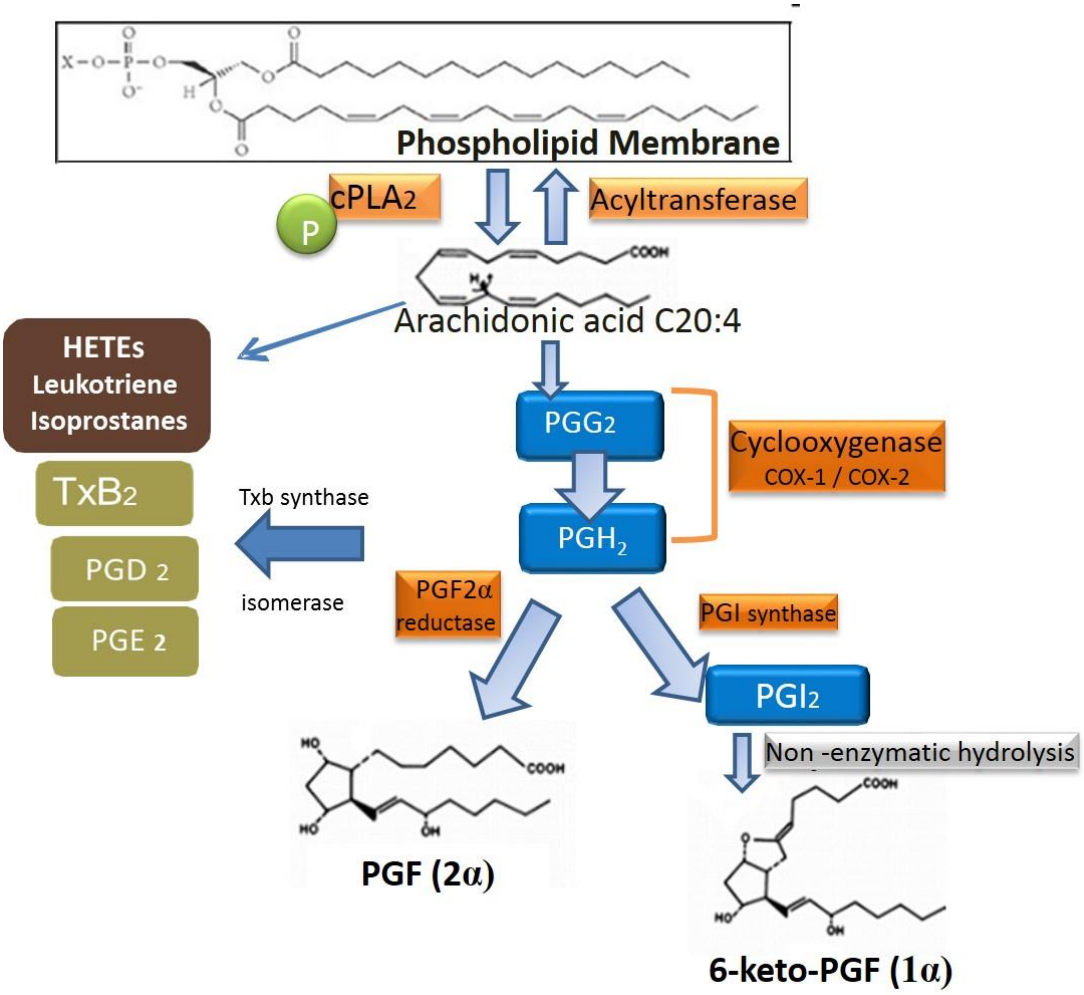

Figure 1.4. Arachidonic Acid Metabolism Pathway and Metabolites.

7.0. Importance of Arachidonic acid metabolism in maintaining cellular homeostasis.

PM exposures induce cellular responses that include disruption or activation of inflammation, oxidative stress and vasodilation. Lipids signals derived from AA metabolism function in similar cellular responses therefore diesel and biodiesel exposure induced changes can be compared by evaluating AA metabolites. Persistent exposure induced inflammation may lead to systemic pathological and physiological changes such as atherosclerosis and hypertension. Progression of disease states such as atherosclerosis and hypertension is indicative of unrestricted inflammation, oxidative stress and alterations of vascular tone, however, the importance of AA metabolites is not well understood. Clinical studies can address the importance of enzymes and AA metabolites.

The role of COX has been evaluated and inhibitors have been developed to suppress the inflammation and vascular tone response. Aspirin and acetaminophen are designed to inhibit the enzyme-active site preventing prostaglandin production (irreversibly and reversibly), thereby minimizing inflammation response via activation of prostaglandin E_2 receptors, EP_2 or EP_4, to initiate cAMP signaling that impairs induction of inflammatory mediators [110, 111]. Mice deficient of COX-2 (i.e., knockout mice) show exacerbated pulmonary artery hypertension compared to wild-type controls without any treatment, suggesting COX-2 has endogenous functions [112]. Additionally, prostacyclin receptor knockout mice were found to have similar responses basally, resulting in pulmonary artery hypertension [112]. Second, iloprost is a drug mimetic of PGI_2 and has been used effectively in ischemic reperfusion to increase blood flow, indicating that the role of PGI_2 is to signal vasodilation [113]. PGI_2 levels and enzyme prostacyclin synthase activity were also reduced in cells from pulmonary artery hypertensive patients relative to normotensive subjects [114], validating that the physiological response of PGI_2 in humans is to increase blood flow. The AA metabolites are important to study because they have recently become implicated in atherosclerosis. Membrane bound release of AA from the phospholipid bilayer is the first step in AA metabolism which is regulated by feedback enzymes phospholipases and acyltransferases. Recent findings suggest treatment for atherosclerosis may rely on the inhibition of acyltransferase enzymes. A drug, eflucimibe, can inhibit acyl-CoA acyltransferase enzyme which is responsible for repopulating the AA and cholesterol in cells [115]. The inhibition of formation of AA and cholesterol esters and subsequent accumulation within macrophages, eliminates the formation of foam cells and

atherosclerotic plaque [116]. These studies validate the importance of AA metabolism response in regulating inflammation, vasodilation (blood flow) and precursory events that generate plaque buildup in humans. The focus of this dissertation is on AA metabolites that function primarily in inflammation and vascular tone changes in response to diesel and biodiesel exposure.

Importance of other endothelial cell functions related to AA metabolism.

Inflammation and vascular tone changes are two of the primary outcomes found with biodiesel and DEP exposure that are linked to AA metabolites. There are, however, other endothelial cell functions related to AA metabolism such as lipid remodeling and balancing pro-coagulation signals. Release of intracellular AA is associated with lipid remodeling enzymes in multiple cell types. In a diesel emissions exposure study of mice, gene expression data collected of lung identified lipid remodeling genes changed significantly relative to air exposed animals [117]. Though the exact mechanism is still unknown this study emphasizes the need for a more comprehensive assessment of the importance of AA metabolism. Free AA can be either re-esterified back into lipid pools (e.g., phospholipids) or metabolized and converted into eicosanoids. Re-esterification of released free AA is important in reducing intracellular levels of fatty acids. Lipid-esterification enzymes are feedback-regulated by endogenous fatty acids as part of the Land's cycle [118]. Release of free AA begins when calcium-dependent cPLA2 or phospholipase C (PLC) is activated by phosphorylation and transported to the phospholipid membrane. Phosphorylation of cPLA2 can occur via a number of mechanisms, but it is most likely initiated by MAPK signaling cascades. Calcium, a co-factor of cPLA2, is either released from intercellular pools or it enters from the external environment. Calcium can also be released via particle interference when local ROS-dependent loss of organelle membrane integrity. The elevated ROS response has been seen with both diesel and biodiesel studies, indicating that this method of cPLA2 activation is likely. Once the cPLA2 reaches the membrane, cPLA2 will cleave at the sn2 position releasing esterified AA (C20:4) into the cell (Figure 1.4). The release of free AA is regulated by multiple pathways and enzymes which can be disrupted by the presence of exogenous free fatty acids [Figure 1.5] [118, 119]. A study by Ciapaite et al found exposure of endothelial cells to free fatty acids (i.e., palmitate (C16) and oleate (C18)) can disrupt NF-κB and alter inflammatory pathways [119]. In addition other studies have found induction of C-

reactive protein expression in endothelial cells suggesting that fatty acid exposure may disrupt cellular homoeostasis [120].

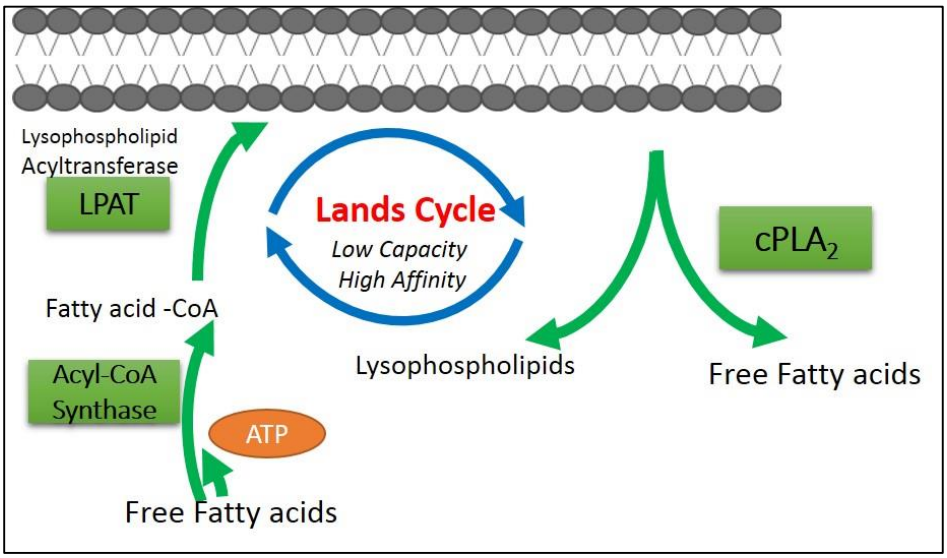

Figure 1.5. Land's Cycle: Deacylation and reacylation (esterification) cycle regulating membrane phospholipids/lysophospholipid balance with arachidonic acid and other fatty acids.

Ambient PM and diesel PM exposure have not been identified to induce immediate changes to the Land's cycle, but ROFA was identified to increase the amount of cPLA2 in airway epithelial cells post exposure, resulting in increased AA levels and some eicosanoids [46]. Several subfamilies of molecules are generated from AA metabolism, including HETE's, leukotrienes, isoprostanes and prostaglandins. The evidence supports the role of PM exposure-induced changes in eicosanoids.

Balancing pro-coagulation mediators via AA metabolism.

As indicated in Figure 1.4, PGH_2 is converted into TxA2 and then thromboxane B2 (TxB2). This is the stable form of TxA2, whose primary function is to initiate platelet aggregation as part of pro-thrombotic/pro-coagulation responses via activation of thrombin. Coagulation, when combined with systemic inflammation, initiates the events for plaque buildup, causing atherosclerosis. *In vitro* DEP-exposure of blood at PM concentrations between 2.5-5.0ug/mL showed that platelet responsiveness to collagen was significantly suppressed in a dose-dependent manner [121]. Although with this study the exact mechanism is unknown, exposure to the diesel soluble fraction was able to induce a reduction in platelet aggregation in blood collected from healthy human volunteers. The results suggested a possible

alteration of TxA2 formation, since the ability to generate thrombin was significantly reduced from control. In contrast, another study examined platelet aggregation with direct DEP exposure *in vitro* using platelet-rich plasma collected from control mice [122] and found increased platelet aggregation in a dose-dependent manner. These results suggest that DEP exposure can directly affect the coagulation response [122]. Previous studies have identified that AA metabolites may affect pro-coagulation due to cross-talk between the coagulation and inflammatory pathways. Studies indicated release of PGI_2 and TxA2 were typically inversely regulated [123], showing platelet aggregation was inhibited when PGI_2 increased. Additionally, the enzyme thromboxane-A synthase is found in platelets and gives them the ability to convert PGH_2 into TxA2/B2 that results in the phenomenon termed "endoperoxide stealing." This allows platelets to intensify TxA2 production by use of extracellular PGH_2 released from other cells including endothelial cells especially if the endoperoxide is not rapidly converted into prostaglandins or PGI_2. Conversely when TxA2 synthase is inhibited, endothelial cells have been observed to utilize platelet endoperoxides. Although this "steal" process was observed *in vitro*, little is known in vivo [124]. The results from DEP exposure suggest a role for TxA2, but the research is still preliminary and more insight would be required on AA metabolism to evaluate a mechanism of action.

The AA metabolites are key players in a few pathways activated with diesel exposure. Shared pathways between biodiesel and diesel include inflammation, oxidative stress and alterations of vascular tone in which AA metabolites were present, suggesting that measurement of lipids can be a useful tool to examine diesel and biodiesel-exposure induced effects. Examination of biological effects induced from biodiesel exposure is a relatively new field and all areas of potential research should be investigated. Additionally, the role of lipids signaling markers generated from AA metabolism should be closely examined, as studies showed that diesel exhaust affects cellular responses regulated by lipids.

8.0. Scope of dissertation.

Reviewing the findings discussed above with biodiesel and diesel exhaust inhalation exposure suggests that these pollutants induced inflammation and vascular tone changes that may involve AA metabolites. The exact mechanisms by which inhalation exposure, of diesel and biodiesel exhaust, induced inflammation and impaired vasodilation is currently unclear, yet evidence suggests translocated bioactive components from the exhaust play a role. Additionally, particles and some soluble bioactive

components from diesel were identified altering lipid mediated cellular responses such as PGE_2 [46, 69, 92, 125], suggesting there is potential for disruption of other lipid signals. My overarching hypothesis for this dissertation project is that AA metabolism plays a role in inducing adverse outcomes from diesel and biodiesel exhaust exposure. The primary focus of my research will be on the role of AA metabolism, in both alveolar macrophages and vasculature endothelial cells. Lipid metabolites from the airways and vasculature can act as inflammogenic mediators and vasoactive regulators. Based on the new information on biodiesel exposure response this dissertation may establish an alternative mechanism of action for impaired vasodilation and identify potential PM differences or similarities in cellular vasoactive signals.

Specific Aim 1. Determine if diesel and biodiesel PM will alter AA metabolites of alveolar macrophages with *in vitro* exposure. Alveolar macrophage exposure to diesel decreased basal PGE_2 levels [92], indicating changes in AA metabolite production, yet nothing is known regarding biodiesel exposure. PM studies have indicated the physiochemical characteristics of PM can determine the cellular response. Most components of exhaust particle do not exert independent cellular responses however some are immediately associated with specific cellular response. For example, metals can induce oxidative stress, elemental carbon can induce inflammation and PAHs can induce both mutagenicity and inflammation. Both diesel and biodiesel vary in the type and amount of elemental carbon, metals and PAHs, suggesting that they could generate different cellular responses. The goal of this aim was to compare biodiesel PM exposure with diesel, to establish how the exhaust particle composition determines differential AA metabolite release.

Specific Aim 2: Develop an extraction method customized for biodiesel exhaust particle based on improved physiochemical characterization and toxicity studies. The purpose of this aim was to establish a method that extracts a wide range of polar and non-polar soluble compounds that are bioactive components of diesel, biodiesel and biodiesel/ diesel –blends exhaust particle. These extractions can be utilized in cell culture for toxicology studies to compare cytotoxicity of biodiesel and diesel. The challenge with current soluble extraction methods is the inherent bias for optimally extracting the organics, typically nonpolar hydrocarbons, found in petroleum diesel not in biodiesel exhaust [44, 67]. In this aim, we will address these challenges of the extraction processes, extract suspensions and develop an appropriate extraction method for biodiesel exhaust PM that can be optimally used in cell culture.

Specific Aim 3. Determine if endothelial cell exposure to the diesel and biodiesel soluble-bioactive components from the novel extraction method, will disrupt AA metabolism. Endothelial cell regulation of vascular tone is mediated in part by lipid AA metabolites (i.e., prostaglandins and prostacyclin). The alterations of synthesis and production of lipid metabolites in endothelial cells can lead to vasoconstriction and potentiate hypertension [114]. Previously reported cigarette smoke reduced production of PGI_2, suggested that exposure to combustion derived PM can alter the production of endothelial cell dependent vaso-active mediators [104]. Based on extract composition mediated cellular responses from biodiesel and diesel emissions, the objective of this aim was to compare if chemical differences result in biological differences. The potency of soluble bioactive components of diesel and biodiesel were used to study the AA metabolites from endothelial cells which function primarily in vasodilation.

Exposure-induced biological responses from complex-combustion emissions has long been a challenge to study, however, based on these three specific aims, a framework has been developed to study AA metabolite changes. Initial response comparisons of diesel and biodiesel PM will be determined in specific aim 1. They will be conducted in AMs, which are the first to encounter inhaled particle. In specific aim 2, physiochemical assessment of PM associated compounds, essentially establishing a unique fingerprint for comparing biodiesel and diesel exposures. Finally, in specific aim 3, I will determine an endothelial cell mediated mechanism of release of prostacyclin that is associated with AA metabolites. The mechanism of action in endothelial cells will be examined with exposure to soluble bioactive components of biodiesel. These studies together will provide new information on biodiesel and diesel exposure-induced endothelial cell mediated vascular dysfunction.

Chapter 2

Diesel and biodiesel exhaust particle effects on WKY rat alveolar macrophages with in vitro exposure

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2.0 Overview

Combustion emissions from diesel engines emit particulate matter which deposits within the lungs. AMs encounter the particles and attempt to engulf the particles. Emissions particles from diesel combustion engines have been found to contain diverse biologically active components including metals and PAHs which cause adverse health effects. However little is known about AMs response to particles from the incorporation of biodiesel. The objective of this study was to examine the toxicity in Wistar kyoto rat AMs of biodiesel blend (B20) and low sulfur DEP particles. Particles were independently suspended in media at a range of 1-500μg/mL. Results indicated B20 and DEP initiated a dose dependent increase of inflammatory signals from AMs after exposure. After 24hr exposure to B20 and DEP gene expression of COX-2 and macrophage inflammatory protein 2 (MIP-2) increased. B20 exposure resulted in elevated PGE_2 release at lower particle concentrations compared to DEP. B20 and DEP demonstrated similar affinity for sequestration of PGE_2 at high concentrations, suggesting detection is not impaired. Our data suggests PGE_2 release from AMs is dependent on the chemical composition of the particles. Particle analysis including measurements of metals and ions indicate B20 contains more of select metals than DEP. Other particle components generally reduced by 20% with 20% incorporation of biodiesel into original diesel. This study shows AMs exposure to B20 results in increased production of PGE_2 in vitro relative to diesel.

2.1. Introduction

Inhaled diesel exhaust particles deposit in the lungs where individual AM engulf particles via phagocytosis. Phagocytosis initiates a response from AM to trigger an inflammatory response which includes release of cytokines, lipid mediators and other signals to recruit neutrophils to deposit site. *In vivo* exposures to DEP particles with guinea pigs and rats revealed phagocytosis by AM and increased inflammation response [88, 126]. Previous studies indicate human macrophages release cytokines IL-6 and TNFα after exposure to coarse and ultrafine particles of diesel exhaust indicating a heightened inflammatory response [127]. Exposure to filtered diesel exhaust and unfiltered resulted in both types causing similar inflammation responses from human AM from BALF, suggesting the particle and its composition plays a leading role in AM response [128]. The composition of the particle and potentially extractable components vary due to incomplete combustion which directly affects inflammation. Inflammation from DEP can be measured with release of arachidonic acid metabolites such as prostaglandins. PGE_2 release indicates an inflammatory response to recruit neutrophils and may also signal helper T cells [129]. Diesel particle exposure interferes with immune responses including AM suppressed phagocytic response to bacterial challenge [130]. *In vitro* diesel exhaust particle exposure has also lead to increased PGE_2 release from monocytes [93]. DEP phagocytosis and inflammation response is well studied however not much is known about B20 effect on AM.

Recent toxicity studies found biodiesel induced similar pro-inflammatory and oxidative stress responses as seen with diesel. The exposure of biodiesel exhaust to rats indicates an increased number of AMs in rat lungs and many had engulfed particles[131]. A rodent study involving intratracheal instillation of both DEP and biodiesel in mice found significantly elevated protein levels in BALF after 24h, indicating an increase of protein inflammation signaling molecules and recruited polymorphonuclear neutrophils [44]. Previous studies indicate increased release of IL-6 and IL-8 with human bronchial epithelial cells exposure to a methylene chloride extract of biodiesel blend *in vitro* [132]. Most studies of cellular responses to biodiesel thus far, are a reflection of the particle not necessarily a reflection of the composition of the biodiesel particle. If we compare the biodiesel particle to the petroleum diesel particle we find there are fewer numbers of PAHs and aldehydes present in the emissions [34, 133, 134]. The biodiesel blend emissions emits fewer acrolein and nitro-PAH's [34, 57]. The particle composition of DEP

consists of an inert carbon core bound with hydrophobic components whereas B100 contains hydrophilic compounds bound to the core [133]. Additionally, incomplete soy biodiesel combustion was identified to emit unique chemical species (ie, methylacrylate & methyl butonate) believed to be fragments of methylated fatty acids esters [34]. The gradual incorporation of biodiesel into petroleum, identified by Brito et al[64], indicates CO_2 and PAH decrease but not all emissions decrease. This non-linear emissions data can imply an irregularity in efficiency of combustion. A study by Tsai et al indicates the physiochemical difference in biodiesel and petroleum diesel exhaust can be due to incomplete combustion of soy methylated esters [57]. In this study, we include composition analysis and cellular responses to petroleum and soy biodiesel blend exhaust particles (B20).

Previous observations of AM initiating an inflammatory response to inhaled DEP particles lead us to hypothesize similar inflammation from our B20 and DEP. We have designed an approach to study freshly collected and cultured WKY rat AM to particle exposure in vitro. At non-cytotoxic particle concentrations AM release cytokines and prostaglandins indicating inflammation. The enzymes COX-1 and COX-2, produce PGE_2 relative to the intensity of the inflammation response. Our objective of this study was to examine macrophage response and the release of arachidonic acid metabolites as a result of in vitro exposure to 20% B20 in comparison to DEP.

2.2. Materials and Methods

Particle collection

The B20 and low sulfur DEP fuels were combusted with a 300hp, 5.9L 2002 Cummins ISB engine. The engine was 2004 EPA heavy duty emission regulations certified. The system was equipped with exhaust gas recirculation (EGR) and high pressure common rail fuel injection. The engine was also fitted with a Johnson Matthey CCRT and consists of a diesel oxidation catalyst (DOC) and diesel particulate filter (DPF). Soot samples were collected by operating the engine at 2000rpm and 61N-m. These operating conditions created low DPF temperature (approximately 190°C) which allowed the PM to accumulate on the DPF without oxidizing. The soot was then collected by back flush with compressed air off the DPF into a collection canister. Soot samples were stored under nitrogen in glass jars until the time of analysis [16].

Particle chemical composition

The study samples and a standard reference material: NIST SRM1649a – urban dust from Washington D.C. were analyzed for acid-soluble inorganic elements, water-soluble inorganic elements, ions and carbon content. Acid digestions were performed with 1.0 mL of 3:1 hydrochloric acid: nitric acid (Optima grades, Thermo Fisher Scientific, Fair Lawn, NJ) for 48 hr at 60°C. Ultrapure deionized water (Millipore Milli-Q, Bedford, MA) extractions were performed with 10mL at room temperature. SRM 1649a was used as an internal control; our averaged values of metals from acid digestion were compared to NIST published values. The mixing cycle was repeated twice, and finished with 1 min vortexing. Supernatants from acid digestions and water extractions were centrifuged at 17,000g, filtered through 0.2µm syringe filters. Elemental analysis was performed using inductively coupled plasma-optical emission spectrometry (ICP-OES), following U.S. Environmental Protection Agency (EPA) Method 2007 revision 4.4 (U.S. EPA 1994), using an axially-viewed, simultaneously-measured instrument (PerkinElmer 4300DV ICP-OES, Bridgeport, CT). Independently-sourced, matrix-matched calibration (VHG Labs, Manchester, NH) and quality control (SPEX Certiprep, Metuchen, NJ) standards were used as specified by the previously mentioned analytical protocol. For carbon analysis, homogeneous suspensions of study samples in 10mL of HPLC-grade methanol were sonicated (Thermo Fisher Scientific, Fair Lawn, NJ) in an ultrasonic bath at 220W for 30 min, then 150 µL was collected onto pre-fired 1.45 cm2 quartz filters and dried in a class 100 clean bench prior to analysis. Carbon fraction analysis was sent to Sunset Laboratories (Hillsborough, NC) which used a thermo-optical method based on sequential pyrolytic vaporization with detection by transmittance (Model 107A, Sunset Laboratory Inc., Tigard, OR) [17].

Endotoxin analysis

Although aseptic technique was followed for both collection and storage of particles, as a precaution a bacterial endotoxin characterization test was conducted by Cape Cod Inc (East Falmouth, Massachusetts). A gel clot method was conducted following Cape Cod standard operating procedures. 5mg of collected PM was re-suspended with 5mL of Pyrotell® for a concentration of 1mg/mL, the particle suspensions were incubated on an orbital shaker for 1 h before analysis. Endotoxin gram positive

contamination was evident if a gel had formed. The assay has sensitivity up to 0.03EU/mL. Both B20 and DEP samples were tested against the positive control the Escherichia coli 0113:H10.

Redox cycling activity of particles

A cell free assay was conducted to assess if particles alone could initiate direct redox cycling. The DEP and B20 were suspended at 1.0mg/mL in DI water. Thiobarbituric acid reactive substances assay was used for (TBARS) quantification reactive species using a previously established protocol [53]. Protocol was modified to include one additional centrifugation at 10,000g for 30 min after initial incubation. Absorbance was detected at 532nm. The positive control used in this experiment was residual oil fly ash (ROFA; a vanadium rich particle) at a 1mg/mL concentration.

Animals

Healthy male Wistar Kyoto (WKY) between 11-17 weeks old, were purchased from Charles River Laboratories, Inc. (Raleigh, NC). All of the animals were placed in an isolated animal room in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC). The animal facility maintained an environment of 21°C (± 1°C), 50% (± 5%) relative humidity and a 12 hour light- dark cycle. The rats were housed two to a cage. Rats were allowed to adjust to their environment for at least a week before AMs were collected. All animals were provided, standard (5100) Purina rat chow (Brentwood, MO) and water ad libitum. The protocol for this study was approved by the U.S Environmental Protection Agency (EPA) Animal Care and Use Committee.

Collection of alveolar macrophages

All animals were anesthetized with euthasol in a 1:1 dilution with saline measured according to body weight, given by intra-peritoneal injection and exsanguinated via abdominal aorta. The trachea was cannulated and the entire lung was lavaged in and out three times and all aliquots were combined for each animal as conducted in a previously established protocol [135]. For H/E (Fisher Scientific Pittsburg, PA) cell staining a portion of each samples was diluted to 2 x 104 cells per 100µL and fixed with Cytospin (Shandon, Pittsburgh, PA) and were photographed with light microscope fitted with a digital camera. The total AMs were diluted at 5 x 105 cells in 1mL of RPMI media (Gibco, Grand Island, NY) with 2.5% FBS (Gibco, Grand Island, NY) and 50µg/mL gentamycin (Gibco, Grand Island, NY).

In vitro exposure

In vitro exposures of both B20 and DEP particle suspensions were conducted on freshly isolated rat AM within polypropylene culture tubes. Particles were weighed out and diluted in RPMI media with 2.5% FBS and 50µg/mL gentamycin (at room temperature). In polypropylene culture tubes the cells were exposed to 100- 500µg/mL of either DEP or B20 and incubated at 37°C with 5% CO2. After 24 h of exposure, the AMs were centrifuged (500x g) for 10 min at 25°C and 800µL of supernatant was collected. In experiments where macrophages were challenged post PM exposure, fresh media (800µL) containing 12.5ng/mL LPS (Sigma Aldrich, St.Louis MO) was added for a subsequent 4 h incubation. In a few experiment particles (100µg/mL) were co-incubated for 30 min with polymyxin B concentration (10µg/mL) prior to adding the modified particles onto AMs. In some experiments the supernatant was collected (stored at -20°C), cells were then lysed and stored at -80°C until mRNA isolated.

mRNA isolation & quantitative real-time PCR

Cytoplasmic RNA was collected using Qiagen's RNAeasy Micro Column kit (Valencia, CA) manufacture's protocol was followed and amended with additional column washes. The mRNA collection includes n=10 animals per dose, quantification, cDNA synthesis and RT-PCR conditions were followed from previously published method [135]. Rattus norvegicus primers PTGS2 (COX-2, cyclooxygenase 2) and Cxcl2 (MIP-2 – macrophage inflammatory protein 2) (ABI; Carlsbad, CA) were measured and data was normalized to the house keeping gene 18S.

PGE_2 ELISA

PGE_2 released into culture media was measured by ELISA (R & D systems Minneapolis, MN) following the manufacturer's high sensitivity instructions. The ELISA kit uses a competitive binding method to assess the sample specific PGE_2 released. An average of three animals were used for media and n=12 for all exposures. Standards were created using culture media and multiple dilutions were studied before reaching optimal absorbance.

Cell viability

AM cells were spun down after 24 h of exposure and 50µL aliquots of supernatant were assayed for cytotoxicity using LDH assay (Promega; Madison, WI). 50µL of post exposure media collected and

assayed with equal parts substrate, after 30 min reaction was stopped and absorbance was measured at 490nm. Trypan blue dye incorporation was used to verify cell death.

3H- PGE_2 binding activity assay

A cell free assay was conducted to estimate the competitive binding affinity of PGE_2 to either B20 or DEP particle. Radioactively labeled 3H- PGE_2 (Perkin Elmer, Boston MA) with specific activity of 200Ci/mmol was incubated in media containing 1-500µg/mL B20 or DEP, for 24h with non-radioactive PGE_2. After 24h tubes were centrifuged at (500xg) and the radioactivity in the supernatant (not bound to particle) was measured by standard scintillation counts (Liquid Scintillation Analyzer TriCarb 1500, Packard). The media contained 2.5%FBS and 50µg/mL gentamycin.

Statistical Analysis

Pair-wise group comparisons were made using Holm-Sidak method and Bonferroni (SigmaStat Software Inc., version 11.0). All graphs and figures are graphed with means and with standard error bars. A value of $P < 0.05$ was considered significant.

2.3. Results

B20 and DEP combustion emissions analysis indicates chemical composition varies

The elemental composition of B20 and DEP greatly differs with a few transition metals and elements (Table 1). Our data indicates Co, Cr, Cu, Fe, Mn, Mo, Ni, and Zn are at least 10-fold greater in B20 than in DEP the acid soluble fraction analysis. Other metals in the acid soluble fraction such as Mg, Sb, and V are only about 15%- 25% more in B20 compared to DEP. Analysis of the water soluble fraction identified 1,623µg/g Zn was present in B20 whereas DEP had only 11µg/g. The toxic metals Mg and Pb were found, in a previous study, elevated in B20 relative to DEP [42]. Measurement of ions from particles dissolved in water indicate increases in PO3+, Na+, Ca2+ and SO42- in B20 relative to DEP. Also in our study, DEP and B20 were analyzed for elemental and organic carbon content. B20 appears to have a higher EC/OC ratio than the DEP (10.7 and 4.8, respectively). B20 contained more total carbon and elemental carbon/g PM than DEP which had more organic carbon/g PM (Table 1).

The 20% introduction of soy based fuel appears to only slightly increase the ability of the particle to generate radicals and thereby producing malonaldehyde (the end product) measured by TBARS absorbance. The TBARS assay using B20, ROFA and DEP relative to water control did not show statistical significance (n=3). The relative fold change of B20 and DEP indicate 3.30 and 3.46 increase from vehicle control. ROFA was about 5.2 fold increased from water control.

DEP and B20 were tested for endotoxin contamination which can occur in the original fuel or from improper storage conditions, which can stimulate AM to induce increased PGE_2 release. Endotoxin testing resulted with no detectable endotoxins present in both B20 and DEP particle suspensions.

In vitro particle exposure of B20 and DEP can lead to cytotoxicity in rat AMs

Cellular cytotoxicity induced by exposure to B20 and DEP is equivalent in rat AMs. Results of lactate dehydrogenase (LDH) released into the media after 24h in vitro exposure indicated dose dependent increase of DEP and B20. However, no statistical significance was detected with DEP and B20 relative to vehicle (Figure 2). Trypan blue measure of cell death indicated similar cell death results. Trypan blue dye indicated dose dependent response but not particle type dependent.

AM respond similarly to B20 and DEP particles in suspension in regards to cell cytotoxicity

Half a million cells were exposed to particles with final concentrations ranging from 1- 500µg/mL in RPMI media. An aliquot of AM were removed after 30 min & 24h incubation and the sample was diluted for optimal visibility. Cells and particles were fixed and stained for image capture using a light microscope. The particle/cell suspension was photographed in which we found particles located externally on the macrophages. Fragments of B20 particles were located within the AM (Figure 1c). At 1µg/mL (Figure1b) the presence of B20 particle had attracted four macrophages to the particle site however the stain indicates AMs remain intact and alive indicating there was no cytotoxicity due to particle. The LDH assay found no statistically significant difference from all concentrations of B20, DEP and vehicle control, indicating both particle types have similar cytotoxic response.

Gene expression studies indicate similar initiation of inflammatory response

Evidence of AM initiating an inflammatory response after particle exposure was collected with elevated gene expression levels of MIP-2 and COX-2 (Figure 2.3). The results are an average of n=10 per treatment. The mRNA levels of COX-2 after exposure to 1,10 & 100µg/mL of B20 and DEP show a dose dependent increase. The highest dose 100µg/mL is significantly increased from media in both B20 ($p>0.001$) and DEP ($p>0.05$). Only B20 exposure appears to increase COX-2 mRNA levels gradually with particle concentration, suggesting COX-2 expression is particle type dependent. MIP-2 expression increased in a dose dependent manner in both B20 and DEP, suggesting MIP-2 expression is concentration dependent and identical with both particle types. DEP at 100µg/mL increased MIP-2 expression significantly, with over four fold increase from media control. However, not even the highest concentration of B20 was able to increase MIP-2 significantly.

PGE_2 *release depends on particle type and dose*

PGE_2 release from AM was measured after exposure to B20 and DEP. The PGE_2 release is elevated at low particle concentrations (1&10 µg/mL) and decreases with high particle suspension (100& 500 µg/mL). At 10µg/mL of B20 PGE_2 release is significantly increased from media ($p\geq0.01$). B20 exposure increased PGE_2 release by 40pg/mL from media control. While DEP exposure also increases PGE_2 release, the values are not statistically significant. Data indicates B20 particle exposure to AMs induced a unique interference which leads to increase PGE_2 release. We also observed the PGE_2 release by B20 at 10µg/mL is independent of the gene expression levels of COX-2 at 10µg/mL.

Particle concentration doesn't interfere with the detection of PGE_2

As we previously noted PGE_2 levels increased initially at low particle concentrations and then decreased with higher particle concentrations. This observation indicates PGE_2 release could be undetected due to particle sequestering (i.e., PGE_2) was adsorbed onto the carbon core. We tested if B20 particle is comparatively less likely than DEP to sequester extracellular PGE_2 and limit detection. Using radio-labeled PGE_2 we conducted a competitive binding assay and identified the two particle types had similar affinity to bind PGE_2 at low concentrations (Figure 2.5). Figure 2.5 shows disintegration counts per

min (DPM) of 3H- PGE_2 for each concentration and particle type. As particle concentration increases there is a decrease in the amount of detectable 3H- PGE_2 available in the culture media indicating particle sequestering does occur. We initially compared particles suspended in 0% FBS and then with 2.5% (Figure 2.5). Without 2.5% FBS the 3H- PGE_2 values remaining in supernatant were five-fold lower than with 2.5% FBS. At 1µg/mL of B20 with 17,728DPM counts B20 was statistically significant from media control. B20 with 100µg/mL had decreased slightly to 16,759 DMP. DEP at each particle concentration shows greater counts than B20 (1µg/mL: 19,204 DPM; 100µg/mL: 17,855 DPM; 500µg/mL: 13,080 DPM). Additionally at 500µg/mL both DEP and B20 indicate a statistically significant decrease in 3H-PGE_2 from culture media indicating dose dependent sequestering. These findings demonstrate there was particle sequestering of the PGE_2 and the results show a difference between B20 and DEP in their ability to sequester.

2.4. Discussion

In our present study we demonstrated for the first time B20 may induce similar cellular disruptions to normal AM function as found previously with petroleum diesel exhaust particles [91-93]. Our experiments with freshly harvested rat AM suggests B20 and DEP exposure initiate different cellular responses related to inflammatory cytokine (MIP-2) and lipid mediator production (PGE_2). Our particle characterization deduced B20 and DEP vary by only 20% in regards to a few particle bound species however there are still many species not yet studied which can vary greatly therefore it is difficult to speculate a direct effect by a single component. However, we have identified a few characteristics specific for B20 which can aid in deducing particle type dependent PGE_2 release and inflammatory response from mature alveolar macrophages with *in vitro* exposure. Data appears to suggest there are components in exhaust which may interfere with PGE_2 release.

Our observation that Zn, a transition metal, was one component uniquely increased in B20 points to possible oxidative stress led mechanism of toxicity. The oxidative stress may induce the elevated inflammatory signal molecules as observed previously with petroleum diesel combustion particles [136, 137]. Previous research indicates metal content in particles especially Zn, Cu and Fe can cause acute inflammatory response [138]. Brito et al [64] indicate in their study, metal emissions released follow a

linear increased directly linked to the amount of petroleum diesel in the original fuel. However our study used low sulfur petroleum diesel containing little Zn, suggesting, in our study the Zn source in B20 emissions is not dependent on petroleum fuel. A study recently found B100 contains high levels of Zn prior to combustion [68]. Other studies found increased levels of transition metals from biodiesel combustion of karanja oil [42] indicating original fuel type may not be the only source of the transition metals found in emissions. We believe in our study however the soluble fraction of transition metals alone in B20 are not sufficient to extrapolate oxidative stress signaling responses in AM. Other studies suggest that AM are capable of responding with induction of oxidative stress [127](measured as chemiluminescence) to very high concentrations of solubilized metals from residual oil fly ash (ROFA); our employed dose and our transition metal content was not sufficient to potentiate the robust inflammatory response seen in B20 exposed AMs. Our study found B20 and DEP have similar cytokine (MIP-2) and COX-2 expression levels therefore ROS mediated inflammatory downstream signaling is not transition metal dependent.

Our study also found elemental carbon varies between the two particle types. Particle composition, specifically, carbon type along with cell type affects the uptake of particles into cells. A previous study compared DEP to carbon black (primarily elemental carbon) uptake in mouse AM and a lung epithelial cell line [139]. The study found a fivefold increase in uptake of carbon black by the mouse AM compared to lung epithelial cells. Essentially a mouse AM had greater uptake of DEP vs. carbon black particles because of the lack diversity of particle as opposed to elemental carbon[139]. We can deduce AM phagocytosis was more efficient with organic carbon or with particle diversity. In reference to our study by replacing 20% of DEP with soy we elevated the EC content of particles and may have decreased particle uptake. Surmising that potentially smaller cellular uptake was more potent for induction of PGE_2 release.

Results found in our *in vitro* exposure study are similar to studies conducted with *in vivo* particle exposure that show inflammatory potential with biodiesel exhaust exposure. Finch et al indicate in a biodiesel inhalation exposure study, AM contain particles demonstrating this cell type as an ideal target [131]. This study also found increased cell density and protein in lung lavage fluid after exposure. Brito et

al [64] also conducted an *in vivo* study exposure via intratracheal instillation of biodiesel and found excess protein in the BALF as well as increased cell density. Increased phagocytosis can initiate increased inflammatory signals and recruitment of other inflammatory cells mediated by release of cytokines. Elevated MIP-2 levels are produced by macrophages and can suggest alveolitis in rats [140]. Our *in vitro* studies MIP-2 gene expression was elevated in a dose dependent manner after 24 h exposure to B20 and DEP. Based on similar findings to our *in vitro* study we can further predict biodiesel may cause protein influx and recruit neutrophils in a whole animal model.

The results of the present study illustrate *in vitro* exposure to B20 and DEP can alter PGE_2 release by AM. Possible causes for B20 exposed cells to have more PGE_2 detected in the supernatant can include: (1) less prostaglandin binding to particle; (2) increased COX-2 gene transcription; and/or (3) alterations in the protein and activity level of COX-2 as well as other enzymes involved in prostaglandin production. Previous research with supernatants from petroleum diesel particle exposed to cells indicates cytokines released from the cell can be absorbed by particles disrupting detection techniques [141]. In our study PGE_2 detection was not biased solely on particle binding (adsorption). We found both B20 and DEP at 100 µg/mL have a similar affinity for PGE_2 (although B20 was significantly decreased from Media Fig. 5). PGE_2 loss (for detection) via binding to PM becomes a significant issue at higher concentrations. A lack of an increased release (relative to control cultures) of PGE_2 from DEP -exposed AM can be due to the specificity and activity level of COX-2. Previous research identified PAHs in petroleum diesel extract, specifically phenanthrene, inhibit COX-2 activity in macrophages [125]. Both diesel extract and phenanthrene alone can inhibit PGE_2 production in macrophages but the exact mechanism(s) is still unclear. This study found DEP inhibits endotoxin-stimulated PGE_2 production in murine macrophages and a cell free (COX-2) enzyme system [125]. These observations are consistent with DEP and low PGE_2 levels in our study suggesting cyclooxygenase activity was disrupted and lead to lower production and release of PGE_2. Similar to our B20 soy combustion particles Ratcliff et al. show B20 soy combustion has 20% lower total PAHs including phenanthrene. The data from our study suggests 20% soy incorporation into diesel fuel can likely lead to less PAH emissions and thereby stimulate PGE_2 production at the lower B20 concentrations used in our studies.

We conducted experiments to identify if the particle exposure had disrupted the arachidonic acid and cyclooxygenase pathways irreversibly. After particle exposure AM were washed and challenge with LPS at 12.5 ng/mL for 4 h. The supernatant was collected and PGE_2 release was measured. Results indicated B20 particle-exposed AM after LPS challenge release more PGE_2 with higher particle concentrations. The opposite occurred with DEP exposure, following challenge less PGE_2 was measured with cells exposed to higher particle concentrations. The results of LPS challenge indicated COX-2 remains efficient after B20 exposure but not with DEP. These experiments informed us particle type is as important as particle concentration when challenging cells and measuring the subsequent PGE_2 release. Previous results from Mundandhara et al. indicated macrophages with LPS challenge would bounce back to normal PGE_2 release after diesel exposure [92]. However the Mundandhara et al study used different combustion particles and conducted experiments with human AMs. We believe in addition to differ particles, our rat AM size, age and previous environment might have altered the reserve of PGE_2 precursors leading to different responses from previous paper. The results suggest rat WKY are more sensitive to particle exposure and sustain irreversible injury to the cyclooxygenase enzymes or to the free arachidonic acid reserve.

Many components have been found in diesel emissions to have biological significance in cells. Analysis of diesel exhaust particles indicates presence of quinones [142], specifically phenanthraquinone which has been identified in generation of ROS and oxidation of sulfhydryl groups. Other diesel exhaust components which have yet been identified to cause a biological consequence are fatty acids. Fatty acid methyl esters (FAMEs) are the building blocks for biodiesel and are suspected to be present in exhaust emissions along with other unique incomplete combustion derived from FAMEs [34]. We believe these two and possibly many other components can disrupt production of PGE_2, via initiation of signaling pathways or suppression of normal functions. Quinone's generate free radicals required for the second step in creating PGG_2 a precursor of PGH_2. ROS generation is necessary for PGE_2 production however with increased lipid peroxidation species can inactivate prostaglandins from extracellular release. Additionally cyclooxygenase -2 has a greater affinity for unsaturated fatty acids than arachidonic acid suggesting less arachidonic acid metabolite production in the presence of unsaturated free fatty acids [143]. Additionally

B20 appears to have more diverse species in reference to detected levels of ions. We believe there are maybe other components of diesel and biodiesel which can generate still unknown interactions within the cell and modulate prostaglandin production or release.

Macrophages along with other cell types can generate differential inflammatory responses to B20 and DEP. Jalava et al [144] conducted studies in which they found AM increased apoptosis, genotoxicity, and ROS production with the use of rapeseed methylated ester (RME) particles in a dose dependent manner *in vitro*[144]. Although cytokines, TNFα and chemokine MIP-2 were elevated from control with RME there was significantly more detected with petroleum diesel fuel combustion particle extract exposure [144]. Rat macrophages were tested for ROS generation and found to increase in RME samples as well as diesel however the response was greater in diesel than RME [144]. Hemmingsen et al [145]conducted *in vitro* exposures with HUVECs and A549 cells to evaluate the strength of gene expression, ROS and DNA damage responses from DEP and B50 (as 50% soy and 100% soy) exposures. A direct comparison between DEP (100%) and B50 generated more than two fold increase in both ICAM-1 and VCAM-1 with DEP but not with B50. Additionally ROS generation was substantially greater (up to four fold higher) with the DEP than B50. The level of response by macrophages after B20 and DEP exposure was similar in both endothelial cells and epithelial cells. The data comprehensively indicates fuel type and particle composition can produce different responses in several cell types.

In conclusion our results indicate exposure to B20 will cause a similar inflammatory response as mediated by DEP with reference to gene expression of MIP-2. COX-2 the enzyme which produces prostaglandins increases with both DEP and B20 however PGE_2 release is significantly increased with only B20 and at low particle concentrations. Our data suggests particle composition is crucial to the AM PGE_2 release. In order to better identify the exact component of exhaust which causes cellular inflammatory signals we need more detailed chemical composition analysis. Independent composition analysis of B20 products and their consequential interactions with rat AM need to be conducted as a means to explain the difference in the response.

	DEP		B20		SRM1649a	
Element ug/g PM	H2O-soluble	acid-soluble	H2O-soluble	acid-soluble	H2O-soluble	acid-soluble
Al	25	189	14	277	809	11,053
As	<6.9	<11.8	<5.9	<5.9	11	23
B	2	5	36	38	5	25
Ba	<1	7	<1	9	50	366 (65)
Ca	<1	367	3,833	6,916	18,602	19,010
Cd	<0.4	3	<0.4	2	23	32.2 (147)
Co	<0.7	2	18	121	3	13.4 (82)
Cr	<0.4	2	4	419	5	90.2 (43)
Cu	<1.2	33	<1	517	80	250 (112)
Fe	<0.4	90	5	4,332	483	24039 (81)
K	3	10	25	33	1,453	2,552
Li	<1	0	1	1	1	15
Mg	<1	49	60	102	2,164	5629 (61)
Mn	0	4	71	145	137	250 (105)
Mo	4	8	17	88	<3	8
Ni	<1.7	3	25	762	67	128 (77)
P	85	142	1,581	1,659	1,475	1,996
Pb	<6.6	<11.3	<5.6	32	2,067	18753 (101)
S (as $SO4^{2-}$)	365	3,313	16,649	16,632	102,021	103247 (105)
Sb	<4.7	<8.1	3	9	<4	23.8 (80)
Se	<6.4	<11.0	<5.5	<5.5	<11	<7.0
SiO2	29	86	29	134	25	1,029
Sr	<1	0	2	4	58	105
Ti	<0.5	10	<0.4	17	<1	934
V	<0.4	2	<0.3	3	126	262 (76)
Zn	11	174	1,623	2,863	1,474	1564 (93)

Ions ug/g PM	DEP H2O-soluble	B20 H2O-soluble	SRM 1649a H2O-soluble
F^-	<41	<35	45
Cl^-	<204	<175	466
NO_2^-	<204	<175	<128
Br^-	<203	<175	101
SO_4^{2-}	<203	18,690	98,373
NO_3^-	220	178	4,889
PO_4^{3+}	<408	4,795	3,105
Li^+	<102	<88	<64
Na^+	<410	382	1,117
NH_4^+	<815	<703	11,883
K^+	<408	<351	1,056
Mg^{2+}	<405	<349	1,721
Ca^{2+}	<2035	5,606	15,860

Carbon type	ug/g by mass	ug/g by mass	ug/g by mass
organic carbon	60,600	36,300	131,700
elemental carbon	292,500	388,000	36,400
total carbon	353,100	424,300	168100 (95)

Table 2.1. Characterization of B20 and Diesel by ICP- Plasma OES. Particles were suspended in distilled water or acid (3:1; HCL: HNO3) for measurement as described in methods. Elemental components, ions and carbon type are measured in µg/g of PM. There are measureable decreases in both metals and carbon when comparing B20 to DEP. We used NIST SRM 1649a particles to verify the acid soluble particle extraction was effective; the percent recovery is given in (). Using our acid-soluble method we had over 93% recovery for Mn, Pb and Zn compared to the NIST published data. Elements such as Fe with our method indicated only 81% recovery relative to NIST published standards.

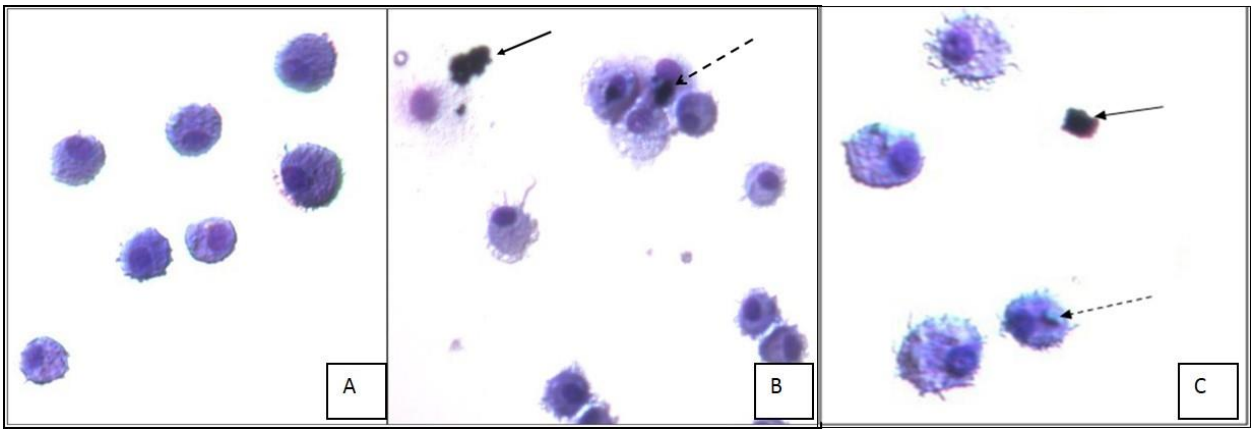


Figure 2.1. DEP and B20 particle suspension with AM. H& E stain of WKY AM with increasing B20 particle concentrations. Density of 2x104 WKY AM incubated 24h with (A) vehicle, (B) B20 at 10µg/mL and (C) B20 at 1µg/mL. All images are of fixed and H&E stained cells. Images taken with Nikon eclipse E600 at 20X magnification. Solid arrow (←) points to unbound B20 (▲‾) indicates B20 within or attached to the surface of the AM.

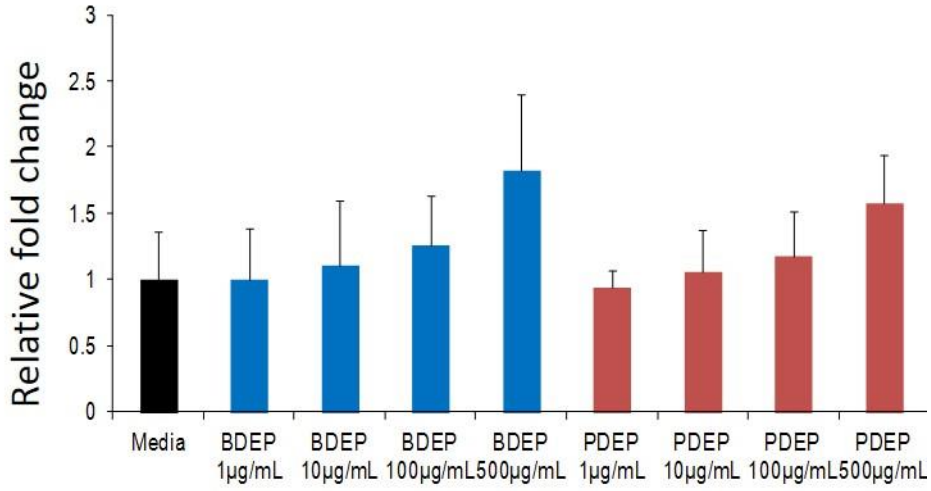


Figure 2.2. Cell cytotoxicity of WKY AM after 24h exposure to DEP and B20 is dose dependent. Cell cytotoxicity of WKY AM after 24h exposure to 1-500µg/mL of B20 or DEP measured by LDH release. Each group represents mean ± SE (n=6). Exposure to B20 or DEP shows no significant cell toxicity as fold change relative to media control.

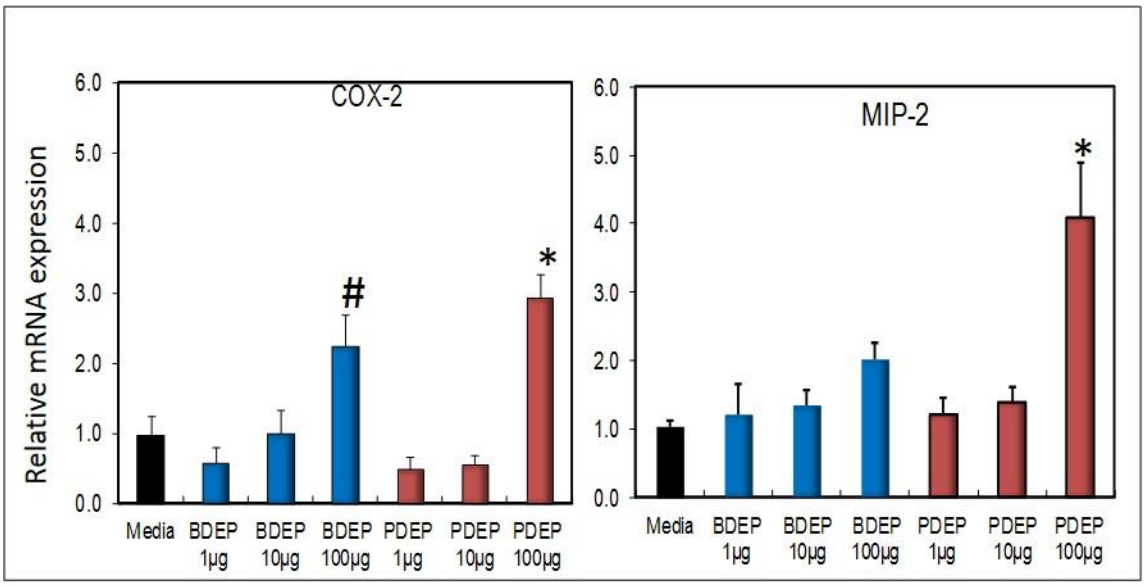


Figure 2.3. Gene expression of inflammation markers, COX-2 and MIP-2, increased with 24hr exposure to DEP and B20 in WKY AMs. Highest dose of diesel alone significantly increased inflammation markers. Gene expression of COX-2 and MIP-2 in WKY AM exposed for 24h to B20 and DEP. COX-2 expression is significantly increased in both B20 and DEP at 100µg/mL; B20 *P< 0.05 and DEP #P<0.001. MIP-2 expression with B20 increases with dose but not significant and DEP at 100µg/mL is statistically significant *P<0.05. Each group represents mean ± SE (n=10).

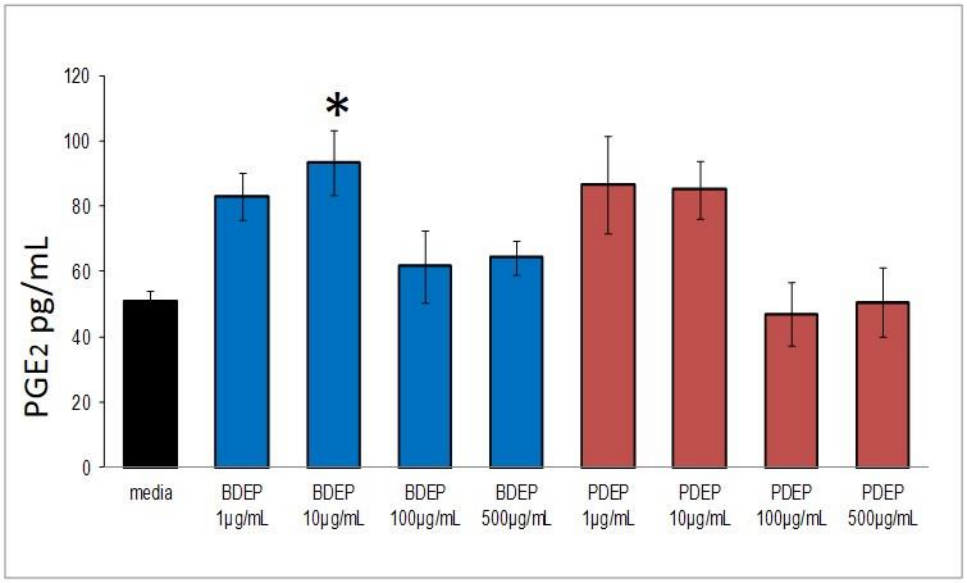


Figure 2.4. Increased PGE_2 release with lower concentrations of B20 but not diesel. WKY AM PGE_2 release following 24h exposure to DEP and B20 was quantified by ELISA. Each group represents mean ± SE of 3-12 animals. B20 concentration of 10µg/mL is statistically significant with reference to media control *P<0.05.

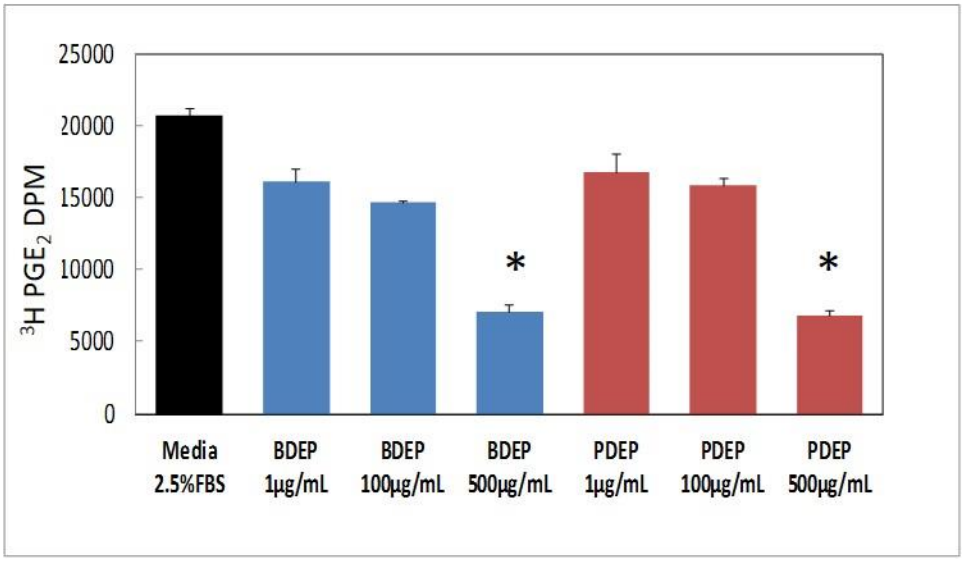


Figure 2.5. Diesel and B20 particle sequestering of PGE_2 is equivalent in a cell free system. B20 and DEP binding of hot (3H-PGE_2) and cold (PGE_2) in a cell free system. Particles were suspended in RPMI media with 2.5% FBS for 24h, particles were centrifuged and supernatant was collected for standard scintillation counting. B20 at both 100µg/mL and 500µg/mL and DEP at 500µg/mL are statistically significant *P < .001 from media control.

Chapter 3

Solvent Extraction of Biodiesel Exhaust Particles for Chemical Analyses and Use with *In Vitro* Bioassays.

3.0. Overview

Air pollution research has been investigating the physicochemical property of combustion emissions composition of different biodiesels and biofuel blends to determine if biological material used in fuel production can generate variable emissions species. This study sought to improve the particle extraction methods currently in use for particle characterization which are optimally developed for petroleum diesel, not biodiesel blends and neat biodiesel exhaust particles. In our methods development, we emphasized solvents which were more appropriate for use with cell culture in order to study biological effects without the solvent itself inducing cytotoxicity. Chemical classes examined included traditional and non-traditional emissions chemical species (i.e., metals, ions, carbon type, free fatty acids and aldehydes). This study identified particle suspensions of different solvents (e.g., tricaprylin, DMSO and water) can illustrate the differences among the three particle types. The results from the soluble fraction analyses suggest an optimal method of extraction for biodiesel (blend and neat) exhaust compounds is different from one optimized for petroleum diesel exhaust. DMSO was chosen as the primary solvent for extraction in DMSO over traditional solvents such as water because biodiesel DMSO extract contained more non-polar species. The DMSO extract when exposed to cells in vitro, induces a dose dependent cell cytotoxicity, which didn't occur with other solvents. The DMSO extract had potent cytotoxicity on EA.hy926 cells compared to the other extracts.

3.1. Introduction

Increasing interest in climate change concerns have highlighted the culpability of anthropogenic sources of air pollution. A substantial contribution of air pollutants were emitted from combustion of fossil fuels [146]. Motor vehicles are predominantly of concern regarding source emissions of particulate matter. The U.S EPA has identified adequate evidence of particulate matter emissions associated with diesel and gasoline emissions from urban roadways [146, 147]. Furthermore, exposure to diesel combustion emissions was shown to be causal to the increase of respiratory and cardiovascular injuries, which include asthma, decreased lung function and inhibition of vascular elasticity [148]. Thus the concern regarding diesel exhaust induced health effects have accomplished development of cleaner alternative energy, such as biodiesel.

Biodiesel is a liquid fuel that is easily substituted into diesel engines. Biodiesel was produced via the transesterification reaction between a plant oil (i.e., triglyceride), an alcohol (i.e., methanol) and a catalyst (i.e., sodium hydroxide) to generate fatty acid methyl esters [41, 149, 150]. The chemical reaction can also be replaced with an enzymatic reaction that exchanges the fatty acids of triglyceride with lighter alcohols leaving mono- or di- glycerides and glycerol as byproducts. Inept refining processes can leave behind the residue that contains intermediates [41]. The transesterification process generates fatty acid methyl esters (FAMEs) that can substitute for diesel in combustion engines. The recent global expansion of biofuels, derived from a variety of feedstock, may alter the pollution profile due to unique exhaust compounds compared to diesel and gasoline emissions. Only a few studies have identified increased NOx emissions, formaldehyde and fatty acid methyl esters released with biodiesel compared to diesel exhaust [34, 151]. Contrastingly, a few exhaust emissions studies indicated combustion of biodiesel neat or blends (i.e., diesel with biodiesel) typically released lower CO, particulate matter (PM) mass, and unburned hydrocarbons [34, 63, 133, 144]. The novelty of renewable energy consumption has generated a lapse in the availability of accurate emissions characterization and insight into toxicity, which can be remedied with accurate examination of particle bound compounds.

Historically, particle characterization and evaluation of toxicity is dependent on extraction methods to identify individual compounds with known mechanisms of cellular toxicity. Some common compounds include PAHs, inorganic ions, metals, and carbonaceous matter both elemental and organic [150, 152]. The direct cellular effects of these specific components have been well studied. PAH emissions can induce genotoxicity and some PAHs are carcinogenic [63, 142, 153, 154]. Metals, specifically transition metals, from exhaust have been identified to induce redox cycling post cellular exposure [53, 155]. Additionally, the organic carbon content can interfere with the immune response and inflammation signals [156-159]. Previously documented methods to study the individual compounds from particle include extraction methods such as Soxhlet, or solid phase extractions [58, 160]. The complexity of the particle and the chemical properties of all components bound to the particle can bias the extraction process. Traditional methods developed primarily for diesel exhaust particles, enriched the extraction of PAHs, with the aid of organic solvents (i.e., dichloromethane (DCM)). These traditional methods are not ideal for biodiesel exhaust particle extraction since there are fewer PAHs previously identified with exhaust. Additionally, the use of DCM would require solvent exchange into a less toxic solvent for use with cell culture for toxicity testing. In this study, we developed a method of particle extraction that is capable of collecting the range of chemical species found on biodiesel exhaust particle and is not susceptible to sample loss that occurs with solvent exchange.

In this report we compared solvent effectiveness to maintain, biologically-active soluble compounds, extracted from biodiesel blend (B20), biodiesel neat (B100) and petroleum diesel (DEP). The goal of this study was to systematically examine extraction quality and determine the extent to which biodiesel and diesel exhaust particle vary to determine if new methods are required for examination of soluble compound of biodiesel that induce cellular toxicity.

3.2. Materials and Methods

Particle sample collection

The biodiesel fuel was purchased from Agland Inc. (Lucerne, CO) and the soy biodiesel blend was made as previously described [161]. Low sulfur DEP, biodiesel blend (B20), and biodiesel neat (B100) combustion particles were collected at the National Renewable Energy Labs (NREL, Golden CO) and were extensively characterized for real time measurements [160]. The three exhaust particle types were conducted using a 2002 model year Cummins ISB engine and samples were a gift to our lab [162].

Particle Reactivity

Identification of exhaust particles reactivity was assessed with all three particles using a thiobarbituric acid reactive substances (TBARS) assay. Each sample particle type was assessed for its ability to induce free radical generation of malonaldehyde. A previously established protocol with a minor modification of an additional particle centrifugation steps for 10 min following particle suspensions, was used to compare and quantitate triplicate sample extractions of 0.5mg sample particle [53].

Dynamic light scattering studies

Measurements of particle size (and agglomerations) in a variety of solvent suspensions were analyzed by dynamic light scattering using the Zetasizer nano ZS with Malvern DTS software version 5.10 (Malvern Instruments, Malvern, MA). Particle suspensions were made with dimethyl sulfoxide (DMSO), water and tricaprylin. Suspensions of particles in all solvents were 10mg/mL, the scattering angle used to measure the light intensity was 90° with a HeNE laser as the light source. Scattered light intensity, specifically polydispersity measurements, correspond directly to size of particles in various solvents suspensions. Polydispersity measurements range from 0 to 1.0, the smaller value indicates particles were more homogenous and particles in the solvent are the same size. All suspensions were measured for polydispersity as opposed to monodispersity. Parameter settings for water were selected from software library, DMSO and tricaprylin specifications were added to the software by our lab. The Zetasizer was used to calculate charge with DLS mode. Water suspensions of sample particles were measured using polystyrene cuvettes. The polystyrene cuvettes were incompatible with DMSO and tricaprylin; therefore

only water suspensions of sample particles were assessed for zeta potential. All measurements were done at 25°C. The following solvent specifications were used: DMSO: refractive index: 1.48; viscosity: 1.99 mPas; dielectric constant: 46.68.; tricaprylin: refractive index: 1.45; viscosity: 20 mPas; dielectric constant: 4.13 [163]. On average 6 runs were combined for mean particle diameter and characterization.

Endotoxin analyses of each particle

Particles were evaluated for endotoxin contamination with an established method using ameobocyte lysate from Limulus polyphemus with a gel clot technique by Cape Cod INC (Cape Cod Inc East Falmouth MA). The bacterial endotoxin assay can detect as low as 0.03EU/mL. All combustion particle samples were identically tested with positive control Escherichia coli 0113:H10.

Soluble extraction species detection by UPLC-PDA

DEP, B20, and B100 particles were weighed out at 10-40 mg and placed in individually labeled 20 mL glass vials and 1 mL of solvent was added. The solvents include: cell culture grade water (Sigma Aldrich, St. Louis MO), hexane (Fisher Scientific, Fairlawn, NJ), tricaprylin (Fluka Chemika no. 91039), acetonitrile (ACN) (Burdick & Jackson, Muskegeon MI), and cell culture grade - DMSO (Sigma Aldrich, St. Louis MO). The vials were sealed and covered from light with foil and placed on a plate shaker at 35 rpm for 48 hrs. After two day incubation with constant shaking at room temperature, the vials were vortexed and 500 µL aliquots were placed into polyallomer ultracentrifuge tubes (Beckman Coulter, Indianapolis, IN). The suspensions were centrifuged at ~60,000 rpm for 2 hrs at room temperature in an ultracentrifuge (Sorvall Wilmington, Delaware). The supernatants was collected and stored in dark vials at room temperature. Supernatants (in 10 µL of each solvent) were injected and separated with a UPLC system consisting of a Waters Aquity UPLC (Milford, MA) fitted with photodiode array (PDA) scanning at 210nm- 500nm, a reverse phase column (Acuity, C18 RP column 2.1 cm x 50 mm), and operated with MassLynx v4 software. The components were eluted in 10 min at 0.25mL/min with a linear gradient beginning with 45% Solvent A (95/5 water/acetonitrile) and 55% Solvent B (100% acetonitrile) ending with 100% Solvent B, followed by 10 min of wash with ACN. All samples were compared to solvent blanks.

ICP-OES for element and ion detection of particle

Element, metal and ion analysis was performed using inductively coupled plasma-optical emission spectrometry (ICP-OES), following U.S. Environmental Protection Agency (EPA) Method 2007 rev4.4 (U.S. EPA 1994). Each particle was dissolved and the measurements were taken with an axially-viewed, simultaneously-measured instrument (PerkinElmer 4300DV ICP-OES, Bridgeport, CT) and done in triplicate. NIST samples Standard reference material Urban Dust from D.C (SRM 1649a) was used as method quality control.

Carbonyl analysis by HPLC-UV

Each exhaust sample was weighed out and processed using a method previously established by Fung et al [164] with 2,4-Dinitrophenylhydrazine (DNPH) as the derivatizing agent for detection of particle bound carbonyls. Sample particle (10 mg each) was weighed out in duplicate in glass 5 mL vials with 1 mL of 100% acetonitrile (ACN). One vial was incubated with 1:1 part of HCl: acetonitrile the other vial was incubated with DNPH derivatizing solution of (8 mg DNPH with HCL and acetonitrile). The samples were allowed to sit at room temperature for 30 mins with vortexing every 10 min. All samples were spun down at 20,000 rpm for 10 min until the particles were pelleted. Supernatant of each sample was collected into a HPLC vial and 10 µL was injected into the Waters Acquity HPLC with Solvent A as 100% water and Solvent B as 100% acetonitrile. The elution gradient started at 50% solvent B and follows a linear gradient to 100% acetonitrile at 0.6 mL/min flow rate for 16 min. DNPH derived carbonyls were detected at 360 nm using one channel detection and relative elution was compared to carbonyl DNPH derived standards actetalaldehyde, propanal, crotonaldehyde, cyclohexane, pentanal, hexanal, heptanal, octanal, nonanal and decanal (AccuStandard, New Haven, CT).

Fatty Acid Detection by HPLC-UV

Fatty acids from particles were derivitized with a chromophore for detection following a previously published method by Putmann et al [165] with slight modifications. Three replicates of each particle type (30 mg) were dissolved in 0.6 mL dichloromethane (DCM) and incubated at room temp on

plate shaker overnight. The suspensions were transferred into centrifuge tubes and centrifuged at 8,000 rpm for 2 minutes at room temperature. The supernatant was collected and dried down using a speed vac (Savant, Farmingdale, NY). The residues were reconstituted in acetonitrile with 40 mM p-bromophenacylbromide and 2 mM 18-crown-6 ether (as a catalyst). Potassium bicarbonate (2 mg) was added to both samples and standards. Samples were vortexed, then heated at 85°C for 45 mins with shaking every 10-15 mins. Samples returned to room temperature, and 20uL was injected and separated via HPLC. Agilent Zorbax Eclipse XBD-C8 column (4.6mm x 250 mm) was used at 2 mL/min flow with Solvent A as 1% glacial acetic acid added to 90% water /10% acetonitrile and Solvent B as 1% glacial acetic acid in acetonitrile. The elution peaks were detected at 254 nm. After separation, the peak elution times and area under the curves were compared with known amounts of fatty acids standards and an internal control (cis/trans C9). The mixture of fatty acids standards (AccuStandard, New Haven, CT) injected ranged from 4.4– 100 µg/mL.

Cell exposure

EA.hy 926 cells, an immortalized epithelial-endothelial hybrid cell line [166], was seeded at 1.2 $x10^5$ cells/well density in six-well tissue culture plates with Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Life technologies) supplemented with 10% fetal bovine serum (FBS) with penicillin/streptomycin and cells were treated with extracts after two days when confluent. Cells were incubated with soluble components or vehicle control at 37°C in a humidified atmosphere with 5% CO_2. Each soluble particle fraction was at a stock concentration of 40 mg particle equivalents/mL in either water, DMSO, or tricaprylin. Cells were treated for 6 and 24 hr with final concentrations between 10- 400 µg/mL. Cell viability was calculated by released lactate dehydrogenase (LDH) activity using a cytotoxicity assay kit Promega (Madison, WI). The trypan blue exclusion dye assay was used after exposure to count dead cells.

Statistics

Statistics were calculated using Sigma Plot version 12.3 (Systat Software Inc). All cytotoxicity data were expressed as mean ± standard deviation. Data was analyzed by one-way ANOVA followed by Dunnett's method for multiple comparisons when appropriate. A pvalue ≤0.05 was considered to be statistically significant.

3.3. Results

Particle chemical profile of the study samples.

Elements, ions, carbonyls and free fatty acids have been identified and measured by ICP-OES from DEP, B20, and B100 combustion exhaust particles. Elemental analysis with water (Table 1A) indicated B100 had the most Cr, Cu, Fe, Mg, Mo, P, Na, SO_4 and V out of all three particles. B20 however had the highest amount of Ca, Co, K, Li, Mn, Ni, SiO^2, Sb, Sr and Zn out of all three samples. Diesel exhaust particles contained the highest amount of Al compared to both B20 and B100. Water soluble particle analysis appears to show a trend of increasing transition metals with the incorporation of biodiesel. The acid soluble particle fraction indicates more elemental species found in greater abundance with B20 relative to B100 and DEP. In general, both water and acid soluble extractions detected higher concentrations of metals from B20 combustion particles with 8 (i.e., water extracted) and 17 (i.e., acid extracted) metals higher than both the DEP and B100 samples. Notable exceptions were that B100 had higher V, Mg, and Fe in some extracts. Comparison of values we obtained with SRM 1649a to NIST website values indicated slightly lower concentrations for several metals [167]. For instance, Cu was 250 and 233 µg/g, Cd was 32 and 22 µg/g, Ni was 128 and 166 µg/g, and V was 262 and 345 µg/g in comparisons of our values with the NIST certificate of analysis, respectively. This lower trend in metals may have been due to decreased detection due to our analyses of several more metals per run than the NIST analyses, such as Ca, Fe, Pb, and Zn.

Ion measurements from ICP-OES were analyzed with particles suspended in water (Table 1B). B20 constituents dissolved in water had relatively large concentrations of $SO4^{2-}$, $PO4^{3+}$ and Ca^{2+}. B100 appeared to only have increased Na+ ions relative to DEP and B20. Lastly, data indicates DEP had more

NO^{3}-, but no other detectable ions, compared to either B20 or B100. All results of ion measurements indicate few ions were present in the particle fractions of all three samples; however, more ions were found in larger quantities in B20 particles. No endotoxin was detected in the three particle types assessed by the Limulus clotting assay (data not shown).

Carbon type analysis measures the amount of elemental and organic carbon which makes up the carbon core of combustion particles, a measure which can alter particle reactivity (Table 1C). The analysis was conducted based on sequential pyrolytic vaporization and detection by transmittance. DEP has the largest organic carbon concentration relative to B20 and B100. The elemental carbon fraction increases with B20 particles relative to B100 and DEP, giving B20 the greatest EC/OC ratio of the three sample particles.

Particles were processed for detection of fatty acid length and quantity. Each particle was identified against the retention time of 11 standard saturated free fatty acids. The total number of saturated fatty acids for each sample type was not dramatically different (Table 2). However, DEP contained more long chain species (> C11) of free fatty acids while more short chain (< C6) fatty acids found in B100. The mass total indicated DEP and B100 had higher detectable levels, but similar species, of the selected free fatty acids compared to B20. DEP also contained higher amounts of the smaller C4-C6 fatty acids than fatty acids >C9. In total DEP had the highest mass total for fatty acids found on 30mg particle.

The gas phase emissions associated with the combustion particles were previously reported [34] and indicated that DEP, B20, and B100 had relatively similar levels of acetaldehyde and formaldehyde. The particles, when analyzed for DNPH derived carbonyls (Table 3), found relatively similar amounts of heptanal, octanal and nonanal in DEP, B20 and B100. The data indicated B20 had the highest total carbonyl amount by mass (i.e., approximately 2.1-fold and 1.4-fold more DEP than B20). Propanal and hexanal were both elevated in B20 relative to B100 and DEP with approximately 12 ng/10 mg of PM equivalence.

The TBARS assay indicated all three particles had relatively the same ability to generate reactive substances through free radical chemistry. DEP was 5.91 ±0.27 SD fold higher and both B100 and B20

were 4.11 ±0.06 SD and 4.96 ±0.05 SD fold greater in TBARS production from water control (i.e., no PM extract). The data suggested DEP particles compared to B20 and B100 can induce greater reactive oxygen species (ROS) generation in a biological system.

The DLS zetasizer results evaluated the ability of each solvent to maintain solubility of each particle type and also to monitor the homogeneity or dispersion pattern of particle in solution. The polydispersity index (pdI) evaluates the ability of the suspension to maintain a homogenous distribution in variable viscous solvents. The value of the pdI indicates that the suspension is more homogenous when the number is closer to zero. B100 and DEP do not have identical particle suspensions in water, DMSO and tricaprylin (Table 4). The pdI values for water suspensions indicate B20 and B100, with 0.658 and 0.608 pdI respectively. These values were more likely to precipitate than SRM 2975 (i.e., NIST standard for high sulfur petroleum diesel exhaust particle) and DEP (0.348 and 0.353 pdI, respectively). In tricaprylin, B100 particles suspended better than B20 and DEP particles according to the data. DMSO appears to maintain most sample particles in stable homogenous suspensions except B100 particles as indicated with a pdI value of 0.711 with B100 in DMSO. The aggregation of the suspension, when variable, can be represented by size of the largest peak and that is a representation of about 70% of the suspension based on Malvern software parameters. This data indicated that when water is used as a solvent for PM suspension, both DEP and B100 are relatively similar with 284 nm and 382 nm size particles but B20 has larger agglomerations (560 nm). B20 maintained an average (particle fraction ≥70%) 256nm size in DMSO, but contained larger particles around 562nm and 743nm in both water and tricaprylin, respectively. Tricaprylin appeared to be the ideal solvent for B100 suspension with average particle size at 489nm and with 0.133 pdI. The solvent with best homogenous dispersion and smallest aggregation was different for each particle type.

Comparison of soluble particle components in different solvents

Solvents with a varying degree of polarity were used to conduct extractions of the three particle types. Several solvents ranging in polarity and stringency were used for the extractions. Figure 3.1 and 3.2 highlight the range of species collected with DMSO and tricaprylin. The other solvents shown in

Figures 3.4, 3.5, and 3.6 show how some solvents are unable to capture the same range of species detected by UV. The data previously indicated that different classes of components were extracted from solvents using our method; the extraction and subsequent UPLC separation and PDA detection was used to further illustrate the compositional differences between the particle types. Extraction of PM with tricaprylin (Figure 3.1) appears to collect non-polar species better than solvents such as hexane or acetonitrile. Typically two relatively large peaks were observed by UPLC analysis of all particle types with the largest peaks eluting approximately at 4.7 and 7.5 mins. The polar components eluting earlier in the run are more abundant with DEP and B100 compared to B20. DMSO, an aliphatic solvent, eluted species many species both polar and nonpolar from all PM types (Figure 3.2). Of the solvents examined, DMSO appeared to extract the most individual substances if each chromatogram peak is considered an individual substance. This was best observed with DEP extractions where 20 resolved peaks were visible. Water and hexane extracted primarily polar and non-polar species, respectively, with varying amounts (Figure 3.4 and 3.5). ACN extracted both polar and non-polar species and indicates peak at 8.64 min may be a specific biomarker of petroleum in the fuel as it does not appear in B100 extraction (Figure 3.6).

Analyses of B100 emissions [160] has found unique volatile fatty acid fragments (i.e., methylacrylate and methyl 3-butenoate) in the gas phase. Unfortunately, we were not able to detect the presence of these components (RT ~ 1- 3 min) in the chromatograms of extracted B100 with any solvent suggesting that those markers may be detected predominantly in the gas phase.

Biological activity of PM extracts

To demonstrate that extracts contain adverse biologically active components, EA.hy926 cells were exposed *in vitro* to extracts of water, DMSO and tricaprylin for 6 hrs. Cytotoxicity was measured with released LDH activity from EA.hy926 (Figure 3.3). Exposure of cells to the tricaprylin extracted particles did not induce changes in LDH values that were statistically significant from vehicle (Figure 3.3A). Cells exposed to water extractions of DEP and B20 indicated low dose (i.e., 10- 50µg/mL) exposures can generate statistically significant ($p \leq 0.007$) cell toxicity. Water extraction of B100 was not significantly cytotoxic even with the highest concentrations (Figure 3.3B). DMSO extractions of B100,

DEP, and B20 induced cytotoxicity at 10-300 μg/mL and were statistically significant from vehicle control (all $p \leq 0.05$) (Figure 3.3C). Data collected with DMSO extracts suggests our lowest dose may also be cytotoxic, preventing visualization of a total dose-response curve. Increased cell culture exposure times of 24 hrs with tricaprylin and DMSO extractions indicated low dose concentrations of DEP were more cytotoxic than B20 and B100 (data not shown).

3.4. Discussion

This study found liquid soluble extraction with DMSO of combustion particles (i.e., diesel, biodiesel, and diesel biodiesel blends) are comprised of biologically active compounds. We initially compared the chemical species such as metals, ions, and organic components of each particle before undertaking the process to create an ideal liquid extraction. An aliphatic solvent, DMSO, was identified to homogenously maintain the soluble compounds extracted from all combustion particle samples without precipitation or bias. The soluble compounds were well sustained in suspension to generate reproducible and dose dependent cellular toxicity response with EA.hy926 cells exposure.

Particle characterization from these samples suggest that diesel particle composition varies not only from biodiesel emissions but also from biodiesel- diesel blend. In this study, the amount of metals and ions varied from B20, B100 and DEP. For example, the metal content of B20 particle was greater than the two neat fuels, B100 and DEP. Sample B20 particle also appeared to be rich in phosphate and nitrate compared to the two neat fuels. The carbonyl content and fatty acid content of the three PM types were relatively similar. Secondary aims of these studies were to evaluate various extraction solvents on their ability to extract both polar and nonpolar species from biodiesel particles specifically, and to then choose a solvent which was relatively non-toxic in order to allow subsequent toxicity testing of the extracted material using cell culture models. In this study, PDA scanning identified unique peaks, indicating different soluble species, from extraction of sample particles with variable solvents (e.g., water, DMSO and tricaprylin). Exposure of the soluble material from each solvent to EA.hy926 cells resulted in the DMSO extract possessing the greatest potency for a dose-dependent cytotoxic response. Thus, the

concept to maintain extraction species in the liquid phase provides to be beneficial for cell culture assessment of soluble compounds toxicity.

Previous studies examining biodiesel combustion have generally indicated decreases in particle emissions, metals, NOx species, carbonyl's and PAHs compared to petroleum diesel yet, most studies show no linear decrease with incorporation of biodiesel into diesel [61, 64, 67]. Our study discovered that the change in emissions, specifically metals, was not associated with the amount of petroleum diesel since diesel had the least transitions metals relative to B20 and B100 samples. Previous reports had shown that metals were found to be elevated in combustion of rapeseed methyl esters (biodiesel) blends [66] , pure waste cooking oil [67, 68] and in kanjar oil [42]. It is still unclear what the reason(s) may be for the increased metal content in biodiesel emissions and the lack of correlation of certain emission components in blends compared to the fuel neat. One study found correlation with the composition of lubricating oil and the metal emissions post combustion suggested that the lubricating oil as the source for metals such as Mg and Cu in biodiesel exhaust [42]. The study proposed elevations of specific transition metals from biodiesel combustion are due to the additives and lubricating oils which help optimize the engine efficiency (i.e., optimize fuel cetane values) that are predominantly used with biodiesel but not with diesel fuel. Additionally, a previous study with rapeseed based biodiesel combustion resulted in particles of elevated Zn compared to diesel. Based on this study, the proposed source of Zn was not found in the lubricating oil, however, it has been proposed that the FAMEs can generate a detergent-type reaction which leaches Zn from the inner linings of the galvanized engine.

Additional differences between biodiesel and diesel include the amount of elemental carbon and organic carbon, fatty acid and carbonyls. Based on our results, the carbon type (i.e., elemental or organic) from B100 and B20 indicate elevated elemental carbon which is synonymous with diesel. However, this is not consistent with previous studies that show combustion of biodiesel increases soluble organic fraction relative to diesel [168, 169]. Additionally, our study analyzed primarily saturated fatty acids from C4-C18, which had not been assessed in combustion particle to this date. The method did not allow for evaluation of unsaturated and methylated, however, we proposed that longer carbon chains are expected in B100 and B20 relative to DEP based on the GC-MS data by Sidhu et al who detected a range

of C8-C30 fatty acids and methylated fatty acids in biodiesel, but not in diesel [40]. Other studies examining air pollutants such as combustion exhaust and ambient air have measured fragmented FAMEs and fatty acids respectively [34, 170], suggesting that there are measureable levels that affect air pollution, but little is known regarding health effects and should be further examined.

Total carbonyls were elevated in both B20 and B100 compared to DEP. Previous studies have identified elevated formaldehyde and acetaldehyde gas phase emissions from B20 compared to DEP [171]. Additionally, studies conducted with multiple vegetable oil type and soy-derived biodiesel found elevated carbonyl levels relative to diesel [154, 172]. The current literature and our study results indicate by blending biodiesel, the emissions will not proportionately decrease carbonyl emissions, from petroleum diesel levels. In contrast, multiple studies of biodiesel blends of soy methyl esters have identified an increase in PAHs [44, 61, 64, 144]. Our DEP, B20, and B100 combustion emissions detected the same PAH decrease with incorporation of 20% and with B100 neat [34]. The emission chemistry may be complex. With fuel blends created with waste cooking oil 20%, 30%, and 100% biodiesel emitted lower PAH levels than diesel, but a 50% blend emitted greater PAHs amounts than pure petroleum diesel [67]. The data suggests all three particles are unique chemical composition fingerprint from each other and particle characterization is largely dependent on both the original fuel type and the percent blend. Our study finds most components bound to exhaust particle are not easy to extrapolate into a linear correlation based upon the proportion of petroleum diesel or biodiesel.

To better evaluate the biological significance of the soluble components they must all be in a stable solution and studied as a mixture. Previous studies have used methods to enrich the extract for specific components using the soxhlet method, dichloromethane (DCM) extraction, and stringent solvents. Additionally, these methodologies were optimized for petroleum combustion related emissions. In our study, we have observed that extraction with DCM, subsequent solvent evaporation with nitrogen, followed by re-suspension in a DMSO can result in up to a 50% loss of chemical species originally extracted, based on peak detection of 200-500nm UV scans (chromatograph not shown). Not only is the extraction method but the extraction solvent is important to collect an accurate sample of the soluble species from particulate matter. A previous study based on water extraction of ambient air (PM 2.5)

identified the insoluble suspension, had the greater potency compared to the soluble extraction at mediating cell death [173] suggest that water soluble components from ambient PM samples may not contain the biologically active soluble species which induce toxicity. Using HPLC separation and photodiode array detection in our study, we identified that DMSO extraction had stabilized numerous non-polar and polar species. Previous studies with lipid mediators indicated that DMSO can keep the easily ionizable carboxylic acid functional group stable in aqueous conditions [174]. Additionally, polar species such as transition metals, are commonly dissolved in DMSO and are stable [174]. The data suggests that there are non-polar species capable of extracting into DMSO from B100, B20, and DEP, though the number can vary by the fuel type.

The extraction and analysis methods proposed in this study offer the potential of standardizing well-defined mixtures which consist of biological active components from exhaust particles. Second, the DMSO extracts used for EA.hy926 cell exposure induced statistically significant LDH release suggesting the compound in solution were toxic. Thus, this method can be used to better evaluate the soluble-species toxicity of new particles including wood smoke and indoor air pollutants of which the range of chemical compounds is still unclear. Typically, many studies have been conducted with extractions to optimize the enrichment of PAHs for genotoxicity studies, or for metals for subsequent redox (ROS) studies. However, in order get a comprehensive understanding of the extrapulmonary cellular response and molecular disruptions to cell functions, a complex mixture of soluble components should be utilized.

Table 1A: Metal concentrations found on DEP, B20 and B100 particles.

	DEP, µg/g (n=4)		B20, µg/g (n=4)		B100, µg/g (n=4)		NIST SRM1649, µg/g (n=4)	
Analyte	H20-soluble	*acid-soluble*	H20-soluble	*acid-soluble*	H20-soluble	*acid -soluble*	H20-soluble	*acid-soluble*
Al	25.0	105.7	14.3	277.1	15.2	116.1	809.3	11,053
As	<6.9	4.7	<5.9	<5.9	<3.4	4.7	10.6	23
B	2.3	5.9	35.6	38.4	8.5	17.4	5.1	25
Ba	<1	4.5	<1	9.2	<0.1	5.8	50.2	366
Ca	<1	230.3	3833.2	6915.8	110.5	1478.8	18602.1	19,010
Cd	<0.4	1.8	<0.4	2.3	<0.3	2.1	23.3	32
Co	<0.7	1.2	17.7	121.1	<0.3	3.2	3.1	13
Cr	<0.4	1.2	4.3	419.5	6.4	32.9	5.2	90
Cu	<1.2	18.8	<1	517.2	4.7	421.1	79.9	250
Fe	<0.4	50.3	5.5	4331.7	15.3	441.2	482.5	24,039
K	3.3	101.3	25.2	32.8	4.4	113.0	1453.4	2,552
Li	<1	0.0	0.7	0.9	0.2	0.2	1.3	15
Mg	<1	32.3	59.6	101.6	180.9	713.5	2164.5	5,629
Mn	0.2	2.1	71.0	145.5	1.4	10.6	137.4	250
Mo	3.8	4.9	16.6	88.4	28.5	49.0	<3	8
Na	48.0	136.7	311.0	343.8	406.2	618.1	1420.8	1,676
Ni	<1.7	1.4	25.3	762.3	<1.6	32.8	67.0	128
P	84.7	74.9	1581.4	1659.4	1946.8	2203.0	1475.0	1,996
Pb	<6.6	7.0	<5.6	31.7	<5.8	9.2	2066.7	18,753
SO4	364.9	1853.5	16648.6	16632.4	1292.2	3042.6	102021.2	103,247
Sb	<4.7	3.6	3.5	9.3	<4.0	3.6	<4	24
Se	<6.4	7.4	<5.5	<5.5	<7.4	7.4	<11	<7.0
SiO2	29.1	47.5	29.0	134.4	30.2	78.4	24.7	1,029
Sr	<1	0.5	1.6	4.1	<1	1.1	57.9	105
Ti	<0.5	5.4	<0.4	17.1	<0.4	5.4	<1	934
V	<0.4	0.9	<0.3	2.7	1.2	19.9	126.0	262
Zn	10.7	96.4	1623.3	2862.9	264.9	1966.7	1474.4	1,564

Table 1B: Ions by ICP-OES

µg/g	DEP_H2O-soluble	B20_H2O-soluble	B100_H2O-soluble	SRM1649_H2O-soluble
F-	<41	<35	<31	45.16
Cl-	<204	<175	<156	465.97
NO2-	<204	<175	<156	<128
Br-	<203	<175	<156	101.20
SO42-	<203	18,689.67	903.40	98,373.37
NO3-	220.30	178.46	<155	4,889.09
PO43+	<408	4,795.49	3,630.85	3,104.62
Li+	<102	<88	<78	<64
Na+	<410	381.53	519.92	1,116.57
NH4+	<815	<703	<627	11,883.19
K+	<408	<351	<313	1,055.88
Mg2+	<405	<349	<312	1,720.81
Ca2+	<2035	5,606.02	<1565	15,860.03

Table 1C: Carbon Fraction Analysis

µg/g by mass	DEP	B20	B 100	NIST SRM 1649a
organic carbon	60,600	36,300	38,200	131,700
elemental carbon	292,500	388,000	118,900	36,400
EC/OC ratio	4.83	10.69	3.11	0.28
total carbon	353,100	424,300	157,100	168,100

Table 3.1. Diesel particle composition varies from both B20 and B100. ICP- Plasma OES analysis of DEP, B20, B100 and NIST SRM 1649 particles. Table 1A of element's includes metals and ions measured with both water soluble and acid soluble (3:1 hydrochloric acid: nitric acid) particle suspensions. Data indicates transition metals Cr, Cu, Fe Mg, Mo and V are highest in B100 acid soluble fraction. B20 has the highest amount of Ca, Mn Ni, Sb, Sr, Zn using acid soluble suspensions. Table 1B consists of strictly water soluble ions. Table 1C contains elemental and organic carbon type data from thermal optical analyzer from Sunset Laboratory Inc. Diesel contains more organic carbon than B20 and B100 however the EC/OC ratio for DEP is only slightly more than B100. B20 appears to have more elemental than both DEP and B100.

HPLC based analysis of fatty acids on combustion PM

Fatty acid	**DEP**	**B20**	**B100**
Butyric acid **(C4)**	13.21	30.62	17.67
Pentanoic acid **(C5)**	16.72	25.71	14.4
Hexanoic acid **(C6)**	121.48	5.3	86.78
Heptanoic acid **(C7)**	N/A	10.14	17.08
Octanoic acid **(C8)**	N/A	14.78	10.96
Pelargonic acid **(C9)**	N/A	14.75	11.42
Hendecanoic acid **(C11)**	N/A	N/A	2.95
Dodecanoic acid **(C12)**	6.03	6.91	N/A
Myristic acid **(C14)**	2.44	N/A	N/A
Palmitic acid **(C16)**	3.72	2.25	3.48
Stearic acid **(C18:0)**	2.73	N/A	2.12
Oleic acid **(C18:1)**	2.43	2.38	2.15
TOTAL	176.31	112.83	169.01

Table 3.2. Particle bound fatty acids totals elevated with B100 combustion. Saturated free fatty acids bound to B100, B20 and DEP were measured using multi-step derivitization process with chemophore and HPLC-UV detection. HPLC Zorbax eclipse column was used for elution with detections at 254nm. Results indicated B100 has more saturated free fatty acid species than B20 and DEP. DEP totals of the detectable free fatty acids are higher than B100 and B20. Data is an average of triplicate runs of particles.

Table 3: DNPH derived aldehydes on collected, suspended, particles

No.	**Aldehyde standards** (ng/10mg particle)	DEP	B20	B100	SRM 2975
1	propanal	4.648	12.208	7.175	5.887
2	crotonaldehyde	3.780	7.420	5.348	4.221
3	cyclohexanal	6.552	24.899	20.391	14.112
4	pentanal	na	na	na	na
5	hexanal	4.242	12.355	6.951	6.671
6	heptanal	3.983	5.341	4.473	4.585
7	octanal	3.794	4.438	4.228	4.277
8	nonanal	4.102	5.110	4.494	4.529
9	decanal	3.633	na	na	na
	TOTAL	**34.734**	**71.771**	**53.060**	**44.282**

Table 3.3. Total particle carbonyls increased with biodiesel and biodiesel blend. Carbonyl analysis of particle was conducted with DNPH derivitizaiton and UPLC- detection. A sample set of nine standards were run and used to detection retention time on C18 column. Internal control of cis-nonanal used per run. Data indicates total detectable carbonyls were greatest in B20. There were relatively fewer carbonyls detected on B100. It appears there are similar carbonyl species profiles for B20 and B100. Hexanal is elevated in B20. Heptanal, octanal, nonanal are approximately the same quantity in DEP, B20, B100 and SRM 2975. Data is an average of duplicate runs of samples. SRM 2975 in duplicates with stdev represented.

Table 3.4. Zetasizer particle suspensions in solvents.

Particle suspension	pdI	aggregation (nm)	zeta potential (mv)
Water			
SRM 2975	0.348 ± 0.002	483 ± 72	−39.6 ± .208
DEP	0.353 ± 0.04	284.4 ± 89	−42 ± .30
B20	0.658 ± 0.17	562.8 ± 422	−32.9 ± .70
B100	0.608 ± 0.13	382 ± 147	−39.6 ± 1.27
DMSO			
SRM 2975	0.309 ± 0.033	4778 ± 357	na
DEP	0.248 ± 0.01	2632 ± 2560	na
B20	0.272 ± 0.02	5055 ± 143	na
B100	0.711 ± 0.02	922 ± 185	na
Tricaprylin			
DEP	0.329 ± 0.061	482 ± 118	na
B20	0.343 ± 0.059	743 ± 343	na
B100	0.133 ± 0.096	489 ± 75.9	na

Table 3.4. Diesel, B20 and B100 particle extraction suspensions vary with solvents and with particle type. Water, DMSO and tricaprylin extraction suspension quality. The three particles types were evaluated with Zetasizer, to identify, if one solvent is more capable of maintaining particle homogenously in suspension. The pdI value describes the width of the particle size distribution curve, ideal dispersion has lower pdI (close to zero). It appears B100 in tricaprylin is the best homogenous suspension. Aggregation data indicates the size of over 70% of the suspension particles. As previously reported Diesel in water [175] contains smaller size particles. DMSO appears to have the largest size particles out of all the solvents. Zeta potential was only applicable for water this value is the particle's ability to conduct charge. It appears all three particle types and the NIST standard have very similar charge potentials in water.

Figure 1. Tricaprylin particle extraction chromatographs

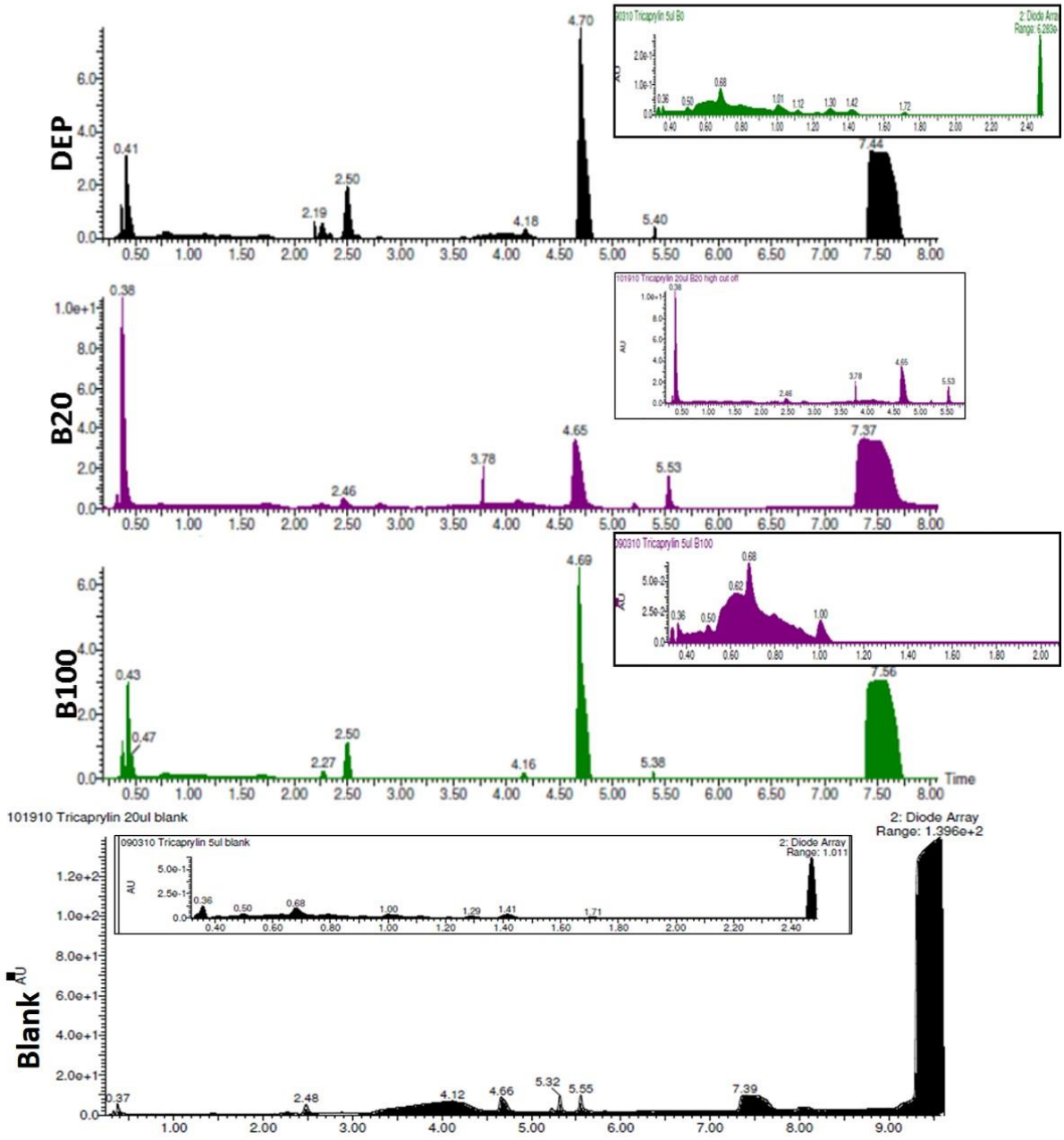
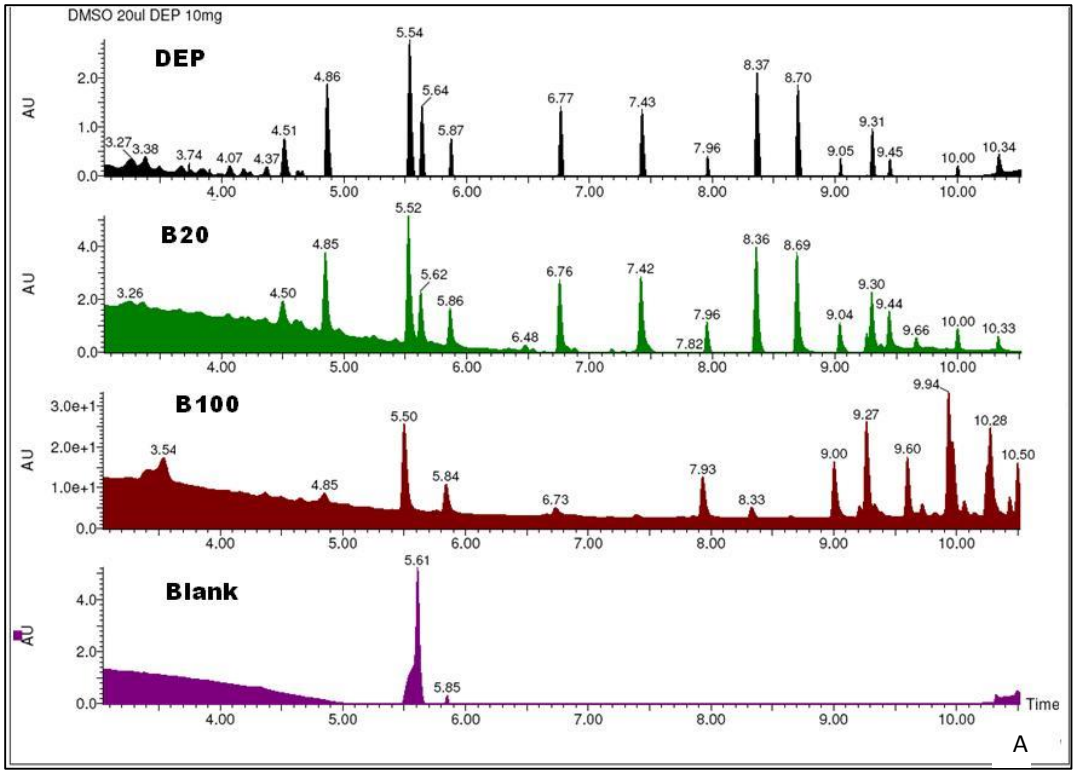


Figure 3.1. Varaible peaks detected from DEP, B20 and B100 Tricaprylin extractions with abundant species detected from B100. 10mg/mL of each sample particle was extracted using Tricaprylin. UPLC –PDA detection scan of 210-500nm. Linear gradient starting with 55% Solvent A (ACN: Water) for 10mins ending at 100% ACN. Smaller boxes are enlarged sections of the polar species which elute quickly. B100 contains smaller polar species found within insert. DEP contains more unique polar peaks 1.12, 1.30, 1.72 mins. B20 had one unique peak at 5.25mins. Tricaprylin extraction represents there is no linear increase of species with increase of biodiesel in original fuel. Petroleum diesel does contain more species as predicted due to its more complex fuel composition.



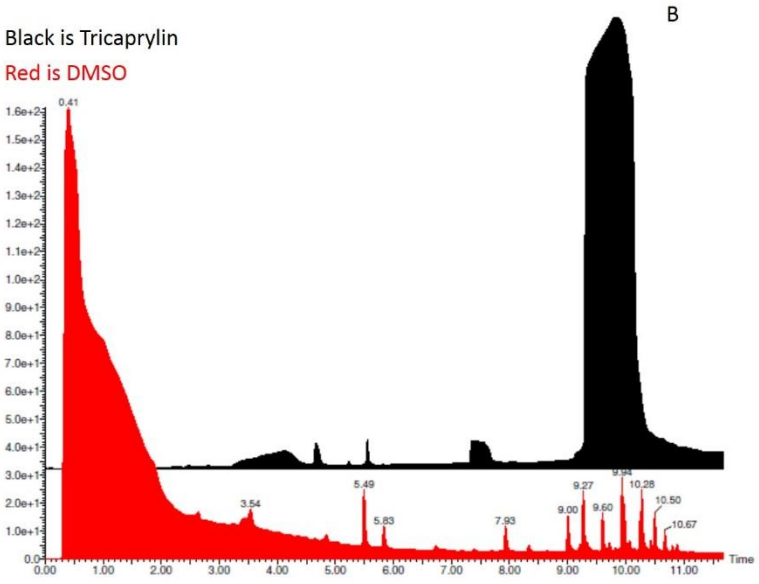


Figure 3.2a,b. DMSO extractions with DEP, B20 and B100 found increased nonpolar species with B100 from PDA- detection of peaks in chromotogram. Chromatogram of DEP, B20 and B100 extracted with DMSO at 10mg/mL. See methods for detail run conditions. Each chromatogram highlights individual species eluting from 3-10mins. Red is B100 chromatograph elutes species later indicating the extraction collected more nonpolar compounds. B20 (green) appears to have equally dispersed peaks 5-10mins. DEP (black) contains more peaks relative to B20 and B100. Figure 3.2B, DMSO and tricaprylin comparison extraction shows bias toward polar with DMSO and non-polar compounds with tricaprylin, due to the solvent nature.

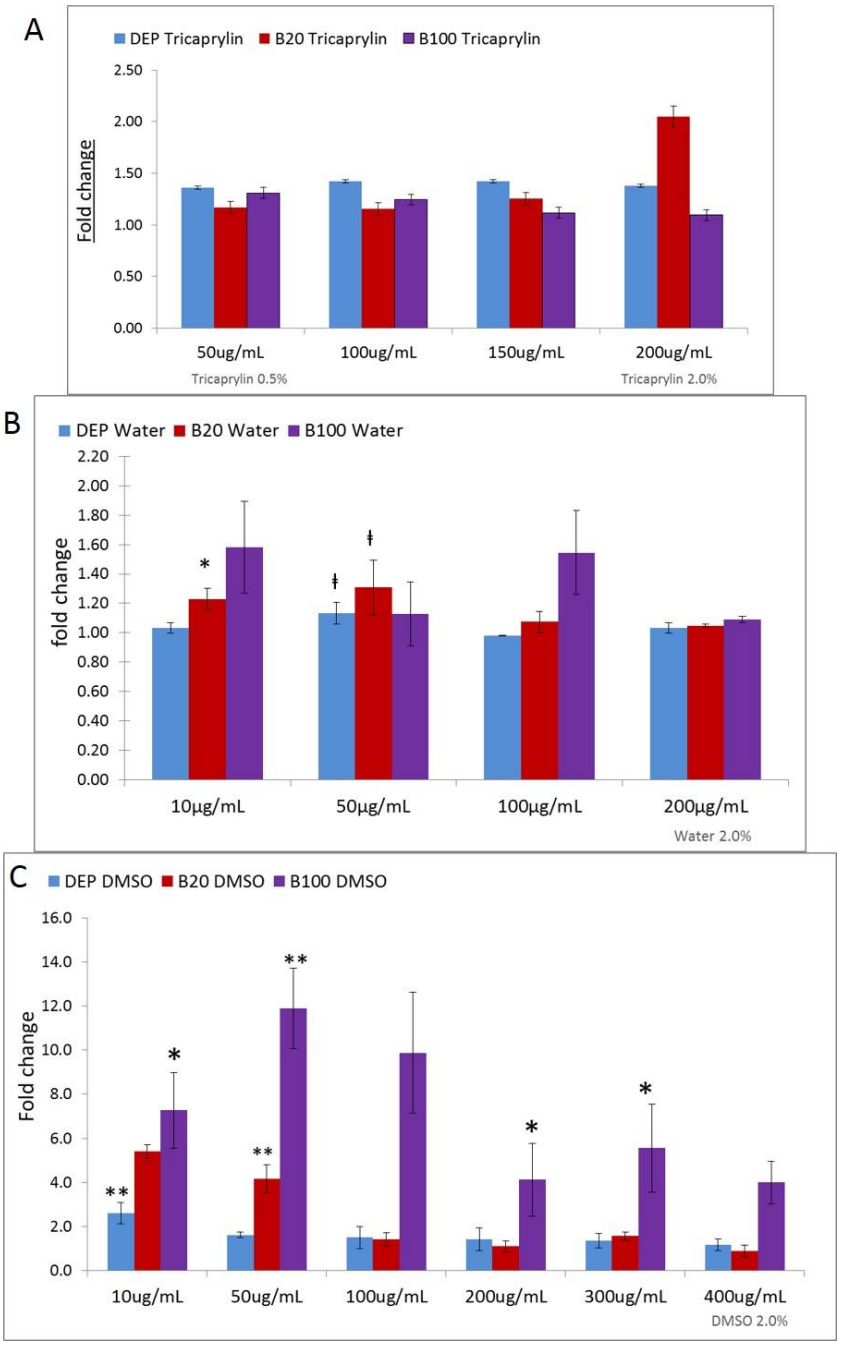


Figure 3.3. EA.hy926 cell cytotoxicity from exposure to different solvent extractions and particle types. Cytotoxicity was measured after 6hr exposure of tricaprylin (Fig 3A), water (3B) and DMSO (3C). Fold change is given relative to each vehicle (i.e., 0.25%- 2% total extracts per well). Data is n=3 with standard error bars. Water extraction of DEP indicates a statistically significant increase at 100µg/mL (**p≤0.001; ǂp≤0.01; *p≤0.05). B20 has statistically significant increases with less than 100µg/mL concentration of DMSO and water but not Tricaprylin. B100 extraction with DMSO indicates a dose dependent cytotoxicity but water and tricaprylin do not appear to have a dose dependent increase in cell cytotoxicity. Data suggests the DMSO extracts of B20 and B100 contain the most biologically active compounds relative to DEP.

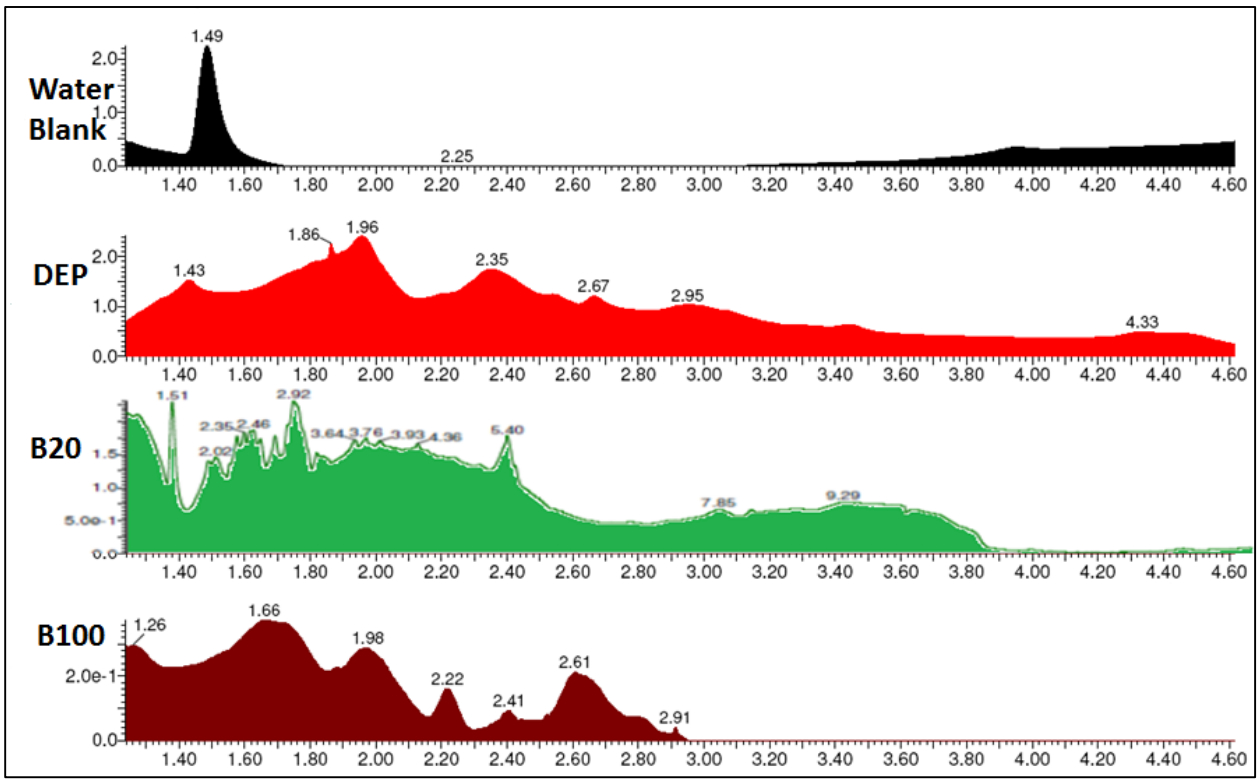


Figure 3.4. Water extractions of DEP, B20 and B100 had poor non-polar species detected in suspensions. Water extractions of all particle with 10mg/mL (particle equivalent). The four chromatographs are particle extractions conducted with 20uL injections of extract. It appears fewer species were collected with water than expected. Some individual peaks are clear however many are engulfed in background. Although each chromatograph appears to indicate the background was high, graphs did not change with lower injection volumes. However, B100 extraction clearly shows few nonpolar compounds were extracted using water as no peaks eluted after 3mins. These data support the idea that petroleum combustion particles have more water soluble components than B100 combustion.

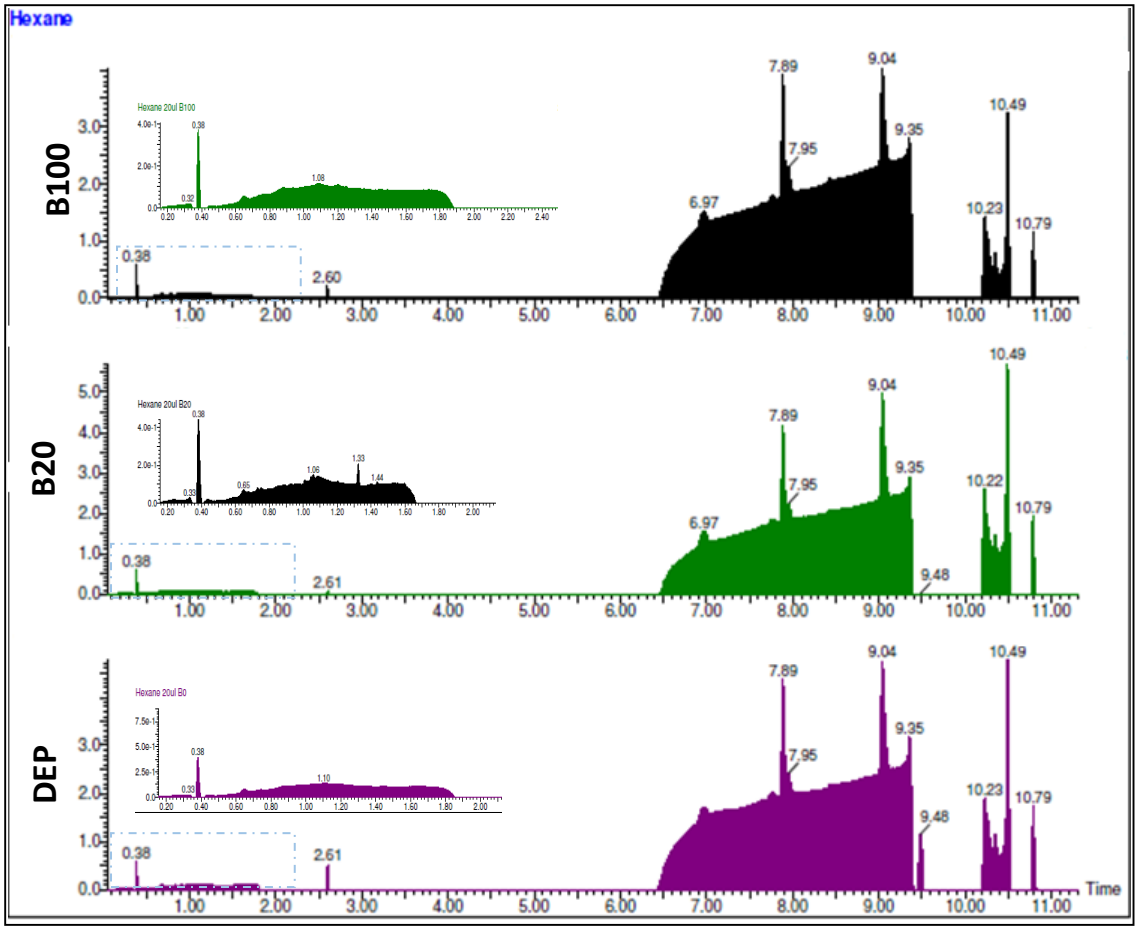


Figure 3.5. Hexane extractions of DEP, B20 and B100 detected identical species and quantities among all samples. Hexane extraction of particle with 10mg/mL (particle equivalent). The large chromatographs are of full gradient elution the inlaid graphs consist of highly polar species eluted before 3mins. The peak at 9.48s in DEP and B20 does not appear in the B100 graph suggesting the compound was extracted with hexane only from DEP and B20 particles. Hexane is a solvent which again shows B20 extraction is unique from either DEP or B100 because of the increase the same volume was injected of all three samples, however only B20 has elevated y-axis values.

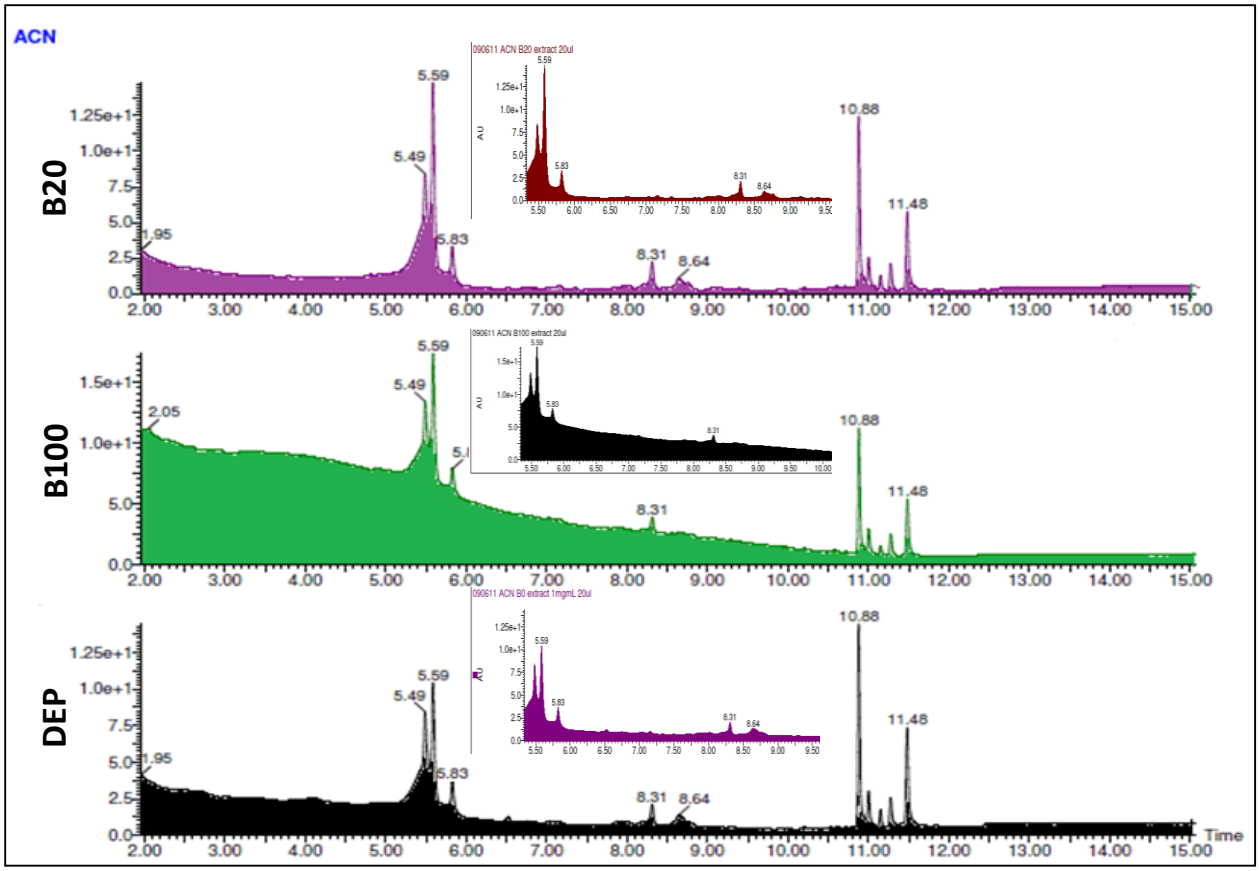


Figure 3.6. Acetonitrile extractions of DEP, B20 and B100 detected similar number and type of species in all samples. Particles were dissolved in acetonitrile at 10mg/mL (particle equivalent). The larger chromatographs contain the full run, smaller inlaid graphs indicate the non-polar species elution between 5-10min. At 8 mins both DEP and B20 elute two similar peaks however B100 has only one. There appears to be more detection of species or background from B100 than from either DEP or B20 with acetonitrile as the extraction solvent.

Chapter 4

HUVEC exposure to diesel and biodiesel exhaust particle generates unique lipid and cytokine profiles.

4.0. Overview

Rising concern for clean alternative energy sources, has led to increased biodiesel production and combustion. However, limited data has indicated, increased biodiesel combustion emissions-induced health effects may potentiate health risks due to incomplete combustion species. To address this need for more data, we analyzed the diesel (DEP) and 100% soy biodiesel (B100) soluble fraction induced Arachidonic acid (AA) metabolism changes associated with inflammation and vascular tone regulation using Human Umbilical Vein Endothelial Cell (HUVECs). The results indicated differences in the cellular responses from these two fuel types, which were likely associated with exhaust composition. Production of lipid metabolites derived from AA which function in signaling vascular tone, both vasodilation (i.e., prostacyclin and stable metabolite 6-keto-PGF1α) and vasoconstriction (i.e., $PGF_{2\alpha}$) were altered. 6-keto-PGF1α, was significantly decreased with B100 but increased over time with DEP exposure. Similarly, even the $PGF_{2\alpha}$ ELISA data found reduced levels with B100 exposure, but an increase with DEP. Further investigation of differential cellular responses with DEP or B100 identified increased pro-inflammatory mRNA expression and protein levels of cytokines IL-6, IL-8 and TNFα with DEP, but not with B100. Our findings suggest that DEP extract exposure induced increased production of 6-keto-PGF1α based on transcriptional activation of inflammatory COX-2. Contrastingly, B100 extract exposure reduced 6-keto-PGF1α levels were independent of COX-1 and 2. B100 exposure indicated reduced free intracellular AA, which was associated with increased re-esterification of the phospholipid membrane. Thus, the reduced free AA was likely mediated by AA esterification enzyme, lysophosphotidyl acyltransferase. The mRNA of three isozymes of this enzyme were decreased. Without AA, the amount available for metabolism into 6-keto-PGF1α was reduced with B100 exposure. The differential cellular responses observed from B100 and DEP extract exposures, were linked with variable soluble DMSO extract. Incomplete combustion emissions

from B100 such as small-chain free fatty acids (i.e., C11-C7) may be responsible for reduced intracellular free AA due to the feedback regulation of acyltransferase. Our study suggests interference of fatty acids from extraction were associated with increased phospholipid re-esterification, thus proposing a possible mechanism for the B100 observed 6-keto-PGF1α reduction. B100 activates unique cellular responses resulting in differential release of lipid mediators and inflammation markers compared to diesel exposure.

4.1 Introduction

Increasing concern of adverse cardiovascular events associated with air pollution has initiated the replacement of petroleum diesel with cleaner renewable biofuels. The use of biodiesel as a cleaner energy source is rapidly growing in popularity within the U.S [1]. Biofuels are uniquely created by the transesterification process. The process begins with plant oil that is reacted with an alcohol and a catalyst to generate fatty acid methyl esters (FAMEs). The FAMEs simply replace diesel in unmodified petroleum diesel engines resulting in fewer particle matter (PM) emissions per gallon of fuel, with 77% reduction [2, 3]. Other emissions including polycyclic aromatic hydrocarbons (PAH), and carbon monoxide (CO) are also reduced [1]. While limited data on the biodiesel combustion emissions exists, studies suggest that the reduced PAH and CO emissions is a tradeoff due to increased levels of NOX, benzene, formaldehyde and zinc content in the exhaust [34, 42, 151]. Although, studies have examined the chemical properties of biodiesel exhaust there is only limited data on the associated health effects.

The sparse number of biodiesel PM studies to date, have found that B100 emissions can induce similar adverse cellular responses as seen with diesel combustion exhaust exposure. Some of the cellular responses found biodiesel also increased neutrophil recruitment [64], oxidative stress [176], mutagenicity [150] and pro-inflammatory responses [159, 177]. Most of these studies suggested that specific cellular responses can be deduced to PM composition. Recently, the high organic matter found in biodiesel combustion may impinge on the oxidative stress response as measured by protein carbonyl data [168] or oxidation with dichlorofluroescin diacetate [90]. Similarly, studies have identified that the inorganic species on DEP such as transition metals are strongly correlated with oxidative stress generation [81]. The use of metal chelators decreased ROS supporting the role of metals in generating oxidative stress.

Transition metals were also associated with increased ROS products from biodiesel exposure to THP-1 cells [176]. The organics exhaust species including PAHs were found associated with increased cellular mutagenicity [150, 154]. The conflicting data of PM composition-dependent effects on cellular responses suggests that multiple compounds may generate similar cellular effects, thus the composition likely accounts for differential cellular responses to diesel and biodiesel particles and particle extracts.

Recent findings demonstrated that controlled human exposure to diesel exhaust emissions reduced brachial artery diameter and impaired vasodilation [77]. Measurement of forearm blood flow remained reduced even after infusion of endothelium dependent vasoagonists such as bradykinin, but not when given endothelium independent vasoagonists. Biodiesel created from rapeseed combustion exhaust exposure generated in the same results [79]. Exhaust composition studies have previously indicated that the gas phase emissions from biodiesel may contain harmful levels of nitric oxide relative to diesel combustion; however in this study gases were diluted to equivalent levels with particle. This suggests the vasodilation signaling disruptions are likely a response to the translocation of exhaust particles or the soluble compounds bound to the particle. The current theories for PM exposure induced cardiovascular effects relies on 1) direct PM contact with endothelial cells lining the circulation or 2) the soluble components are translocated into circulation leading to alterations and/or 3) systemic inflammation markers originating in response to PM from the lung or extrapulmonary tissue disrupting endothelial cells homeostasis [5]. Although, multiple factors are responsible for vascular tone regulation, mediated by endothelial cells, the exact mechanism by which DE and BD exhaust altered vasodilation is not clear. There is also little evidence to support the same physiological response pathway seen in both diesel and B30.

Endothelial cells contain peptides, NO and lipids to regulate the vasodilation function, however little is known regarding the lipid mediator, prostacyclin (PGI_2) that is specific to endothelial cell-mediated vasodilation. PGI_2 is generated from free arachidonic acid (AA) released from the lipid bilayer when signaled by cPLA2. The stable metabolite, 6-keto-PGF1α, is typically measured because of the short half-life of PGI_2. The limiting factor for PGI_2 production is the availability of intracellular un-esterified AA, which is also feedback regulated by lipid remodeling enzymes (e.g., acyltransferases and

phospholipases). Although, no studies have associated diesel or biodiesel exposure with PGI_2, studies with cigarette smoke exposure showed reduction of PGI_2. Chronic smokers had significantly reduced 6-ketoPGF1α relative to the non-smokers [14]. Additionally, smoke condensate was incubated with HUVECs and resulted in significant dose dependent reduction of 6-ketoPGF1α, suggesting that there were soluble compounds in the condensate responsible for disruption of endothelial cell homeostasis [15]. The particle and the soluble compounds alone may not be the only exhaust component responsible for alterations in 6-ketoPGF1α. Studies have indicated common combustion–related gas pollutants (e.g., ozone and nitrogen dioxide) can also alter lipid levels [178]. Previous exposure research with diesel and biodiesel have not yet addressed if lipid vasodilation signaling is disrupted in endothelial cells.

These vascular tone signals include a gas (-NO), protein (endothelial-derived hyperpolarization factor (EDHF)) and peptide (endothelin-1 (ET-1)). Some studies showed that nitric oxide (NO) was reduced and ET-1 was elevated from basal levels with diesel exposure. Diesel exhaust exposure in rat coronary arteries (CA) was associated with reduced NO release, relative to air [96]. A fluorescence detection assay identified CA generated superoxide (O^{2-}) suggesting that eNOS was unable to uncouple and therefore NO was not released to initiate dilation response on possibly that O^{2-} reacted with NO. Another diesel exposure study found that the human subjects had elevated levels of plasma endothelin-1 (ET-1) which is associated with constriction [76]. Although the exact mechanism by which EDHF established a vasodilation response is unclear there is evidence for hyperpolarization of smooth muscle cells concurrent with vasodilation [179]. This was initially identified with 1hr incubation of arterial cells to cigarette smoke DMSO extract suggesting there are PM soluble compounds that can alter the EDHF mediated vasodilation response in rats [179]. Based on these studies with diesel exposure and vasodilation signals, there is maybe multiple pathways responsible for vasodilation and thus pollutant exposure induced effects are not limited to single markers.

The role of lipid mediators in diesel and biodiesel exhausts-induced endothelial cell- mediated vasodilation has not been well studied. We hypothesized that the AA metabolite pathway of endothelial cells is susceptible to interference from DMSO-extractable fractions of diesel and biodiesel exhausts. The primary objective of this study was to use the soluble extractable fraction from diesel and biodiesel

exhaust particles to evaluate the lipid 6-ketoPGF1α production regulation and inflammatory response in endothelial cells.

4.2. Material and Methods

DMSO particle extraction containing the soluble fraction

Biodiesel fuel soy methyl ester from Agland (Eaton, CO) and petroleum diesel from Haltermann Products (Channelview, Texas) were combined with additive and lubricants according to previously reported methods [161]. These fuels were combusted at National Renewable Energy Labs located in Golden CO, under EPA AVL 8-Mode Heavy Duty Cycle conditions [161]. The exhaust particles were stored in glass jars at 25° C. Particles were weighed out and dissolved in DMSO as previously described in chapter three. The extracts of diesel (DEP) and biodiesel (B100) were also stored in glass vials at 25° C.

Cell culture and exposure

Human umbilical vein endothelial cells HUVECs (Lonza) were seeded in either a 100mm dish or 12well plate with approximately 6.0 x 105 or 12 x105 cells, respectively, for most assays. Cells were grown in endothelial cell growth media supplemented with hydrocortisone, human epidermal growth factor, human fibroblast growth factor, fetal bovine serum, gentamicin and amphotericin, heparin, human recombinant vascular endothelial growth factor, ascorbic acid and recombinant insulin like growth factor (Lonza). Cells were 70% confluent before pre-treatments and exposures, using passages 3-7. Cells were incubated at 37°C under 5% CO2 with and without IL-1B from Cell Signaling at 1ng/mL concentrations for the length of the extract exposure. Total amount of DMSO in all experiments was maintained at 0.25% of media.

Cell viability and cytotoxicity detection

HUVECs were incubated with DEP and B100 at concentrations of 40-100µg/mL for 6 or 24hrs. After exposure media was aspirated, cells were rinsed and incubated with formzan dye as per manufacturer's instructions (MTT assay) (Promega, Madison, WI). Cells were lysed prior to absorbance reading. Cell viability was assessed with mitochondrial enzyme activity post 6hr or 24hr exposure. Cell

cytotoxicity was determined by measuring the activity of released lactate dehydrogenase (LDH) using cytotoxicity detection kit (Promega, Madison, WI).

Quantitative real time PCR

Total RNA was isolated using RNAeasy Mini Columns (Qiagen). A total of 1.5µg RNA was reverse transcribed into cDNA using Random Primers from the TaqMan kit (Life Technologies). RT-PCR was performed in 96well plates in 20uL total volume using manufacturer conditions with an ABI prism 7900 HT quantification system (Applied Biosystems, Foster City, CA). Human primers for COX-1/2, PGI-synthase, SOD-1, NOX-4, NQO-1, THBD, VWF, tPA, HO-1, VCAM-1, ICAM-1, TIMP2, MMP2, IL-6 and TNFα (Life Technologies) were used and samples were run in duplicate. All gene primers were normalized to human β-actin and relative fold change was calculated for each treatment (ΔΔ Ct).

Western blotting

Cells were lysed on ice using RIPA buffer. Proteins were separated on 8% reducing SDS-PAGE (Pierce) and transferred onto nitrocellulose membranes before blocking with 5% non-fat milk and incubated overnight with primary antibody in 4˚C. The incubations with the primary antibodies for COX-1,2 (Cell Signaling, Mass), PGI-synthase (Cayman Chemical Ann Arbor, MI) and β-actin (Fisher Scientific) were followed by incubations with horseradish peroxidase – conjugated anti-mouse or anti-rabbit immunoglobulin G. membranes were developed using enhanced Pierce ECL system (Thermo Fisher Scientific).

Cytokine, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ ELISA measurements

The Human Proinflammatory II kit (MesoScale Discovery, Gaithersburg MD) was used to evaluate cytokine levels of IL-6, IL-8, TNF-α, and IL-1β released into media from HUVECS post 6hr exposure. MesoScale Discovery (Gaithersburg MD) ELISA plates were prepared according to manufacturer's protocol. Standards were created with diluted cell culture media and analyzed on the MS2400 Imager (MesoScale Discovery). The levels of 6-keto-PGF1α and $PGF_{2\alpha}$ were examined in the media using EIA kits purchased from Cayman chemical (Ann Arbor, MI) and samples were processed according to

manufacturer's protocol. PGF2a was measured in media with an ELISA kit from R&D Chemicals (Minneapolis, MN).The manufactures instructions were followed for both kits.

Cyclooxygenase, PGI synthase, and cPLA2 activity enzymatic activities

Cells were challenged after extract exposure with 10uM AA (Sigma), and 1.0uM PGH2 (ENZO). COX-1/2 and PGI synthase inhibitors were used for assay verification; (acetylsalicylic acid was purchased from Sigma; NS-398 and U51605 Cayman Chemical). The calcium dependent cPLA2 activity was assayed in lysate after exposure. Cellular extracts were collected using HEPES buffer at 10mM pH 7.4 with 1mM EDTA and incubated on ice for 30mins. Cell debris was precipitated by centrifugation 10,000x g for 10mins at 8°C and supernatant was collected. The lysate was used in a cPLA2 enzyme activity kit according to manufactures protocol (Cayman Chemical). Each sample was done in duplicate post incubation with bromophenol (calcium independent enzyme activity inhibitors). Each reaction was allowed to progress for one hour and the data were normalized to blank.

HPLC analysis of extracellular eicosanoid release from HUVECs

HUVECs were incubated with 0.5uCi [3H] AA (ARC, St. Louis, MO) prior to extract exposure for scintillation based detection of AA metabolites. Cells were incubated with [3H] AA for 18hrs for cellular incorporation then cells were rinsed and replaced with fresh media, and incubated with extract and/ or IL-1β for 6hr. After exposure media was collected in glass tubes and acidified to pH 3.0 with 9% formic acid. Remaining cells were rinsed twice with PBS and collected in RIPA buffer for quality control of total tritum in cells. Eicosanoids were extracted from acidified media using 1:1 cyclohexane: ethyl acetate extraction as previously described (Suggs, Madden et al. 1986). Aqueous layer was collected in new tube then repeated and pooled fraction was dried using N2 gas at 4°C overnight. Samples were re-suspended in 140µL (1/1: v/v) methanol: water vortexed thoroughly and transferred into HPLC vials. Internal standards of PGF2α and 6keto PGF1α (Cayman Chemical) were added at 4µg/mL per sample. HPLC buffers were prepared as solvent A (9/1/0/0.1% H2O/methanol/acetic acid) and solvent B 100% methanol. A C18 reverse phase column was equilibrated with 43% solvent A and 57% solvent B. A gradient of 43% Solvent A for 27mins followed with 34% for 57 min and finally 0% modified from Henke

et al 1986. Twenty second fractions were collected and mixed with 1:4 with liquid scintillation cocktail and radioactivity measured by standard scintillation counting.

Measure of free (un-esterified) arachidonic acid

Free arachidonic acid (AA) was measured with cellular incorporation of ^{3}H-AA pre-exposure followed by lysis, extraction preparation and HPLC separation with scintillation counting of eluted fraction. The HUVECs were given 0.5 µCi universally labeled ^{3}H-AA (American Radiolabled Chemicals, City ST; 200mCi/mmol) per 10cm dish at 70-75% confluence for 18hrs after which labeled media was removed and cells rinsed thoroughly. To ensure all the free AA is measured prior to metabolism, we inhibited acyltransferase, lipoxygenase and cyclooxygenase using inhibitors Triascin C nordihydroguaiaretic acid (NDGA), acetylsalicylic acid and NS398 (Sigma Aldrich, St. Louis, MO). Cellular lipids were extracted using 2mL methanol both for lysis and extractions. Samples were dried down using N_2 gas and re-hydrated in 90uL water: methanol 1:1 (v/v). A Waters Acuity HPLC fitted with a C18 reverse phase column (HiCHROM Ultrasphere 5ODS dimensions 250 x 4.6mm) with an added guard column was used for sample separation and the retention time of non-radioactive lipid standards were identified by dual UV-VIS detection (193nm & 292nm). Solvent A was 90/10/0.1 (Water/ Methanol/ acetic acid) and solvent B 100% Methanol using a previous method. Gradient for solvent A and B is as follows (47:53) for 30 min; (40:60) 30-45min; (27:73) 45-60min;(13:87) 60-80min;(0:100) for 80-110min. Elution fractions were collected and triatium detected using standard liquid scintillation counting (Perkin Elmer). Values were calculated as percent 3H-AA from total titrated lipid intracellular pools including lysophosphotidylcholine (LPC) and glycerophospholipids (Standards were purchased from Cayman Chemical).

Statistical analysis

All experiment values are expressed as means ± SEM unless indicated. Statistical analyses was conducted with, SigmaPlot v 12.3 by Systat (San Jose, CA). The tests used included one way ANOVA analysis followed by Dunnet's or Holm-Sidak methods. In some experiments paired-t tests were used when appropriate.

4.3. Results

HUVECs viability and cytotoxicity after exposure to DEP and B100

The effects of DEP and B100 extracts (up to 200 µg (PM mass equivalent)/mL) on cell viability and cytotoxicity were evaluated under identical exposure conditions with MTT and LDH assays. The cell viability data generated from the MTT assay indicated no distinct changes with B100 and DEP relative to vehicle with extract concentration of 10-150ug/mL for 6hr exposure (Figure 4.1A). With 200ug/mL DEP but not B100 exposure, there was a 50% reduction in HUVECs viability ($P \leq 0.01$). MTT based cell viability was represented as fold change and has been normalized to amount of protein. Trypan blue measurements confirmed ~60% cell death with the highest DEP concentration (data not shown). No cytotoxicity was found with LDH release into media after 100µg/mL DEP and B100 exposure from 6- 24hs (Figure 4.1B). An additional MTT assay and trypan blue incorporation with 100ug/mL for 1, 6 and 24hrs also resulted in no significant change in viability after DEP or B100 exposure (Figure 4.1C).

Changes in mRNA expression of inflammation, coagulation, thrombosis and tissue repair markers in endothelial cells incubated with DEP and B100 extracts.

Endothelial cells respond to extracellular influence and therefore gene expression changes were measured to evaluate exposure induced response. Gene expression values were normalized to B-actin and represented as fold change from vehicle after 6hr exposure (Table 1). There were no significant changes with DEP and B100 in 100µg/mL gene expression associated with coagulation cascade such as thrombomodulin (TMBD), von Willenbrand factor (vWF) and tissue plasminogen activator (tPA). No significant changes were found in matrix metalloproteinase (MMP-2) and tissue inhibitor matrix metalloproteinase- 2 (TIMP2) expression after B100 and DEP exposure. However, expressions of genes involved in inflammation response were induced with DEP but not B100 exposure. Pro-inflammatory markers such as tumor necrosis factor (TNFα) increased significantly with DEP with ($p \leq 0.05$). Adhesion molecules ICAM-1 and VCAM-1 and COX-2 mRNA also increased with DEP. Interestingly, only the B100 exposed cells responded with significant increases in expression of genes for antioxidant enzymes,

hemeoxygenase-1 (HO-1) and NADPH-oxidase-1 with approximately two fold increase from vehicle. This suggests that unlike DEP, B100 at non-cytotoxic levels HUVECS can mediate an ROS response

Selected cytokines reflect differences in extract response

Cytokine proteins were released from cells and were detected in media. Cytokines proteins were measured after 6, 8 and 24hr exposure to B100 and DEP (Figure 4.2). With only 6hr exposure there was significant decrease of IL-8 and TNFα with B100 exposure relative to vehicle and a non-significant decrease noted with IL-6 production as well. With 8hr B100 exposure statistically significant decreases were found for all three cytokines. These decreases of cytokines did not persist at 24hrs time point, suggesting the B100 extract potency dissipated in media or. in time the cells were able to overcome the suppression induced by B100 exposure. The data presented here highlights a decrease with B100 but almost no change with DEP.

Eicosanoid production responses vary with extract and IL-1B stimulus

The AA metabolism derivatives responsible for vascular activity are PGF2α and 6-keto-PGF1α (vasoconstrictor and vasodilator, respectfully) were analyzed. The amount of 6-keto-PGF1α and $PGF_{2\alpha}$ release was measured in media after 6hr exposure to B100 and DEP to evaluate endothelial cell mediated response. Lipids were measured with both ELISA and radioactivity detection as described in methods. Similar to the reduction seen with cytokines after B100 exposure 6-keto-PGF1α and $PGF_{2\alpha}$ a were reduced relative to vehicle with 6hr exposure (Figure 4.3A). In contrast DEP exposure indicated a significant increase ($p \leq 0.05$) of 6-keto-PGF1α with 8hr exposure and consequently $PGF_{2\alpha}$ was found significantly reduced ($p \leq 0.002$) with only 6hr exposure using ELISA detection (Figure 4.3B) Data from radioactivity assays was represented as raw 6-keto-PGF1α DMP. The HPLC based data confirmed ELISA data of statistically significant increase of 6-keto-PGF1α with DEP exposure with 8hr exposure (Figure 4.3C). HUVECs were co-exposed to extract (100ug/mL) with 1ng/mL of IL-1B to simulate a biological systemic response in endothelial cells. The data indicated that IL-1B induced the production of 6-keto-PGF1α in all cells by at least 80pg/mL, but B100 exposure at 4,6 and 8hr remained reduced relative to vehicle and with 8hr exposure there was a trend toward significance with $p=0.058$ (ANOVA subject test Holm Sidak)

(Figure 4.3D). Furthermore, a non-significant reduction was noted with 6-keto-PGF1α with B100 exposure and IL-1B relative to diesel and was found with radiolabeled HPLC based method (Figure 4.3E) suggesting IL-1B stimulation was not sufficient to increase 6-keto-PGF1α levels relative to vehicle. PGF2α production was also unaffected by IL-1B stimulus as noted (Figure 4.3F). There was a significant reduction of B100 relative to diesel, thus suggesting that the precursor to both 6-keto-PGF1α and PGF2a must be deficient after B100 exposure because both metabolites are created from the same precursor using different enzymes.

Cyclooxygenase activity, mRNA and protein levels were elevated with DEP not B100

Precursor enzymes in the production of 6-keto-PGF1α, were evaluated with mRNA, protein and activity assays after exposure. The mRNA results identified that COX-2 was significantly increased with DEP exposure after 6hrs exposure (Figure 4A). This was also found true at earlier time point of 4hr exposure (data not shown). The COX-1 gene after 6hr exposure shows no change with B100 or DEP however COX-2 is significantly increased with DEP, thus indicating an inflammation response increased transcription. The COX-2 protein was increased with DEP when stimulated with IL-1B compared to B100 with and without IL-1B as quantified from the blot with densitometry (Figure 4.4B). The figure shows a visible increase in the western blot of COX-2 protein in cells exposed to DEP, and the densitometer indicates an approximate 100AU increase from vehicle; however there was no statistical significance. The enzyme kinetics were calculated using amount of product (6-keto-PGF1α) relative to the amount of challenge (Figure 4C). In the activity assay the cells were challenged to increasing concentrations of AA for 10min. The results of the exposures suggest there is immediate production of 6-keto-PGF1α measured from supernatant collected using the ELISA method. The 6hr DEP exposed cells had responded to the challenge the fastest as noted by the steep slope (m=42.41). The B100 exposure created an environment which almost matched DEP with Veh exposure that had the lowest slope (m=19.26). The activity of COX-1 and COX-2 are measured together based on the amount of 6-keto-PGF1α and therefore the increased COX-2 levels in DEP exposed cells may have increased the rate of 6-keto-PGF1α production.

Enzyme activity of acyltransferase and release of free AA altered by B100 extract.

The rate limiting step for production of 6-keto-PGF1α is the availability of free AA which occurs when released from lipid cellular pools. Acyltransferase is a prevalent enzyme in HUVECs which can repopulate the lipid membrane with AA preventing its use by COX1/2. The cells were pre-treated for 4hours with COX inhibitors and NDGA alone or together with acyltransferase inhibitors for 6hrs incubation before adding B100 and DEP extracts. The results were converted from DPM, using 20 second fraction collections, into percent of free AA calculated as DPM of AA over the total phospholipid and triglyceride pools (Figure 4.5A). The first set of data with COX and LOX inhibition, indicated significantly lower levels of AA post exposure with DEP and B100 (*); $p \leq 0.001$. The data also indicated with vehicle about 50% of AA was available for metabolism into 6-keto-PGF1α from measurements of AA fractions over total DPM of all phospholipids and triglycerides. The expected decrease found with DEP exposure and inhibitors suggests there were potential interactions regarding inhibitors and the AA availability not seen with B100 exposure. The LOX inhibitor, NDGA, was previously used as an anti-oxidant which might reduce the availability of free AA or interfere with COX-1/2 inhibitors, thus keeping them active. The second set of free AA measurements include pre-treatment with acyltransferase inhibitor; Triascin C in addition to LOX and COX inhibitors. The inhibition of acyltransferase enzyme followed by B100 exposure resulted in a statistically significant increased with a t-test ($p=0.002$) relative to without inhibition of acyltransferase. This doubling of the amount of available free AA from B100 cells suggests the inhibition of acyltransferase activity was directly responsible for amount of AA available for metabolism. Acyltransferase genes and isoforms were examined relating to gene expression to assess the feedback regulation. However, mRNA levels of lysophosphotidylcholine acyltransferase (LPCAT1-4) were not significantly elevated with exposure to B100 alone (Figure 4.5B). It is likely that other lipid pools can release free AA not maintained by LPCATs.

HUVECs exposure to free fatty acid leads to increased LPCAT and COX-2 levels

Since B100 exhaust can contain free fatty acids (i.e. unsaturated, saturated and methylated fatty acids), the effects of free fatty acids in altering acyltransferase gene expression levels was examined as a possible mode of action. HUVECs were incubated with free fatty acids, stearic and oleic acids to assess if exogenous fatty acids can alter acyltransferase mRNA transcripts. Cells were exposed to 0, 5,10,25,50 and 75uM of stearic acid and oleic acid alone, for 6hr and cytotoxicity was evaluated using LDH assay of the media after exposure (Figure 4.6a). Cells were also stained with trypan blue confirming the LDH results that 0-25uM were non-cytotoxic with 6hr exposure. However, there were some exposures with 50-75uM concentrations which resulted in over 20% trypan blue (not shown) stained cells suggesting cell death; therefore the two highest doses were not considered for subsequent analysis. HUVECs were exposed to 0-25uM concentrations for 6hrs before mRNA was collected for further gene expression analysis. LPCAT3 levels increased significantly ($p \leq 0.01$) from vehicle with stearic acid but not with oleic acid. Inflammation markers COX-2 and TNFα levels also increased significantly with stearic acid and seem to be dose dependent. Oleic acid exposure alone increased TNFα expression. Exposure to oleic acid did not significantly increase gene expression of LPCAT3, COX-2 but did increase TNFα. There also appears to be a dose dependent increase of LPCAT3 transcript levels with stearic acid (Figure 4.6b) and suggests that increased free fatty acids can stimulate the transcription of re-esterification enzymes to reinforce the lipid membrane.

4.4. Discussion

The use of biodiesel continues to grow worldwide while the health effects impact remains unclear. Health effect issues are complicated by the mixture of emission compounds released from biodiesel and diesel combustion. The results from this study identified that multiple pathways are likely affected by the soluble constituents from PM emitted from B100 and DEP. Studies have proposed that soluble compounds may interfere directly with the endothelial cells lining the vasculature via translocation, which was modeled in this study. This study established exposure to soluble compounds from diesel and biodiesel particles can affect the levels of inflammatory mediators of vascular tone

regulation in endothelial cell culture. The findings in this study compliment the hypothesis that different pathways are activated with DEP and B100 exposures. The study results found inflammation was largely dependent on DEP exposure, not on B100. The data suggested that B100 exposure disrupted lipid signaling by restricting the availability of free intracellular AA, thereby potentially reducing the endothelial cell's capability to initiate vasodilation responses.

Oxidative stress response dependent on variable exhaust compounds

The exact mechanism by which pro-inflammatory or vascular tone responses are mediated is unknown. Previously, studies hypothesized the mixture of biologically active species adsorbed to the core of both, biodiesel and petroleum DEP can affect cellular responses. Reports specified increased aldehydes and nitro PAH's from biodiesel combustion both with and without a filter and an exhaust gas recirculation chamber [33, 61] suggesting cellular responses may be mediated by these compounds. In one study a common PAH, phenanthrene, was compared to diesel and the results found a prostaglandin reduction with increased phenanthrene concentration but not DEP exposure in human macrophages [125]. This study proposes that phenanthrene exposure can affect the release of prostaglandins.

Additionally, a recent study found B100 combusted under Euro2 conditions emitted double the amount of zinc as Diesel combusted under the same conditions [157]. Comparison of the health effects of these samples resulted in increased inflammation with both B100 and diesel based on rodent exposure studies, thus proposing transitions metals can also impact exposure responses. Transition metals have a direct cellular response that can be measured via elevation of antioxidant gene expression. In our study B100 characterization indicated elevated levels of zinc, copper, iron and vanadium relative to DEP (Table 3.1). In consequence HUVECs exposure to B100 resulted in significantly elevated gene expression levels of HO-1 and NADPH-1 that are both related to cellular oxidative stress (Table 4.1). Other studies have corroborated these findings, with other antioxidants enzymes gene expression studies. For example, Hawley et al found B99 exposure to human Bronchial Epithelial Cells resulted in elevated SOD gene expression [82].

Other combustion compounds can also be associated with oxidative stress, such as chemicals with ester groups. In a previous biodiesel study, approximately half the B20 exhaust particles were identified to contain an ester group [176]. The exposure of the ester –rich PM resulted in significant ROS generation in TPH-1 cells after only 1hr incubation suggesting that multiple individual chemical species can induce oxidative stress and inflammation not just one type of emissions compound.

Composition based cellular responses differ with Biodiesel and Diesel

Composition differences from biodiesel and diesel suggest that unique cellular responses are expected. In addition to our study, previous studies have found differences in pro-inflammatory response from diesel and biodiesel exposed cells. In this study with exposed HUVECs, we found significantly decreased amount of IL-8 with 6 and 8 hrs B100 exposure (Figure 4.2). Though both protein and mRNA levels indicated cytokines were decreased with B100 exposure, DEP exposure resulted in IL-6 increase in protein levels after 24 hr exposure. In contrast, studies with airway epithelial cells (i.e. BEAS2B cells) exposed to biodiesel resulted in an elevated IL-6 transcripts from diesel [43, 89]. Previous THP-1 cells exposed to RME found no significant increase of IL-8 mRNA over a 24hr exposure and BEAS2B cells exposed to increasing concentrations of B50 also did not find significant increase of IL-8 gene expression levels [43]. It is possible not all cell types respond similarly to biodiesel exposures. These differences in cellular responses from diesel and biodiesel might relate to lipid mediators.

The lipid signals, 6-keto-$PGF_{1\alpha}$ and PGF2α production varied with exposure to diesel and biodiesel in HUVECs. Further examination of the enzymes involved in the production resulted in variability with COX-2 gene expression, protein and activity. DEP exposure increased the COX-2 expression and activity but B100 exposure did not. Previous studies have indicated increased COX-2 activity, generally increase the 6-keto-PGF1α production. To verify the connection with induction of COX-2 and 6-keto-PGF1α we stimulated the cells with IL-1B along with exposure to the study samples. This cell culture model represents the cooperative role of PM soluble compounds and the biological global inflammation response, which is typically associated with PM inhalation exposure *in vivo*. Studies indicated HUVECs

treatment with pro-inflammatory cytokine IL- 1B increased protein levels of both COX-1 and COX-2 but without the stimulus only COX-1 is present (as it constitutively expressed) suggesting that the increase in COX-2 with diesel exposure is associated with increased inflammatory cytokine response [180]. A previous study found biased release of vasodilator, 6-keto-PGF1α not PGF2α or TXB_2 with IL-1B stimulus [109]. In our study, we have seen significant increases of 6-keto-PGF1α with IL-1B stimulus only with DEP at 6 hrs but not with B100. Since PGF2α was also increased after DEP and with IL-1B stimulus, but B100 was not, we examined the upstream precursors of the AA metabolism pathway.

Free fatty acid and acyl transferase association

The differential 6-keto-PGF1α production with IL-1B stimulus from DEP and B100 exposure raised questions about where in the AA metabolism pathway exposure caused a disruption. In this study, we examined the gene expression and activity of enzymes involved in the arachidonic acid esterification, which is the limiting agent in production of 6-keto-PGF1α production. The lysophospholipid acyltransferase is the principal enzyme in esterification of lipids and cPLA2 is the principal enzyme in the release of lipids. In this study gene expression levels of four acyltransferase gene transcripts were elevated with B100 exposure compared to vehicle and diesel, but fold change was not significant suggesting that a combination of decreased activity of multiple gene transcripts and isozymes were needed to determine an inhibited response of acyltransferase activity (Figure 4.5b). The acyl transferase enzyme is only one of the esterification enzymes associated with the Lands cycle (Figure 1.7). In the esterification process the acyltransferase is secondary to the acyl-CoA synthetase enzyme. Though previous studies have indicated that acyltransferase activity was directly correlated with active phospholipid remodeling [181]. This was evaluated with cells incubated with either media alone or media enriched with free AA, thus the results indicated in media alone the activity of acyltransferase functions at a lower rate [181]. Our study also measured the induction of acyltransferase gene expression with HUVECs exposed to stearic and oleic acid (Figure 4.6ab) and found that stearic acid (i.e., C18:0) dose dependently increased acyltransferase gene expression. Supporting data showed with HUVEC exposure to saturated fatty acids, mRNA levels of esterification enzymes were altered.

For a comprehensive understanding of the Land's cycle, enzymes that function in releasing lipids from the variable lipid pools were examined. Gene expression data did not show any difference from DEP and B100 exposed cells regarding cPLA2 expression (data not shown). Additionally, cPLA2 activity found B100 exposure was increased activity (data not shown), thus flooding the intracellular environment with free AA yet there is depletion of AA metabolites. This increased cPLA2 activity with B100 can explain how inhibition of acyltransferase with inhibitor Triascin C generated more free AA than vehicle exposure (Figure 4.5a) in 6hrs.

This study suggests the enzymes associated with phospholipid remodeling can alter the feedback activity maintained by the Lands cycle. Additionally, other studies found acyl-CoA synthetase enzyme has specificity for fatty acids of varying length with the greatest affinity for arachidonic acid followed by linoleic acid, oleic acid , palmitic acid and stearic acid [118]. The measure of acyl-CoA synthetase activity was previously identified to be directly correlated with amount of substrate (i.e. free fatty acids). In a previous study HUVECs were given substrates, palmitic acid (C16:0) and stearic acid (C18:0), and the amount of CoA- derived product was found increased relative to initial substrate concentrations. Additionally, there was a bias which resulted in a 6-fold increase of palmitic acid derived CoA product and only a 4-fold increase with stearic acid derived CoA product [119]. The previous study suggests acyl-CoA synthase has a greater affinity for palmitic than stearic acid. Our previous PM characterization studies found both palmitic and stearic acid present in our exhaust particle (Table 3.2), which allows us to propose that fatty acids from B100 particle interfered with the re-esterification regulation. These enzymes and the pathways are critical for cellular homeostasis and B100 exposure disrupts this process suggesting the B100 soluble extract contains more complex emissions compounds which interfere with lipid AA metabolites.

DEP exposure indicated increased AA metabolites with IL-1B stimulus relative to B100 suggesting different DMSO extractable fraction compounds were activated in diesel and biodiesel exposures. The combustion emissions from biodiesel with diesel are largely unique and can generate variable cellular responses. The variability is not only a factor when comparing diesel with biodiesel but is a factor comparing one study with another. Research with combustion emission's induced cellular responses are

challenged by the diversity of compounds. Increased variability, from one study to another, is likely a result of the range of engine conditions and different biofuel feedstock. A possible reduction in variability was created by standardizing the combustion and collecting the particulate matter for distribution. Though, there are diesel exhaust standard reference material, none currently exist for biodiesel, thereby variability in cellular responses from biodiesel studies can be a result of the lack of ability to standardize biodiesel combustion emissions from one study to another. In addition, we acknowledge that in ambient air, PM emissions are largely reduced with biodiesel relative to diesel and exposures per mass are not interchangeable with diesel and biodiesel. Conversely, in this study PM soluble fraction per mass induced significant alterations of the AA metabolism pathway with B100, but not with DEP. Thus, the PM soluble compounds from B100 are more potent at lower concentrations compared to diesel. In summary, we have shown that exposure to the soluble fraction of DEP or B100-induced different cellular responses. The data further suggests that a COX-2 dependent increase of 6-keto-PGF1α occurs with DEP but not with B100. Our findings also suggest that B100 DMSO extract can disrupt lipid remodeling at non-cytotoxic doses. Thus, it is imperative to identify the active compounds that lead to the observed physiological changes. To date, most research examining vascular tone has focused on the roles of NO, ET-1 and EDHF with diesel and biodiesel exposure. Based upon our research, the lipids involved in endothelial cell mediated vascular elasticity regulation should be considered for causal physiological effects from B100 exposure.

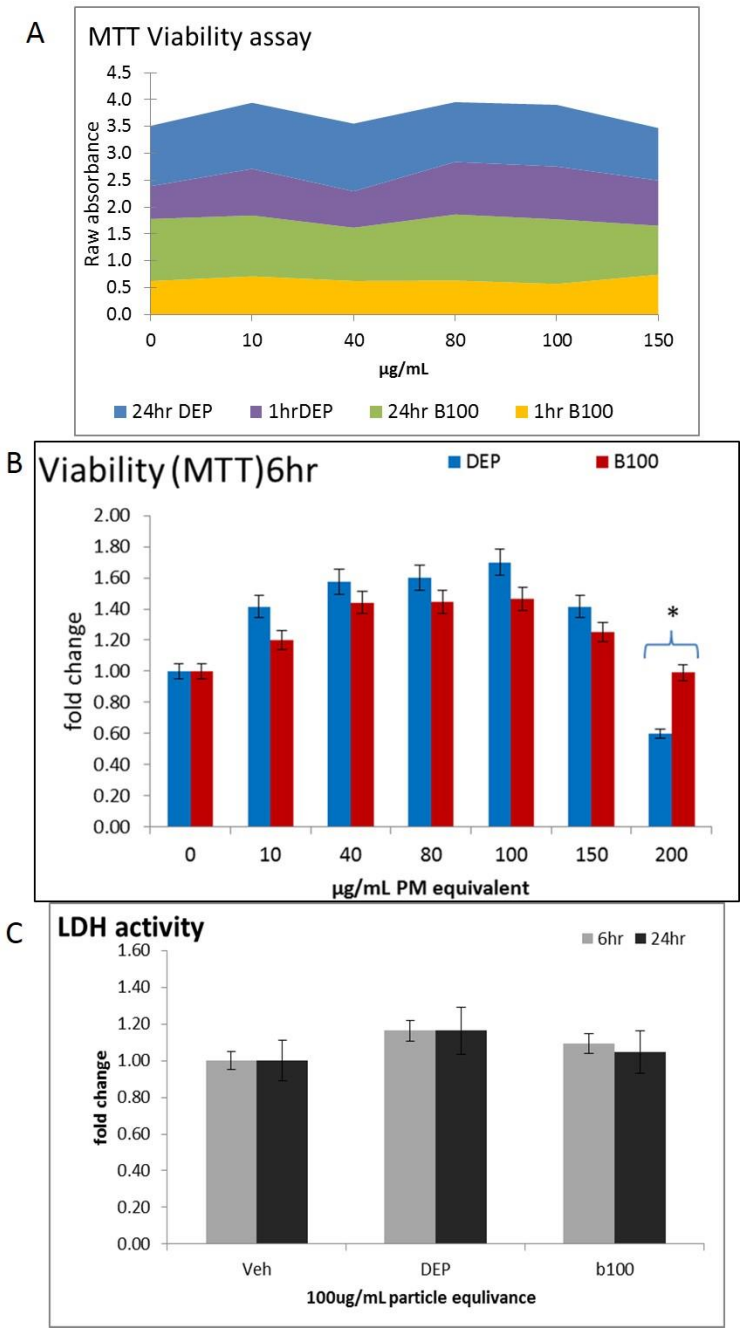


Figure 4.1. DEP extract but not B100 induced time dependent toxicity with HUVECs exposure. Mitochondrial activity of 1 and 24hr exposure was compared with B100 and DEP extract (A), and results show DEP has increased activity with 24hr exposure than B100. With 6hr exposure, MTT results indicate DEP exposures do not vary from B100 exposures (B). There was no significance between DEP and B100 concentrations of 10-1500ug/mL for 6hrs, but at 200ug/mL DEP significantly varies from B100 in HUVECs; n=3; (* P< 0.01). T-test comparisons were only made between B100 and DEP of the same concentrations. LDH release was measured after 6hr exposure with 100ug/mL DEP and B100 (C) which resulted in no statistical significance between the two extracts ;n=3.

Table 1. Gene Expression overview of multiple signaling pathways.

6hr extract exposure 100ug/mL	DEP	B100
Coagulation	HUVECS	
TMBD	1.049 ±0.1	1.230 ±0.2
tPA	1.052 ±0.1	1.049 ±0.2
VWF	0.910 ±0.4	0.919 ±0.4
Injury and Repair		
MMP-2	1.182 ±0.1	1.373 ±0.2
TIMP-2	0.798 ±0.2	0.906 ±0.4
Inflammation		
Cytokines		
IL-6	1.105 ±0.3	0.691 ±0.2
TNF-α	3.399 ±0.8*	2.263 ±0.6
Adhesion Signals		
ICAM-1	1.977 ±06*	0.861 ±0.3
VCAM-1	2.070 ±0.3*	0.642 ±0.2
Eicosanoid enzymes		
COX-1	1.527 ±0.2	1.552 ±0.3
COX-2	1.771 ±0.4*	0.531 ±0.2
Oxidative Stress		
HO-1	0.908 ±0.3	2.107 ±0.4*
SOD-1	0.913 ±0.1	1.770 ±0.6
NADPH-1	1.580 ±0.6	2.460 ±0.8*

Table 4.1. Survey of inflammation and oxidative stress markers vary with DEP and B100 exposures. Gene expression of HUVECs with 6hr exposure to DEP and B100. Endothelial cell adhesion molecules: ICAM-1, VCAM-1 were significantly increased with DEP exposure for 6hr with 10ug/mL extract. Additionally TNFa and COX-2 were also significantly increased in expression with DEP exposure. Only HO-1 and NADPH-1 were significantly increased with B100 with over two-fold expression changes from vehicle. Values are mean ± SE (n=6). The data is presented as fold change from vehicle and previously normalized to B-actin. There was statistical significance was conducted with ANOVA (*$P<0.05$) and compared to vehicle control.

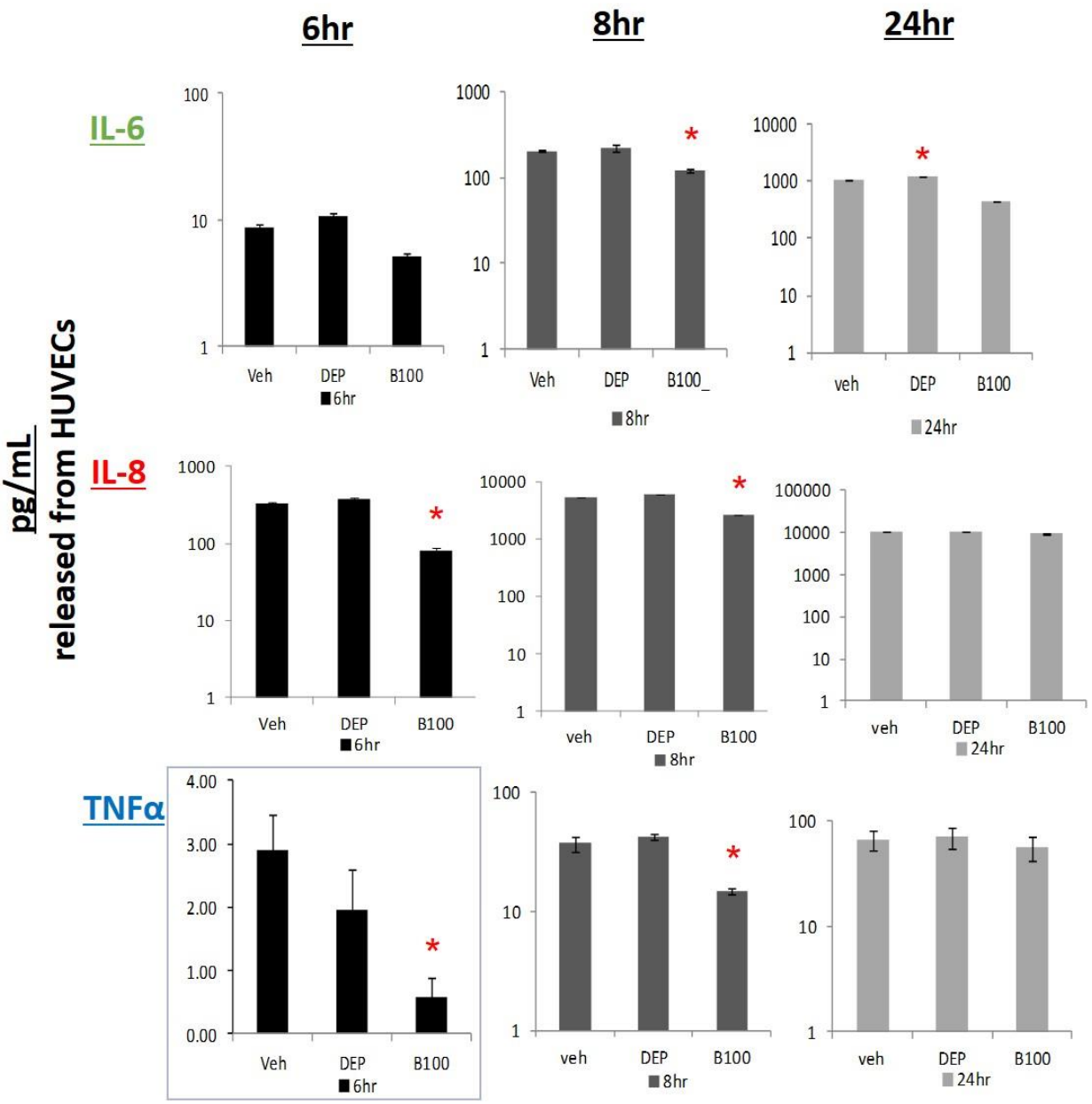


Figure 4.2. B100 exposure produced significant decrease in cytokine production. Cytokine release post 6,8,24 hr exposure to DEP and B100 with HUVECs. There was detectable decrease in the amount of IL-8 and TNFα released after 6hr and 8hr exposure to B100 with (*P<0.05). DEP at 100ug/mL for 24hr lead to a statistical increase of IL-6, but no changes in cytokine before 24hrs. The cytokines IL-6, IL-8 and TNFα are all decreased with B100 exposure after 8hrs using MesoScale Discovery ELISA plate (n=6).

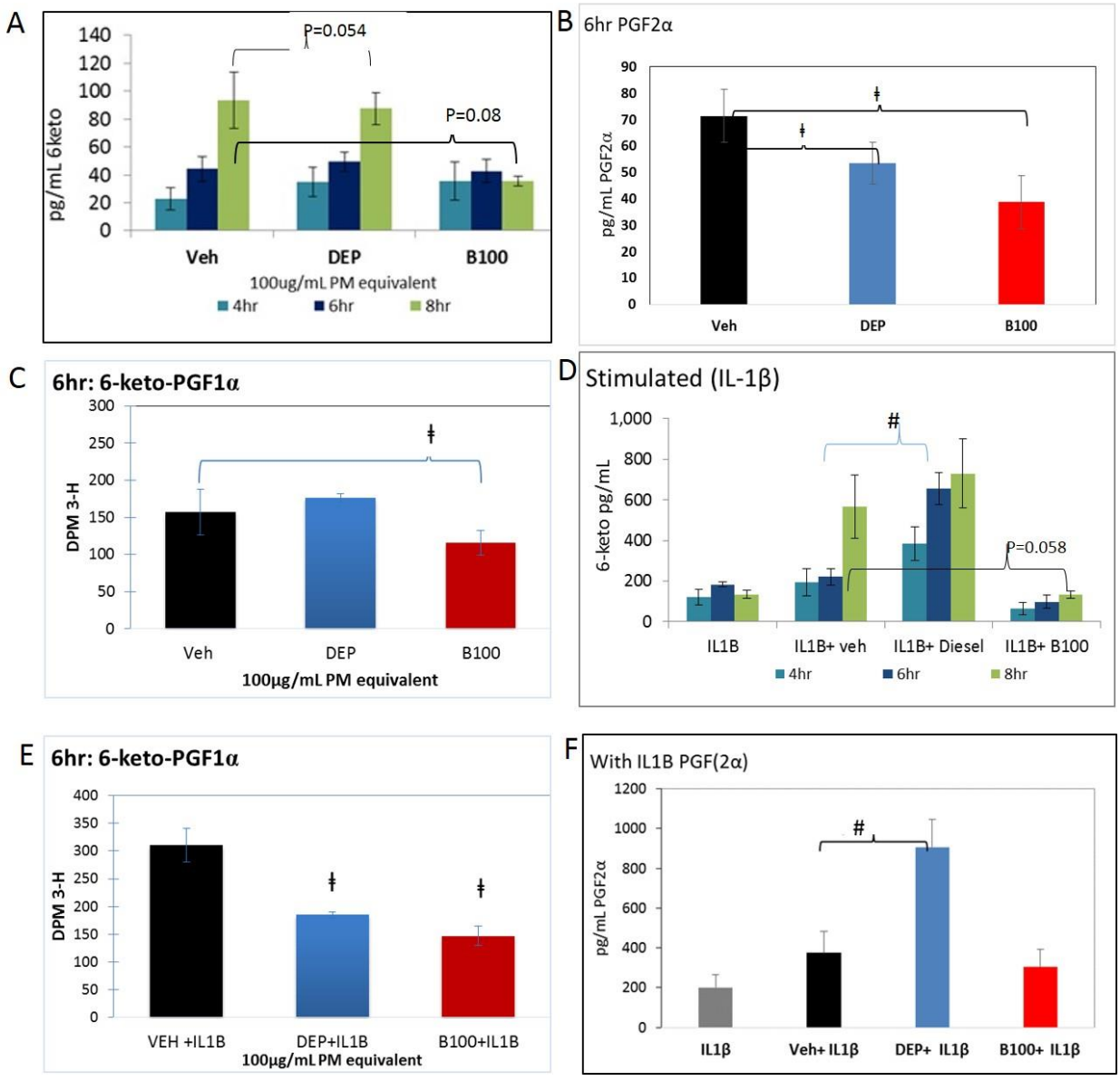


Figure 4.3. Prostacyclin detection with and without IL-1β stimulus remains decreased with B100 exposure. 6-keto-PGF1α release into media with and without IL-1β in HUVEC with 6hr exposure to 100ug/mL of DEP or B100. A, B, C (n=7) represent 6-keto-PGF1α and PGF2α released from cells exposed to extract alone measured with the ELISA and HPLC methods. HUVECs were concurrently stimulated with IL-1β and measured for 6-keto-PGF1α and PGF2α release in figures D,E F. All values represented as mean with SE. ANOVA # P ≤0.002; ǂ P ≤0.05

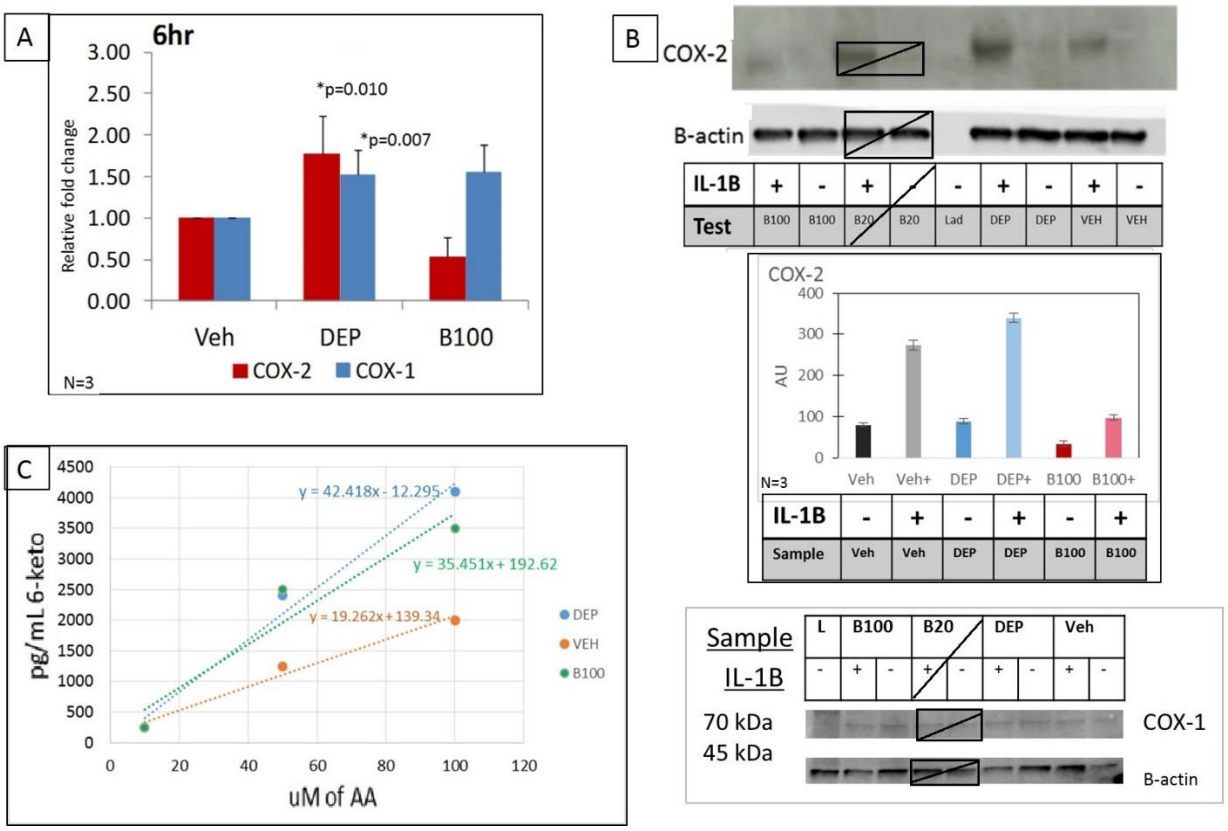


Figure 4.4A-C. B100 exposure induced prostacyclin production is independent of COX-2 expression and activity. Cyclooxygenase activity was evaluated in HUVECs with 6hr exposure to extracts DEP and B100. Figure 4A) RT-PCR data is represented as fold change from B-actin with COX-1 (blue) and COX-2 (red). There is significant increase with DEP exposure (*) p value $p<0.01$ using ANOVA. N=3 with standard error bars. The decrease of COX-2 found with B100 exposure indicates there were no inflammation based activation of transcription factors for COX-2 induction. Figure 4B) Protein detection of COX-2 with stimulated and unstimulated cells after 6hr extract exposure. COX-1 protein detection indicates there is no detectable difference with B100 and DEP. A densitometer measurement of COX-1 (not shown) found no significant changes[ǂ]. Chart below the blot is adjusted densitometry calculated as (arbitrary units) average of n=3 passages 5-7 of HUVECs. Figure 4C) Activity of COX-1/2 post exposure suggests COX-1 plays a dominant role in B100 exposed cells. Cells were pulsed with AA substrate for ten minutes before media was collected immediately and frozen. DEP has the greatest slope almost double the vehicle slope indicating there were more enzymes present to generate product 6-keto-PGF1α (and cofactors). If not expressly stated the data represents 6hr HUVEC exposure with B100 and DEP).

ǂ B20 data has been removed from blots, in light of recent literature which states biodiesel blend fuel is unlike either diesel or biodiesel neat. The unique physiochemical properties of B20 were also found in Chapter 2 particle characteristics.

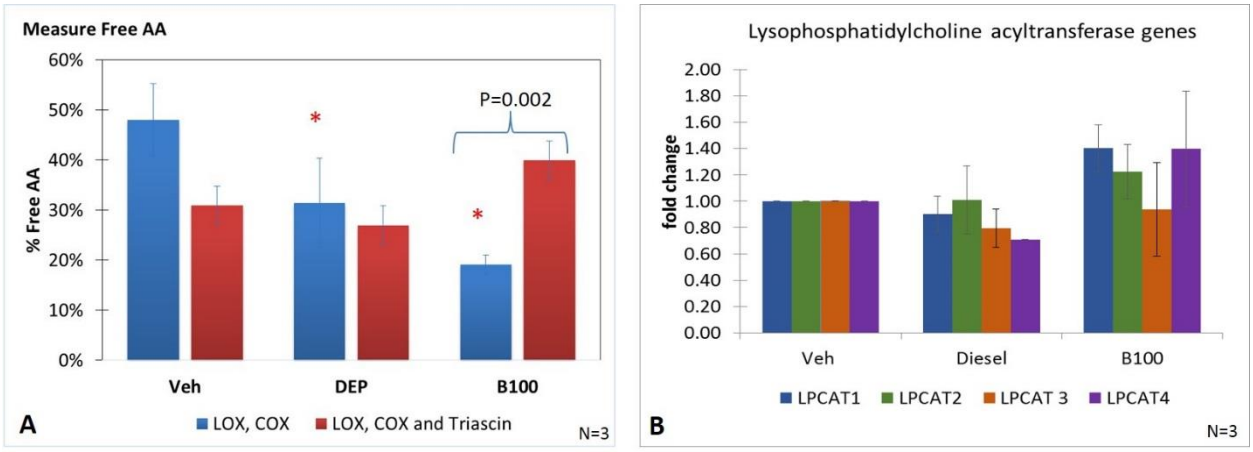
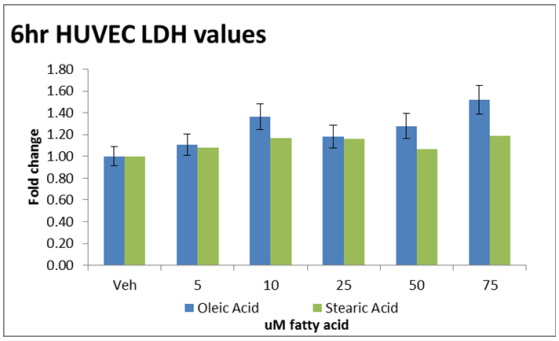


Figure 4.5.a,b Acyltransferase function increased with B100 exposure but not with DEP. Evaluation of re-esterification enzymes (LPCAT1-4) and product (AA) with DEP or B100 6hr exposure in HUVECs. Inhibition of lipoxygenase and cyclooxygenase were conducted simultaneously to prevent AA metabolism. Esterification enzyme, acyltransferase was also inhibited with LOX and COX to measure free AA alterations due to extract alone. Evaluation of free AA was measured in cells after DEP and B100 6hr exposure (A). Data shows cellular scintillation counts as percent of total free intracellular AA over total lysophatidylcholine pools. The available free AA after pre-treatment with COX and LOX inhibitors (blue bars) indicates significantly lower lipid availability after DEP and B100 exposure relative to vehicle (*P< 0.001) ANOVA with Holm-Sidak test. The red bar represent free AA after pre-treatment with LOX,COX and Triascin C (acyltransferase inhibitor), which shows that B100 exposure when re-esterification enzymes are inhibited releases double the amount of AA than B100 exposed cells without re-esterification enzymes inhibited. Free AA of B100 exposed cells with and without Triascin C are statistically significant from each other. The figure suggests B100 treated cells had an increased rate of re-esterification. The re-esterification enzyme expression analysis did not indicate significantly elevated mRNA transcripts of LPCAT1-4 (B) from vehicle however there is a trend observed of elevated expression of all LPCAT1-4 genes only with 100µg/mL B100.

A



B

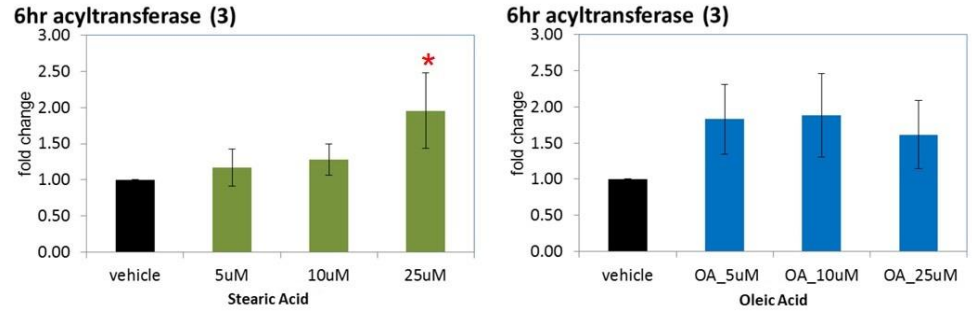


Figure 4.6a,b. HUVECs exposure to stearic acid and oleic acid finds increased acyltransferase transcripts and increased COX-2. The fatty acids were incubated with HUVECs at concentrations ranging from 0-75uM for 6hrs. Following 6hr exposure the supernatant was collected and measured for LDH activity. There is no significant difference in cytotoxicity from stearic acid compared to oleic acid and there is no difference from vehicle control. However at 75uM the trypan blue incorporation into cells indicated over 25% cell death with oleic acid which forced the use of 25uM as the maximal concentration in all the following experiments. Gene expression data of stearic acid and oleic acid 6hr exposure using HUVECs. Stearic acid (C18:0) is a saturated fatty acid we incubated independently with HUVEC, in contrast we also incubated unsaturated fatty acid oleic acid (C18:1). There is dose dependent increase of acyltransferase (LPCAT3) that is significantly increased from vehicle with 25uM stearic acid with $p=0.007$ but not with oleic acid. The corresponding increase of acyltransferase limits the amount of free AA present for use with COX-1/2. (*) indicates Pvalue $\leq$ 0.01 with ANOVA.

Chapter 5
CONCLUDING REMARKS

5.1. Review of Global Hypothesis.

Ambient air pollution PM remains a critical factor in inducing adverse health effects worldwide. Air pollution particles can originate from natural occurrences (i.e., volcanic eruptions and wildfires), however a substantial portion of $PM_{2.5}$ are released from anthropogenic activities [147]. Petroleum diesel combustion is one of the most abundant sources of ambient PM [146, 182]. Recent global air quality and health initiatives have promoted the use of alternative renewable energies. In 2012, the U.S government passed the Energy Policy Act, that announced an expansion of the renewable fuels initiative, which increases biodiesel production up 9 billion gallons from the previous year [183]. Combustion studies comparing biodiesel with diesel indicate fewer PAHs, CO and PM emissions with biodiesel but increased metals, aldehydes and NOx species suggested that there are tradeoffs from introducing renewable energy sources [33, 67]. Regardless, continued production and consumption of biodiesel will eventually alter the current vehicle derived air pollution profile that is currently dominated by diesel and gasoline vehicle emissions. The change in the ambient air-pollution profile may adversely affect respiratory and cardiovascular systems however health consequences are not well studied.

It is still currently unknown if introduction of biodiesel combustion products may alter human health. Currently diesel combustion emissions (DE) are strongly associated with adverse cardiovascular events. Inhalation of DE was previously shown to increase the incidence of myocardial infarctions [184] and impair brachial artery vasodilator function, in humans [77] which contributed to the increase in hospital admissions for cardiovascular events, following exposure to traffic-related air pollution [71]. Biodiesel combustion emissions effects on human biological responses and health effects are limited. No field epidemiology studies have addressed biodiesel exposure induced adverse health effects, but *in vitro* toxicology studies have indicated increased pro-inflammatory [43, 132] and oxidative stress responses [176]. Furthermore, rodent studies with biodiesel exposure via intratracheal instillation identified pro-inflammatory responses including increased protein and increased cell

density in BALF [64]. These studies suggested that there biodiesel exposure can induce potential toxicity and activate lung neutrophil recruitment as an inflammatory response. Though there are no field studies, a preliminary controlled human-exposure study indicated significant alterations of vascular tone induced by exposure to both neat and biodiesel blend emissions [79]. This study found biodiesel induced impairment of vasodilation that was linked to endothelial cell dependent mediators. Both lung inflammation and endothelial cell mediated vascular tone regulation required lipid metabolites of AA metabolism and to date no studies have examined if biodiesel exposure alters lipid signaling. In this research we examine the changes of lipid inflammation signals from alveolar macrophages and we look at lipid vasodilation and constriction signals released from endothelial cells in response to biodiesel and diesel exposure. The goal of this dissertation research was to uncover if biodiesel and diesel particle or their soluble extractable fractions affect AA metabolism, thus, causing a disruptions in inflammation and vascular tone, using *in vitro* cell culture models.

Review of Chapter 2 Results

The goal of the research was divided into three sub aims and in the first sub-aim we compared the differences between diesel and biodiesel exhaust particle exposure and production of PGE_2, an AA metabolite. Using rat alveolar macrophages we found PGE_2 levels varied with exposure to biodiesel and diesel. COX-2 was increased in both samples, but there was a detectable increase of PGE_2 only from biodiesel exposure, which suggested that biodiesel exposure may activate different cellular pathways from diesel. This was evident with pro-inflammatory marker MIP-2, that was significantly increased with diesel, but not with biodiesel exposures. The results in Chapter 2 suggest that there are PM component differences that induced the differential cellular response from exposure to biodiesel blend and petroleum diesel. PM characterization of the samples showed that biodiesel blend emissions contain more of select metals than diesel, but overall biodiesel emissions were reduced by 20%. The other physiochemical properties of the two PM samples did not vary significantly, including fatty acid methyl esters as noted in chapter three. No detectable change was measured when comparing B20 and DEP PM binding of PGE_2 in cell free media. However, PGE_2 release was significantly increased with B20 at lower concentrations than with DEP exposure. In both PM sample exposures there was induction of COX-2 and dose dependent increase of PGE_2. This study suggested that lipid production can increase due to specific exhaust

compounds unique to biodiesel blend. This study raised multiple questions regarding lipid-signaling molecules associated with exhaust composition exposure, which were further evaluated in Chapters 3 and 4.

Review of Chapter 3 Results

The complex composition of DEP and biodiesel emissions (i.e., blend and neat) and the extraction of biologically-active compounds from PM, were addressed in Chapter 3. In this chapter, a number of standard air pollution PM characterization tests were performed using DEP, B20 and B100 combustion particles. Metals were found elevated in both B20 and B100 compared to DEP, but carbonyls and ions were consistent among all three samples. Traditional characterization tests do not usually include measuring free fatty acids present on the particle; thus we adapted a method to quantitate saturated and unsaturated fatty acids from diesel and biodiesel. Furthermore, traditional particle characterization studies utilize extraction methods which generally have limitations, including a bias toward enrichment of nonpolar species bound to the particle, specifically PAHs. Therefore, we developed an extraction method, ideal for biodiesel that maximized the collection of both non-polar and polar-soluble compounds. The extraction process involved dissolving particles in solvents in an attempt to leach the soluble species off the carbon core, creating a liquid suspension of stable biologically-active metabolites. The suspension was centrifuged to pellet the particles and only the soluble fraction was collected. The stable homogenous liquid suspensions were critical for generating reproducible results when cell culture models are treated. The solvent's ability to maintain soluble compounds in suspension depends on two measurements 1) polydispersity of suspension and 2) surface charge of particles. Our results identified DMSO is better than water at preserving soluble compounds. The DMSO extracts also had the greatest potential to induce dose-dependent cytotoxicity when exposed to cell lines suggesting this solvent is ideal for 1) maintaining a diversity of biologically active compounds in biodiesel PM and 2) generate homogenous suspensions for reproducible, repetitive evaluation.

Review of Chapter 4 Results

In sub aim three, we examined B100 and DEP induced changes to production of lipid AA metabolite, 6-keto-PGF1α, which is the stable metabolite of PGI_2 that regulates vasodilation in endothelial cells. A reduction in production of, 6-keto-PGF1α, was found with B100 exposure relative to DEP. Chapter 4 also identified a strong

association with COX-2 dependent production of 6-keto-PGF1α from DEP exposure but not with B100. An overview of cellular responses from B100 identified that antioxidant gene levels, but not cytokines or adhesion molecules were significantly elevated. Evaluation of AA esterification enzyme, lysophotidylcholine acyltransferase, shows B100 exposed cells had increased re-esterification activity, thus reducing the amount of free AA available for metabolism into 6-keto-PGF1α. There are several other enzyme associated with the re-esterification process, but it appears that increased free fatty acids can increase the feedback regulation mechanism reducing free AA in cells. This study revealed that B100 exposure can generate differential cellular responses from DEP and that lipid metabolites which regulate vasodilation are altered with B100. This research suggests there are variable cellular responses from DE and B100 that are likely associated with the soluble biologically active compounds extracted from particle. Cumulatively, the research conducted for this dissertation generated substantial assessment of biodiesel PM exposure and potential adverse health effects. Second, significant contributions were made for studying PM mixtures regarding 1) development of a method for mixtures utilizing liquid suspensions of particle extracts and 2) discovery that lipid metabolites derived from AA are potentially good markers for biosensing and exposure models.

5.2. Significant Biological Effect findings from this dissertation research

Health assessment implications of biodiesel exposure

A study involving healthy volunteers found that both B100 and B30 exposures can induce impairment of vasodilation and were associated with regulation mediators originating from endothelial cells (EC) [79]. There are multiple EC specific molecules responsible for vasodilation including prostacyclin. The Chapter 4 results from HUVEC exposure to BD suggested a plausible mechanism associated with BD exposure and impairment of prostacyclin, a lipid vasodilation signaling molecule. The pilot study was conducted with whole BD exhaust, but in Chapter 4 the focus was on PM due to predicted impact of soluble PM constituents on vascular health [5]. Results from Chapter 4 with vascular cells combined with results from Chapter 2 of AM exposure to BD particles suggests that there are species specific to biodiesel, that are soluble, which can cause disruption of AA metabolism. The exposure to BD not only proposed a unique lipid signaling response, but complemented previous work, that found BD induced inflammatory responses.

Risk Characterization/ Assessment value of this work

Research regarding BD exhaust is limited, but a number of studies have indicated exposure to BD can result in altered cellular responses. There are no epidemiology or occupational health studies to date which directly assessed if BD exhaust induced health effects or surrogates of health effects. There are, however, a handful of in vitro studies that suggest PM from BD exhaust can initiate several molecular initiating events associated with adverse outcome pathways (i.e., genotoxicity, oxidative stress and inflammation). For example, in one study the S9 fractions after exposure to BD resulted in significantly increased DNA adducts and the exposure was comparable to diesel fuel [150]. In another study both THP1 cells and normal human bronchial epithelial (NHBE) cells increased ROS and gene expression levels of antioxidants (i.e., HemeOxygenase -1) with BD exposure [82, 176]. Pro-inflammatory markers were also elevated in BEAS-2B and HUVECs with BD exposure [43, 90, 132]. Collectively, these studies along with the work conducted in this dissertation support the risk characterization process to identify if BD emissions are potentially hazardous.

The disruption of AA metabolism highlighted a plausible BD specific cellular response which indicates the importance of this pathway. The enzyme PGI synthase which is responsible for production of 6-keto-PGF1α has been reported to be a candidate gene for cardiovascular disease. Studies have identified a nonsense mutation in exon 2 of the PGI synthase gene that was identified in patients with essential hypertension. In a separate study, patients with EH were found with three different genotypes and out of the three, a single genotype resulted in significantly reduced 6-keto-PGF1α plasma concentrations. These findings, suggest that EH patients can be identified as a sensitive population to BD exposure. Hypertension is a complex disease and is controlled by multiple pathways and organs but the plasma reduction of 6-keto-PGF1α suggests lipid AA metabolites may play an important role.

Issues to consider regarding Human Risk assessment

The research conducted in this dissertation is isolated to the PM component from exhaust emissions. The exposures with both DE and BD were conducted with the same mass concentration of PM or soluble fraction, however studies have indicated that BD combustion emits fewer PM than diesel. Previous studies have indicated that PM decreases with increasing biodiesel in diesel/biodiesel blends suggesting the B100 would emit far fewer

PM per gallon of fuel than diesel. The results from this study may indicate adverse health effects from 100µg BD with regards to changes in AA metabolism, but nothing was identified with DE at the same concentration, suggesting that lower BD particles mass concentrations may potentially induce more response. Another difficulty with BD exposure health assessment is the variability of emissions with different biodiesel feedstocks. For example, some studies have suggested that soy biodiesel emits more metals than waste cooking oil [67, 68]. These complexities with risk characterization of BD suggest that further research is required to accurately evaluate the health effects of biodiesel neat and blend combustions.

Future Directions

Research within this dissertation has raised several questions pertaining to uncertainty factors for evaluating biodiesel exposure risk, such as emissions characteristics, exposure conditions, and identification of potentially sensitive population. Not all of the uncertainty factors can be addressed, but with future work, clarity may be achieved regarding a sensitive population. This study proposed hypertensive subjects with genotype variations of the PGI synthase allele may have increased risk of cardiovascular injury with BD exposure. Future work with rodent models of hypertension and BD exposure followed with examination of 6-keto-PGF1α in blood and or urine may elucidate if there is any potential risk. Additional experiments can utilize biosensing methods with human exposures and plasma for treatment of HUVECS. The HUVECs can be pretreated with acyltransferase and cPLA2 agonists (e.g., melittin and calcium ionophore) to further elucidate the mechanism previously proposed. More research is required to extrapolate data collected from in vitro exposures for use in biodiesel hazard identification.

The dissertation research has provided limited emissions data which can potentially be incorporated into modeling software for examination of how biodiesel emissions may alter the air pollution-profile as popularity of biodiesel grows. Modeling the air pollution profile changes and quantitatively addressing the amount of incomplete combustion emissions may encourage development of engine aftertreatments that will reduce the metals emitted from biodiesel combustion. Biodiesel is a relatively new fuel with limited research of exhaust composition and health effects. Further research is required before the information can be used for regulatory

decision making on the safety of renewable biofuels and address, in part, whether global increase in biodiesel production is acceptable for public health.

5.3. Significance discoveries from this dissertation research

Significance and Rationale for Novel extraction method.

Combustion exhaust is a mixture of gas and PM and to reduce the complexity we focused on the PM emitted from the engine that was collected on a glass ceramic filter. We developed the novel extraction method for biodiesel because its PM mixture differs from diesel and ambient air PM by the presence of fragmented and whole fatty acid methyl esters (FAME) [34, 40, 170]. While previous studies did not detect FAME in diesel emissions, studies have identified fatty acids in ambient air [170] and FAMEs in biodiesel [40]. The biofuel before combustion contains FAMEs and fragmented FAMEs or incomplete transesterification compounds such as fatty acids suggesting the source of these unique emission is the fuel itself. Other exhaust components found in both in diesel and biodiesel exhaust include PAHs, nitro-PAHs, aldehydes and alkenes [38]. The increased diversity of compounds unique to biodiesel exhaust resulted in an effort to find a new extraction method that would optimally collect all biologically active soluble compounds, including the fatty acids. Importantly, this optimized method is not limited to use with biodiesel, but can be used for possibly better extraction of unique species from other combustion emissions, such as woodsmoke, indoor air pollutants and cigarette smoke.

Our novel extraction method incorporates the beneficial aspects of several previously established particle-extraction methods and improves the efficiency of extraction without changing the chemistry. Throughout this process we eliminated include 1) use of potent cytotoxic solvents for extraction, 2) solvent exchange of the collected fraction and 3) use of solvents that resulted in non-homogenous suspensions. These more established methods have worked in the past primarily with gasoline and diesel exhaust PM to identify the genotoxic combustion emissions compounds that have been identified as PAHs. Recent advancements in engines have reduced the PAHs emissions by 70% with the help of a catalyst chamber and other technological interventions. Thus, the PAHs have been reduced in emissions and are not by themselves a high priority for extraction from particles. Our method focuses on the attempts to collect both the non-polar and polar species bound to the

particle with an emphasis on the FAMEs and fatty acids emitted as incomplete combustion emissions from biodiesel.

Advantages of DMSO based PM extraction

The solution-based method that was developed allows for further manipulation of the soluble composition. For example, Chelex bead-extraction can be used to remove metals from solution. Chelex 100 beads function as chelating agents, in which the beads bind metals for removal. We examined the HUVEC responses to exposure of Chelex-treated DMSO extractions of DEP and B100. The process involved 16hr incubation of extract with 20mg beads, followed by centrifugation to pellet the beads that were bound with transition metals. The purified extract was added to HUVECs and evaluated initially for antioxidant gene expression changes and 6-keto-PGF1α changes (Appendix Figure 1a,b). Removal of the metals seems to have affected mRNA levels of HO-1 (Appendix Figure 1c,d), bringing the levels significantly lower than vehicle ($p=.007$). HUVEC exposure to the purified extract increased 6-keto-PGF1α up to 100pg/mL from untreated B100 exposure (77pg/mL), so B100 was no longer statistically different from DEP or vehicle. This experimental manipulation, was possible because we have a liquid suspension that will not disintegrate or chemically alter the Chelex beads. DMSO extract is also unable to disintegrate C18 beads found to bind nonpolar species with carboxyl groups, which suggests that this method can be utilized for other bead cleanups. The advantages for using this type of method for PM extraction with DMSO and with no further need for solvent exchange shows consistency with the lipid signals from whole-particle exposure.

The chelated extract was also used in cell culture with HUVECs [Appendix Figure 1a,b], which resulted in substantial decrease of 6-keto-PGF1α release from both diesel and biodiesel. The amount of lipid signal detected from 6hr exposure was indistinguishable from the control, suggesting that the chelated DMSO extract removed the biologically active soluble compounds that had previously mediated a change in 6-keto-PGF1α release. The role of metals was previously shown with metal-rich PM, ROFA, and resulted in increased PGE_2 release [46]. In this study, significant exposure-induced changes were identified, but no clear mechanism was found. The conversion of AA into metabolite signals is mediated by several enzymes with minimal disruption from excess intracellular metal or fatty acids. However, the availability of free AA from the esterified state, which is mediated

by acyltransferase, was possibly regulated by the presence of free fatty acids [118]. In one study the fatty acids induced an increased affinity for esterification. The study found a range of affinities depending upon fatty acid length and saturation: AA(C20:4) ≥ eicosapentaenoic acid (C20:5) ≥stearic acid (C18:0)≥ Palmitic Acid (C16:0) [118]. Some studies suggest free fatty acids increase cellular uptake of iron when challenged with exogenous fatty acids [59] with possibly altered oxidative stress. However, the increased influx of oxidative-stress responses have not been confirmed by increased ROS products. Nevertheless, the studies suggest that 1) a correlation between free fatty acids and metals may exist and 2) the biodiesel response may result in part, from combining fatty acids with metals. Mode-of-action studies were made possible with use of this novel extraction method and the ability to maintain species in solution. This method is ideal for assessment of mechanisms induced from exposure. Specifically, this method examines the impact of soluble compounds from PM and the lipid-signaling mechanisms that participated in regulating vascular tone and inflammatory responses.

5.4. Lipids are potential markers for biosensing and effects biomarkers.

A secondary accomplishment of this research is to build the foundation for potential study of lipids as biomarkers and biosensing markers. Lipid signals function in a wide range of intracellular pathways. For example, PGE_2 release from AMs can signal pro-inflammatory signaling in the lung. Other examples include vascular-tone regulation via 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$. Other studies not discussed in this dissertation include activation of the coagulation cascade via lipid signals such as TxA2. Although most pathways consist of multifactorial signals, lipids are ideal because they are synthesized de novo and can be detected immediately. The schematic [Appendix Figure 2] illustrates the detection capability of lipids, genes and proteins in the time from initial response or stimulus. Other cellular signals such as proteins typically require time for transcription and translation from initial stimulus before an increase can be measured. In addition to an extended detection time, there are several other advantages for using lipids specifically for exposure assessment. The versatility of lipid markers does not end with cell culture use; it extends to plasma and urine, and 6-keto-$PGF_{1\alpha}$ can be quantitatively measured in both. AA metabolites appear to be versatile markers for use with both in vitro and in vivo exposure studies indicating that further development of these markers would be of benefit for occupational exposures or for a new type of co-culture model called biosensing.

Biosensing

One of the newer methods to identify the mode of action from exposure is coined "biosensing," by Campen et al, which is an unbiased assessment of endothelial cell response to complete plasma compounds. This method varies slightly from previously established endothelial co-culture models since it is not limited to discrete cellular components of plasma. A typical endothelial co-culture models consists of AMs, where were previously isolated from in vivo exposures, exposed to a singular toxin released from an immortal cell culture. However, in one proposed biosensing model all the plasma or sera soluble compounds from PM inhalation along with circulating factors, were exposed to primary endothelial cells in culture. The biosensing method attempts to bridge clinical samples, such as serum and plasma, with primary cell cultures to evaluate cellular mechanisms of toxin exposure with greater sensitivity. An example of a biosensing model was conducted by Channel et al using coronary artery endothelial cell cultures (hCAEC) and human plasma collected from subjects exposed to diesel, NO_2 or air with intermittent exercise for 2hrs [28]. The results found statistically significant elevated VCAM-1 mRNA levels in hCAEC after exposure of plasma collected from subjects exposed to the diesel and NO_2 but not to air [28]. This study clearly supports the use of a biosensing method for studying potential biological effects in air pollution exposure studies. It also demonstrates endothelial cells are sensitive enough to react to 1) the soluble species from diesel exhaust inhalation exposure, or 2) peripheral tissue-activated signals from exhaust exposure.

Endothelial cells may be ideal for biosensing with soluble compounds from PM inhalation exposure, but they are not the only cells used for evaluation of soluble compounds in circulation. In this dissertation, it has already been established that exposure to PM from diesel and biodiesel alters lipid mediators in AMs. Thus, demonstrating that AMs could be useful in exposure biosensing. The use of lipid signals to detect exposure induced changes is effective in not only endothelial cells, but also in hybrid endothelial, epithelial cells and coronary artery cells. We found that 6-keto-$PGF_{1\alpha}$, was also increased with diesel and decreased with biodiesel in primary human coronary artery endothelial cells (hCAEC) [Appendix Figure 3]. The physiological functions or role of vein and artery cells may differ based on nutrient-rich or nutrient- depleted blood and how much pressure the cells can withstand but results show that endothelial cells, in culture, have a similar response to B100 and DEP. The data suggested that lipid responses were consistent in multiple cell lines as indicated in exposure studies

conducted with hCAEC and HUVECs [Appendix Figure 3]. The 6-keto-$PGF_{1\alpha}$ results resemble the responses from HUVECs, though there may be increased sensitivity with the EA cells to B100. Endothelial cells from the coronary artery are among the first vascular cells to receive fresh blood from the lung and are accordingly exposed to high concentrations of soluble components that cross into circulation from the alveoli, suggesting that hybrid cells response is variable from that of primary cells due. The released lipid measurements indicated an increased amount of 6-keto-PGF1α from the hybrid cell line with exposure to DEP, followed by the primary cells. The hybrid cell line response to DEP and B100 is similar to the primary cells, however, hybrid cells have a greater sensitivity to exposure than the primary cells, suggesting variable cell derived phenotypic responses from biodiesel and diesel exposures. Most lipid metabolites are found not only in cell culture, but also in serum and urine. Detection of 6-keto-$PGF_{1\alpha}$ in these bodily fluids also makes lipid metabolites ideal for use as occupational exposure biomarkers.

Traditional exposure biomarkers

Ideally, biomarkers can be evaluated in human clinical samples directly after exposure. Typically, they include proteins found in excess in blood after stress exposure. For example, c-reactive protein (CRP), a protein inflammation marker was found to be a good predictor of the risk of cardiovascular events [80, 185]. In fact, air pollution exposures induced elevated levels of CRP and cytokines in blood in humans [185]. Cardiolipin is a phospholipid and a common indicator of mitochondrial dysfunction. Its mass is decreased after structural damage to the mitochondria caused by ROS generation. This type of damage has been associated with heart ischemia reperfusions, thus, establishing cardiolipin as a lipid marker for cardiovascular injury. Although current research has not detected AA metabolites as potential markers for inflammation or aberrant vascular tone, potentially 6-keto-$PGF_{1\alpha}$ could serve as one.

Several key characteristics of AA lipid metabolites such as PGE_2 and 6-keto-$PGF_{1\alpha}$, make them ideal for use as exposure biomarkers. First, lipids are synthesized *de novo* and are not dependent on enzyme transcription or translation, making lipids independent of multiple enzymes and processes. As previously stated, lipids are immediately released in response to stimulus or exposure and are maintained at elevated levels, unlike proteins which require time to be altered from endogenous levels. Additionally, the lipid remodeling processes, which

utilize AA metabolisms, are evolutionary conserved therefore the same lipid structures can be studied in both humans and mouse models.

Though the lipid structures are structurally conserved, there are differences in PGI synthase expression within the population. Amano et al proposed the variable expression was associated with variable number tandem repeat polymorphisms in the promoter region of PGI synthase [186]. These variations were found in both normotensive and hypertensive individuals, suggesting a disease such as hypertension is a complex disease and may not be associated with only one gene. Among the hypertensive cohort distribution of genotypes was associated with significantly decreased plasma concentrations of with 6-keto-$PGF_{1\alpha}$. These findings combined with our in vitro study suggests with 6-keto-PGF1α may be both a diagnostic and exposure biomarker for hypertension. A PGI_2 drug mimetic, (i.e., Iloprost and cicaprost) has long been used to reduce hypertension [187]. This extends the benefit of lipids not only as exposure biomarkers but translatable to identify risk of hypertension.

Challenges of biodiesel exhaust and exposure biomarkers.

The first challenge to substantiating an exposure biomarker relates to the large number of assumptions made to extrapolate from *in vitro* research. One assumption is most *in vitro* exposures are several fold higher than what is observed in the environment. However, controlled human-exposure studies are also typically several fold higher than daily ambient exposures [188]. Currently, there is little modeling or particle-equivalence data exists to assess the relevant dose for use in cell culture that is comparable to real environmental exposures. The second challenge for use of 6-keto-$PGF_{1\alpha}$ as a biomarker is the assumption that no changes with soluble extract from biodiesel occur as it enters the lung. It is likely that because the lung contains phase I and phase II metabolizing enzymes (P450s) some components of PM might be altered within the lung.

The second challenge for use of 6-keto-$PGF_{1\alpha}$, as a biomarker is that, we have to assume there are no changes with soluble extract from biodiesel as it enters the lung. Third, we have to assume the essence of the extract will not differ with interaction of multiple cells in the vasculature or in the microenvironment of circulation. There is still difficulty in quantifying the number of uncertainties associated with extrapolation of cell culture results to human exposure. The largest hurdle for determining 6-keto-$PGF_{1\alpha}$ as an exposure biomarker would be

that if the levels are decreased with an exposure instead of increased due to detectability issues. The use of 6-keto-$PGF_{1\alpha}$ may be restricted, however, it remains to be a new mechanism of action to focus on for future research.

5.5. Significance of Research and Future Implications.

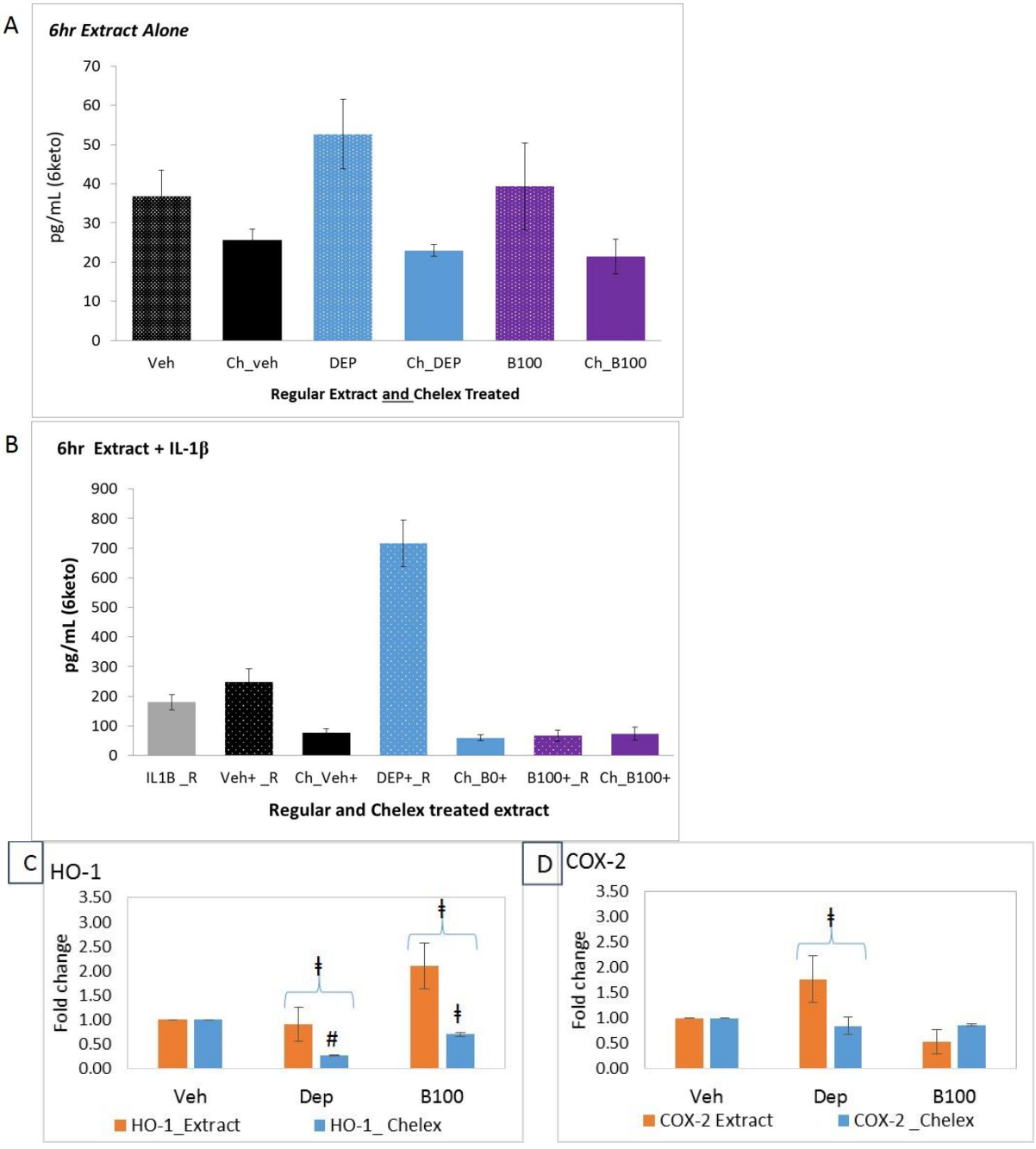
Growing global public concern over health implications of occupational or unintentional exhaust exposure to biodiesel can potentially increase the risk for cardiovascular events. This dissertation research highlights one of multiple mechanism by which diesel and biodiesel exposure may disrupt endothelial cell mediated vascular tone. Further evaluation of exposure composition associated responses is required.

The method developed for biodiesel particle extraction is applicable for other exhaust particles rich in lipids. This can include woodsmoke and other biomass combustion emissions. DMSO extraction and the stability of the suspension that indicated accurate and consistent sampling. This method also minimized the focus on PAHs since advanced engine technology has been successful in reducing PAH by 70% with the use of a catalyst and particle filter. Not only will our extraction method be ideal for biodiesel created with rapeseed, corn or waste-cooking oil, but may prove useful with other natural combustions and e-cigarettes.

While this dissertation research suggests that lipid AA metabolites can be exposure biomarkers for inflammation and vascular-tone alterations, we acknowledge that these studies are not able to dismiss the participation of other signaling pathways. Many more endothelial cell-dependent pathways are involved in altering vascular tone (e.g., eNOS, ET-1 and 12(S)-HETE). However, it can be postulated that lipid mediators are capable of altering the vascular tone as observed in individuals exposed to biodiesel.

In summary, the research within this dissertation added to the limited studies that evaluated exposure-induced biodiesel health effects. In addition this research has led to two major findings 1) development of an extraction method which is a better tool to address the role of singular compounds in a mixture, and 2) AA metabolites may provide practical exposure biomarkers for future research with PM exhaust. In moving forward, the future of examining vascular tone changes with B100 exposure relating to AA metabolism looks promising. Future studies should utilize biosensing as a novel tool to evaluate air pollution-induced adverse health effects.

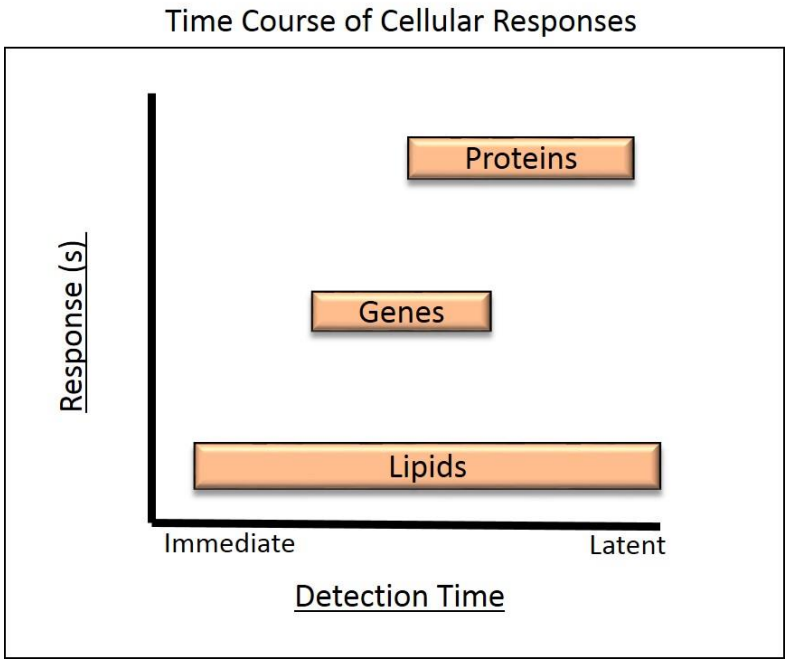
Appendix 1.0. Chelex Treated Extract Exposure Responses and 6-keto-PGF1α Production.



Appendix Figure 1a,b,c,d. Chelex 100 Resin-treated extract exposure for 6hr to HUVECs. Figure 2A) ELISA data representing the release of 6-keto-PGF1α with exposure to regular extract (Veh_R) and Chelex treated extract (Ch_Veh . The levels are subtle and cells with stimulus IL-1B are also represented in Figure 2B. The B100 levels of 6-keto-PGF1α appear increased or have returned to control levels.

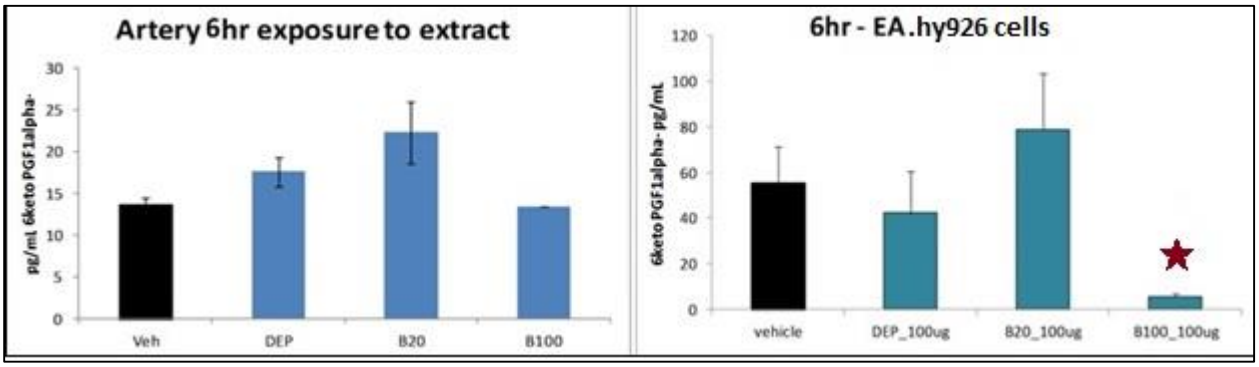
Figure 1c,d. Gene expression changes of COX-2 and HO-1 with Chelex treated extract. The COX-2 and HO-1 gene expression levels changed with Chelex (blue). DEP chelated extract had reduced levels of COX-2 and significantly reduced levels of HO-1. ANOVA of Chelex treated extracts with vehicle # P=0.001; (ǂ) P=0.05 N=3. T-tests used to compare DEP and B100 extracts with chelated extracts.

APPENDIX 2.0 Timecourse of Cellular Signals



Appendix Figure 2. Schematic of intracellular responses to stimulus and the time it takes for detection. Lipids are detected almost immediately, followed by genes and finally after translantion proteins.

APPENDIX 3.0. Human Arterial Cells and Immortal Hybrid Cells 6-keto-PGF1α Response to Extract Exposure



Appendix Figure 3. Six hour exposure to hCAEC and EA.hy926 cells with B100, B20 and DEP. ELISA data is represented as pg/mL of 6-keto-PGF1α. Significant decrease detected with B100 exposure relative to vehicle. ANOVA p value ☆ =0.004. N=4. Same 100ug/mL PM equivalence was used for all cell exposures.

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