# Mechanisms and Markers of the Cardiovascular Response to Traffic-Related Air Pollution in Humans

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## Abstract

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Exposure to traffic-related air pollution, a problem in city centers worldwide, is associated with an increased risk of cardiovascular morbidity and mortality. Though adverse health effects associated with these inhaled pollutants are well substantiated, the mechanisms between exposure and outcome remain unclear. Identification of molecular and physiological processes is critical in distinguishing susceptible populations and advancing prevention efforts. The studies in this dissertation integrate basic science with clinical measures to examine aspects of the vascular response in humans exposed to diesel exhaust (DE), a model traffic-related pollutant. This work combines three unique opportunities: an on-going genotype-stratified controlled human exposure study, a novel particle deposition device for *in vitro* modeling at the air liquid interface (ALI), and an inhalation facility designed to generate combustion-based pollutants. The studies examine multiple clinical and cellular responses to DE in humans under highly controlled conditions. Studies were designed based on the hypothesis that inhaled particles interact with nociceptive receptors within the airway epithelium, leading to the release

of circulating vasoactive and inflammatory mediators; this in turn induces vasoconstriction and oxidative stress, raising the risk of cardiovascular events. Aims include investigation of 1) the effect and mechanism of DE inhalation on blood pressure and transcriptional outcomes in human subjects, and whether this effect is modified by genotype for the nociceptor TRPV1; 2) the primary response to DE exposure in human bronchial epithelial cells, using a state-of-the-art electrostatic deposition chamber; 3) the secondary response using human coronary artery endothelial cells cultured with DE conditioned media and human serum; and 4) the effect of DE on the ratio of reduced to oxidized glutathione (GSH/GSSG) in healthy adults, and whether pre-exposure antioxidant supplementation modified this response. This dissertation provides data to support the hypothesis that the acute hemodynamic effects of DE act via alpha adrenergic system, and evidence for the involvement of TRPV1; provides evidence that the lung epithelium may play an initial role in inflammatory processes and barrier dysfunction in coronary artery cells; and demonstrates that an acute exposure to DE is associated with a reduction in the GSH/GSSG ratio, consistent with shift in balance toward an oxidized state. Combining *in vitro* biomarkers with clinically-observed findings may provide key insights into understanding how exposure to traffic-related air pollution can promote or exacerbate the development of disease.

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| | |
|---|---|
| **ACTB** | Beta actin |
| **ALI** | Air liquid interface |
| **Ang II** | Angiotensin II |
| **ANS** | Autonomic nervous system |
| **BP** | Blood pressure |
| **CALM1** | Calmodulin-1 |
| **CCL2** | Chemokine (CC motif) Ligand 2 (MCP-1) |
| **CCL5** | Chemokine (CC motif) Ligand 5 |
| **CO** | Carbon monoxide |
| **$CO_2$** | Carbon dioxide |
| **COX2** | Prostaglandin-Endoperoxide Synthase 2 (PTGS2) |
| **CVD** | Cardiovascular disease |
| **DBP** | Diastolic blood pressure |
| **DE** | Diesel exhaust |
| **eNOS** | Nitric oxide synthase 3 |
| **EPA** | Environmental Protection Agency |
| **FA** | Filtered air |
| **FDR** | False discovery rate |
| **GAPDH** | Glyceraldehyde 3-phosphate dehydrogenase |
| **GCL** | Glutamate cysteine ligase |
| **GCLc** | Glutamate-Cysteine Ligase, catalytic unit |
| **GSH** | Glutathione |
| **GSSG** | Glutathione disulfide |
| **GSTM1** | Glutathione S- Transferase Mu 1 |
| **HCAEC** | Human coronary artery endothelial cell |
| **HF** | Heart failure |
| **HIF-1α** | Hypoxia Inducible Factor 1, alpha subunit |
| **HMOX-1** | Heme oxygenase 1 |
| **ICAM-1** | Intracellular Adhesion Molecule |
| **IHC** | Immunohistochemistry |
| **IL-1β** | Interleukin 1 beta |
| **IL-6** | Interleukin 6 |
| **MI** | Myocardial infarction |
| **MMP2** | Matrix Metallopeptidase 2 |
| **MT3** | Metallothionine 3 |
| **NAC** | N-acetyl cysteine |
| **NACIVT** | Nano-aerosol chamber for in vitro toxicology |
| **NF-κB** | Nuclear factor-kappa B |

| | |
|---|---|
| **NHBE** | Normal human bronchial epithelial cell |
| **NO** | Nitric oxide |
| **$NO_2$** | Nitrogen dioxide |
| **$NO_x$** | Nitrogen oxides |
| **OCLN** | Occludin |
| **PM** | Particulate matter |
| **$PM_{2.5}$** | Fine particulate matter |
| **PPIA** | Peptidylprolyl isomerase A |
| **ROS** | Reactive oxygen species |
| **SBP** | Systolic blood pressure |
| **SLPI** | Secretory Leucocyte Protease Inhibitor |
| **SNP** | Single nucleotide polymorphism |
| **SNS** | Sympathetic nervous system |
| **$SO_2$** | Sulfur dioxide |
| **TBP** | TATA-binding protein |
| **TGFβ** | Transforming growth factor beta |
| **TRAP** | Traffic-related air pollution |
| **TRPA1** | Transient Receptor Potential Cation Channel, Ankyrin 1 |
| **TRPV1** | Transient Receptor Potential Cation Channel, Vanilloid 1 |
| **UFP** | Ultrafine particulate matter |
| **ZO-1** | Zona occludens 1 (TJP1) |

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## **Chapter 1.** Background

Exposure to outdoor air pollution is associated with a wide range of adverse health effects, yet of the 3.7 million premature deaths attributed to outdoor air pollution in 2012, 80% were due to cardiovascular or cerebrovascular disease.[1,2] Epidemiologic studies have found the strongest associations with exposure to fine ($PM_{2.5}$) and ultrafine (UFP) particulate matter, which, in urban areas, is generated predominately by motor vehicle emissions. Though average pollutant levels in the US have decreased following the implementation of the Clean Air Act, heavy commercial trucking and traffic congestion still lead to peaks in pollutants inhaled by commuters, pedestrians, and those living near major roadways. Stagnant weather patterns can quickly lead to pollution "emergencies", such as those recently occurring in Paris and Beijing, where $PM_{2.5}$ levels were nearly 60 times the US annual standard.[3] With rapid economic expansion driving increased use of pollution-generating coal and an enduring dependence on internal combustion vehicles worldwide, worrisome air pollutant levels continue to persist. Over 95% of the urban population in major cities is exposed to pollution levels exceeding the WHO's air quality guidelines. Furthermore, recent evidence has shown that pollutant concentrations below current EPA standards induce negative effects in susceptible individuals.[4]

Scientific understanding of the pathophysiological underpinnings of cardiovascular disease development, and triggering of disease events, has benefited from extensive basic scientific, translational, and clinical research. There is little or no reason to believe that the increased cardiovascular morbidity and mortality from environmental agents ultimately results from activation of distinct biological pathways. Rather, environmental factors likely work via augmentation of well-characterized "final common pathways" of cardiovascular

disease such as the processes underlying atherosclerotic plaque development, triggering of ischemic events, development of arrhythmias, and alterations in cardiac function or structure. These final pathways to the most prevalent cardiovascular outcomes are common to both well-understood and novel cardiovascular risk factors. A general framework for how environmental factors can lead to these pathways is shown in Figure 1.1.[1] As can be seen, downstream pathways leading to clinical events can be augmented by a number of potential processes.

Clinical, epidemiological, and experimental studies suggest that environmental effects occur via pathways known to affect cardiovascular risk including changes in blood pressure, blood lipid levels, and vascular function. The end effects and processes involved, therefore, are likely to be similar to those associated with other variant and invariant risk factors. Further work has elaborated potential "upstream" processes and advanced our knowledge of the early physiological responses to exposure. Our understanding of these early responses to the body's intake of the environmental agent and the initiating pathophysiologic event are still largely the domain of *in vitro* and animal models.

Exposure to environmental pollutants occurs via three general routes: inhalation, ingestion and dermal absorption. Exposure assessment in humans is often a significant challenge for environmental researchers; in contrast to pharmaceutical dosing, exposure patterns for environmental agents are rarely consistent among individuals. Variations in behavior (e.g. time spent indoors vs. outdoors, water consumption, physical activity level) influence the amount and/or duration of exposure. Though health effects are assessed based on measurable environmental concentrations, characteristics such as breathing rate (inhalation) or skin permeability (dermal) modify an individual's dose. The physiological

response to a similar dose may also vary, based on health status, genetic variation in activating or detoxifying enzymes, effects of co-pollutants and other factors.

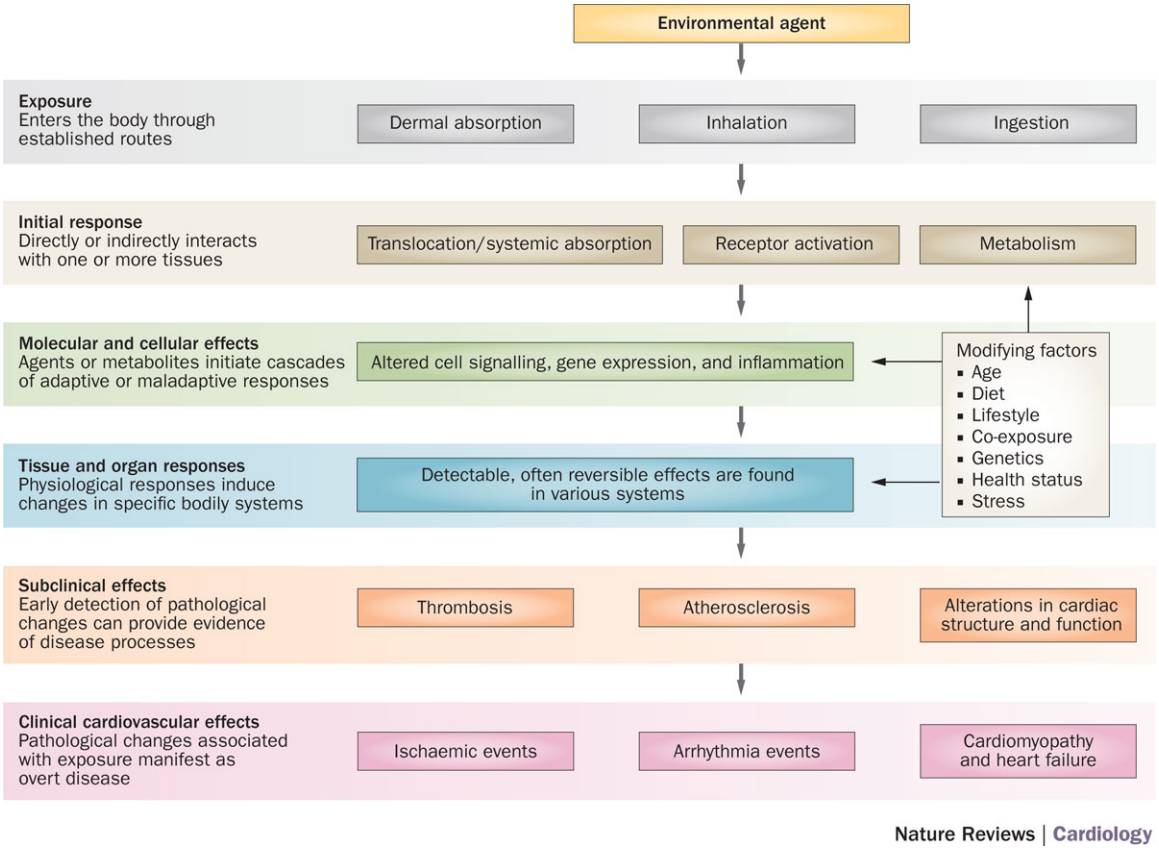


**Figure 1.1 A framework for the characterization of the effects of environmental factors in cardiovascular disease.** A general framework can be constructed to follow the pathways by which the effects of agents are seen. Agents enter the body through established routes, interact with one or more organs and tissues, initiating signaling cascades and physiological responses, leading to subclinical and ultimately clinical pathological changes.[1]

## TRAFFIC-RELATED AIR POLLUTION

In large cities, motor vehicle emissions are the dominant source of air pollution. Traffic-related air pollution (TRAP) is a complex mixture of combustion-generated gases and particles, principally nitrogen oxides ($NO_x$), particulate matter (PM), volatile organic compounds, carbon dioxide ($CO_2$), sulfur dioxide ($SO_2$), carbon monoxide (CO) and hydrocarbons.[5] This research focuses on the effects of PM, for which the evidence is most consistent, however, the effects attributed to TRAP are likely the result of interactions between gases and particles, and between inorganic and organic components.

Particulate matter is generally categorized by mean aerodynamic diameter, into $PM_{10}$ (<10 μm mean aerodynamic diameter), coarse (<10 μm and > 2.5 μm diameter), fine ($PM_{2.5}$; <2.5 μm diameter), and ultrafine (UFP; <0.1 μm diameter) fractions (Figure 1.2). Non-exhaust sources such as brake wear and road dust generate most roadway $PM_{10}$ particles, whereas combustion sources—predominantly vehicle-exhaust emissions - generate $PM_{2.5}$ and UFP.[6] Upon inhalation, particles <10 μm diameter can deposit in the airways and lungs, with smaller particles effectively reaching the alveolar periphery. It has been proposed that from here particles can activate sensory receptors, induce inflammatory and stress responses, or cross the permeable epithelial and endothelial barriers into the systemic circulation.[7] Research suggests, however, that the effects elicited by particles are not solely based on size. Factors such as surface chemistry, co-pollutants, and charge can influence particle translocation as well as the cellular response to exposure.[7-8] Though experimental and personal monitoring allow for differentiation between UFP and $PM_{2.5}$, the majority of air monitoring networks categorize air particulate only as $PM_{10}$ and $PM_{2.5}$, restricting population-based research to these classifications, and therefore much of the data to date.

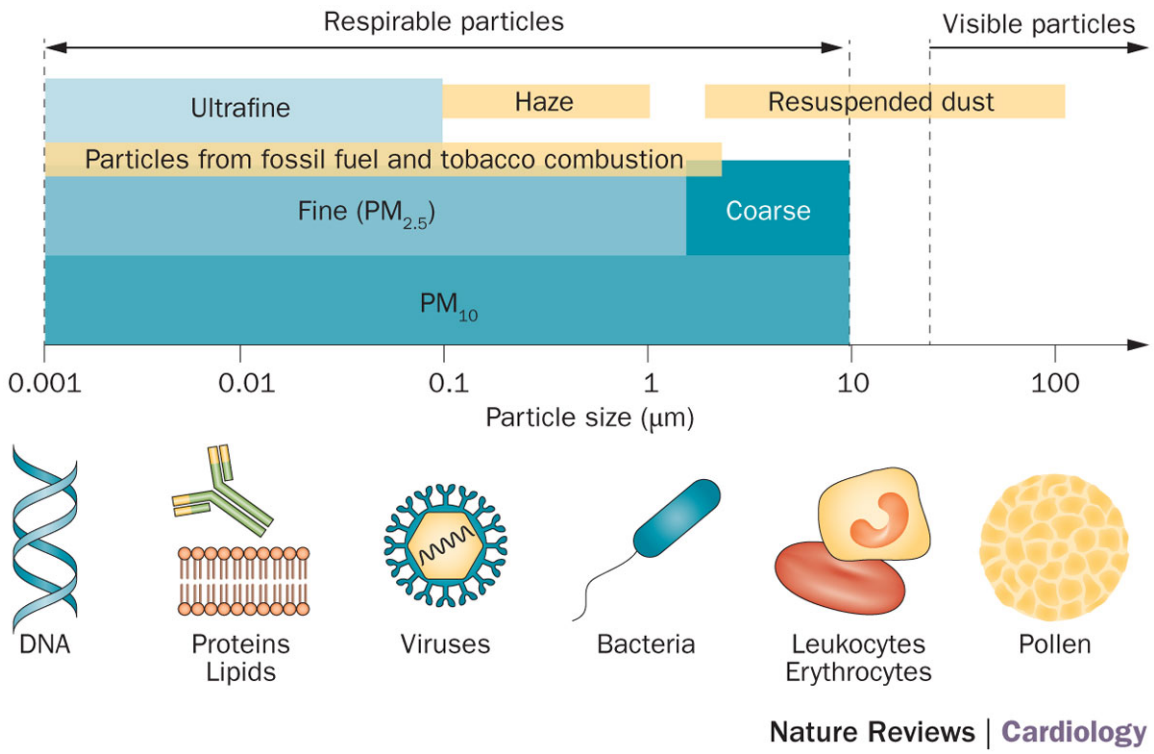


**Figure 1.2 Particulate matter size categorization.** Biological references are shown below for size comparison.[1]

Unlike many areas of scientific research, human studies comprise the majority of air pollution research studies. Human-centered research often produces the most convincing evidence, however, air pollution studies face multiple challenges. The lack of unexposed controls combined with small effect sizes necessitates exceptionally large cohorts. Population-based studies must also employ surrogate measures of exposure, as personal monitoring is cost-prohibitive in prospective cohorts, and cannot be done retrospectively. Individual exposure estimates are therefore most often derived from stationary monitors within a set proximity to each subject's residence, or based on residential proximity to roadways (well-characterized gradients in pollution levels exist within approximately 50 – 1500 meters of major thoroughfares).[8] Monitoring data has historically been sparse in countries outside of the U.S., and remains limited in developing countries, however, recent

large scale research efforts in the EU have been established to address this gap in knowledge. Expanding air monitoring networks and advances in spatial and temporal modeling have significantly improved the amount and quality of exposure data in the U.S. as well. Such progress has increased the precision of results and has led to the detection of health effects at concentrations below current regulatory standards.

Controlled human inhalation studies address many of the challenges faced by epidemiologists. Specialized facilities created to generate model TRAP exposures allow researchers to investigate physiological effects of exposure under highly regulated and replicable conditions. Studies are designed to examine within-subjects differences in response to pollutant vs. clean-air inhalation sessions. While limited to the detection of minor, transient effects of acute exposures, the controlled nature of the exposure and ability to randomize study parameters significantly reduces the variability associated with population-based research. Furthermore, controlled exposure studies allow researchers to investigate effects of specific exposure components in isolation, and efficiently test the efficacy of interventions, such as particle filters, on health outcomes.

It is important to note that the health effects of air pollution are likely to be underestimated. Participants in controlled-exposure studies are generally healthy adults, and although population-based cohorts include a much broader range of individuals, some groups (such as children and the very sick) remain underrepresented. In addition, the majority of studies in this field have been conducted in high-income countries with relatively low pollution levels; risk is likely greater in highly polluted cities or in conjunction with other risk factors such as poverty, psychosocial stress, and exposure to other environmental pollutants.[9,10]

## EFFECTS OF LONG-TERM EXPOSURE

Over twenty years ago, findings from two large cohort studies, The Harvard 6 Cities (HSC) Study and American Cancer Society (ACS) Study, drew needed attention to the adverse effects of urban air pollutants. [11, 12] Follow-up studies found the impact on cardiovascular health was particularly striking: each 10 $\mu g/m^3$ increase in daily mean $PM_{2.5}$ increased risk of cardiovascular mortality by 8-28%.[13, 14] Effect estimates reported in subsequent research have been fairly consistent, with increased precision in results, primarily due to improvements in exposure assessment and modeling. A pooled analysis of studies conducted worldwide estimated excess risk of cardiovascular mortality per 10 $\mu g/m^3$ rise in $PM_{2.5}$ at 11%, with stronger associations tied to mortality from ischemic heart disease (IHD).[15] While TRAP-related mortality risk in highly polluted cities is substantial,[16, 17] data suggest that associations with morbidity and mortality persist, and perhaps may be greater, at lower exposure levels.[6 4] To date a threshold under which long-term TRAP exposure has no effect on health has not been detected.[18]

Heart failure (HF) and IHD are the primary clinical outcomes associated with long-term TRAP exposure.[19-21] Risk for IHD death has been estimated to increase 10-30% per 10 $\mu g/m^3$ rise in residential $PM_{2.5}$, with significantly higher mortality among those living within 50 meters (m) of a high traffic roadway.[20, 22, 23] The prospective Nurses' Health Study reported risk for sudden cardiac death (SCD) increased an average of 6% with each 100 m decrease in residential distance from a major roadway.[24] Though more commonly associated with short-term exposures, recent evidence suggests risk for acute myocardial infarction (MI) is also linked with higher long-term $PM_{2.5}$ levels, as well as with residential relocation to more polluted neighborhoods.[25, 26] In addition, chronic exposure to TRAP plays a role in the

reoccurrence of stroke, HF and MI,[27] and contributes to post-event mortality rates.[28] A recently described exposure-response relationship between first-time MI and occupational exposure to vehicle exhaust reported the promising finding that, as with smoking, risk appears to decrease with time after exposure cessation.[29]

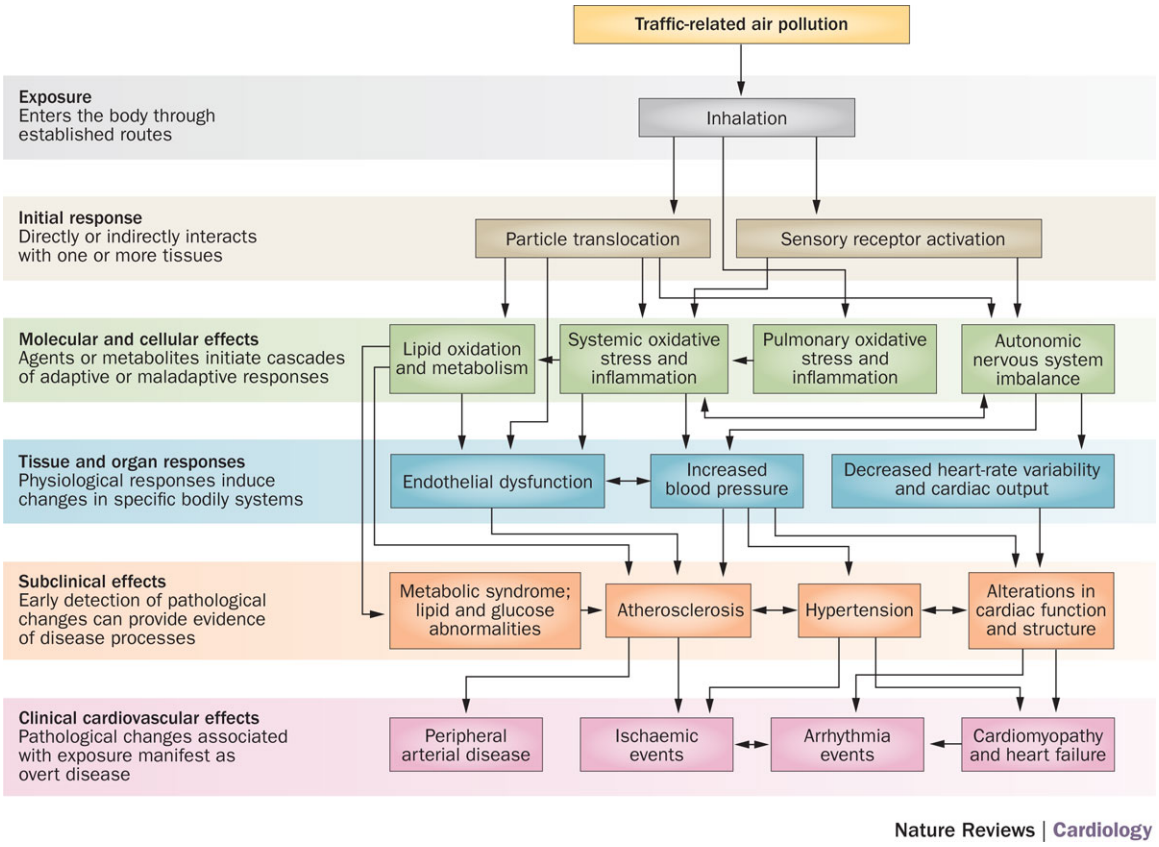


**Figure 1.3 Cardiovascular effects and proposed mechanisms of chronic exposure to traffic-related air pollution.** Multiple pathways have been hypothesized to underlie the long-term effects of exposure to TRAP, including oxidative stress, inflammation and dysregulation of the autonomic nervous system.[1]

Recent evidence has emerged suggesting inhaled pollutants may activate or contribute to pathways known to promote obesity and type 2 diabetes, important risk factors for CVD.[30] Ambient pollutant levels have been associated with both prevalent[31] and incident diabetes,[32, 33] as well as with cardiometabolic effects such as insulin resistance and increased serum leptin levels.[34] Findings on whether or not those with existing diabetes[35] or metabolic syndrome[36] are more susceptible to the effects of pollutants have been somewhat inconsistent.[37]

The relationship between TRAP and pregnancy-related hypertensive disorders is a new focus in air pollution research. Levels of $PM_{10}$, $PM_{2.5}$ and $NO_2$ have been associated with increased risk of pregnancy-induced hypertension, gestational hypertension, and preeclampsia, a leading cause of maternal death.[38, 39] A 5 $\mu g/m^3$ increment in $PM_{2.5}$ exposure was found to elevate risk for preeclampsia and hypertensive disorders of pregnancy by 30-47%.[40] Notably, associations have been independent of confounding factors such as maternal age, ethnicity, and socioeconomic status, and for exposure levels within EPA standards.[41-43] Higher exposures to air pollutants have also been associated with low birth weight, preterm delivery, and future risk for CVD.[44-47]

Researchers have also investigated a possible relationship with thromboembolism. Retrospective and case-control studies have reported positive associations between TRAP and venous and pulmonary thrombosis, as well as associations with decreased prothrombin time [48-50] however prospective studies have not produced similar findings.[51, 52] Though the biological mechanisms are plausible based on coagulation and inflammatory effects induced by PM, results to date remain inconclusive.

### ACUTE EFFECTS

Short-term elevations in TRAP have also been associated with increases in mortality and morbidity (Figure 1.4). Recent studies in the US and EU estimate mortality rates rise by 0.6 – 1.7% with a 10 $\mu g/m^3$ increase in PM ($PM_{10,10\text{-}2.5,2.5}$) in the preceding days, and a similar or greater impact on cardiovascular mortality.[53-55] Though some variability is based on the definition of onset (symptom onset vs. hospital admission), studies investigating ischemic events have predominately found the strongest effects related to increased exposures occurring hours to a day prior to the event, indicating that inhaled pollutants may act as a trigger.[56-58] Risk for onset of MI was found to increase nearly four-fold within one hour of exposure to urban traffic.[59] In fact, two large meta-analyses estimate 0.6 – 7.4% of acute MIs can be attributed to TRAP/traffic exposure.[60, 61] While associations with $PM_{2.5}$ are most commonly reported, MI onset has also been related to other combustion-related pollutants including carbon monoxide (CO), $NO_2$, $SO_2$ and $PM_{10}$.[60]

Refinements in exposure and outcome assessment have led to more precise, and in some cases greater, risk estimates associated with short-term exposures.[62, 63] Detectable differences in clinical outcomes have been attributed to relatively minor fluctuations in pollution levels; a recent study found risk of acute ischemic stroke was 34% higher when prior day $PM_{2.5}$ levels exceeded 15 $\mu g/m^3$, compared to days with lower ambient concentrations.[62] Newer data also suggest effects vary by clinical subgroup, which may explain discrepancies among earlier studies. Research on stroke hospitalization and mortality has found exposure to TRAP increases the risk of ischemic, but not hemorrhagic, stroke[64], specifically large artery occlusive stroke.[62] Similarly, elevated $PM_{2.5}$ levels have been reported to increase risk of ST-elevation MI but not non ST-elevation MI (NSTEMI).[65]

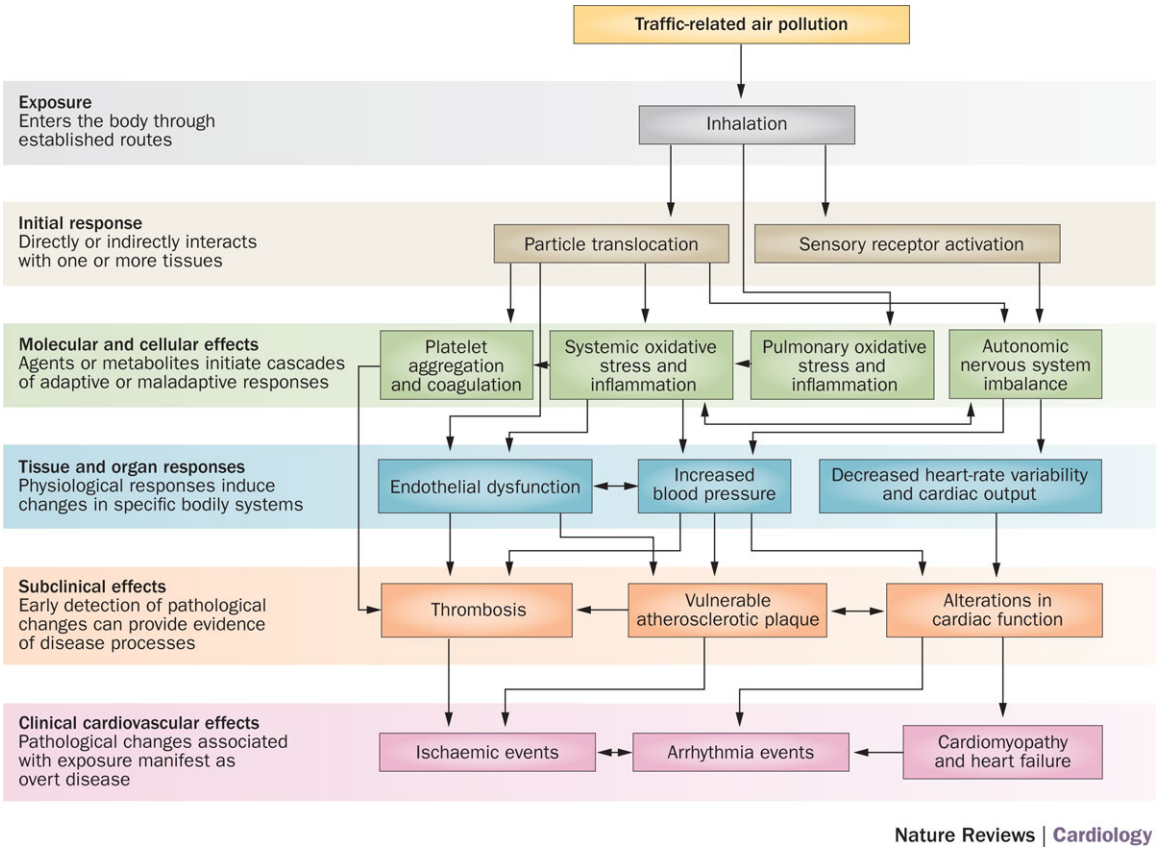


**Figure 1.4 Cardiovascular effects and proposed mechanisms of acute exposure to traffic-related air pollution.** Inhaled pollutants can activate receptors in the lung, or potentially cross at the alveolar level to enter the systemic circulation. Molecular and cellular effects lead to responses in various tissues and organs, to subclinical effects, and eventually to clinical cardiovascular effects.[1]

Short-term peaks in $PM_{2.5}$ and $PM_{10}$ have also been reported to increase risk of cardiovascular hospitalizations,[66] specifically for HF,[67] arrhythmia,[68] atrial fibrillation,[69] diabetes and cerebrovascular-related neurodegenerative disease.[70] Though data on acute exposures has previously been sparse outside the US, recent studies conducted in the EU have found similar effects for PM-associated cardiovascular hospitalizations,[71] while smaller associations have been reported in China.[72] Interestingly, new data suggests that the dose-effect relationship becomes steeper with decreasing PM concentrations, which may explain

the variation in results.[71] This finding further underscores the point that pollution levels at or below current standards continue to adversely impact public health.

## SUBCLINICAL EFFECTS

Exposure to urban air pollution is associated with a number of pathophysiological changes that are known to precede—or contribute to—CVD and mortality. In this context, commonly assessed outcomes include blood-pressure modulation, alterations in cardiac rhythm and structure, and the development or progression of atherosclerosis.

### *Blood pressure and vascular function*

Controlled human exposure studies have demonstrated that acute exposures to TRAP can prompt a rapid and moderately sustained increase in systolic BP (SBP).[73, 74] While the magnitude change (4-8 mmHg, on average) would have a negligible effect in healthy adults, a similar peak in susceptible individuals could induce atherosclerotic plaque disruption, ischemia and MI. Smaller increases in diastolic BP (DBP) have also been reported, though less consistently.[75] The acute BP response has been noted to vary between urban, suburban, and rural-based exposure locations,[76] suggesting that hemodynamic effects may be source or concentration specific.

Chronic exposures to TRAP have also been associated with elevated BP, though not exclusively.[77-79] BP increases have been associated with exposure to combustion-generated pollutants[80] and residential traffic density,[78] with stronger effects reported among elderly subjects[81] and persons with CVD.[82] A study on residential roadway proximity found prevalent hypertension was 9% higher in those living ≤ 100 m from a major road than in those

living ≥ 1000 m.[83] Interestingly, individuals not on hypertensive medication appear more susceptible to TRAP-related BP changes,[78, 82] indicating the pollutant-induced physiological response likely occurs via pathways commonly implicated in hypertension targeted by existing therapies. Though the biological mechanisms leading to HF and MI differ, both can be caused by physiological changes induced by air pollutants, such as increased blood pressure, disturbances in heart rate, myocardial ischemia and cardiac impairment.[84] In addition, both may be exacerbated by ventricular hypertrophy or oxidative injury.[85, 86]

Hemodynamic changes in animal models are challenging to measure, however, murine studies suggest $PM_{2.5}$ synergistically enhances the effect of angiotensin II (AngII), a common target for anti-hypertensive drugs, on SBP and mean arterial pressure. Significant increases in plasma AngII concentrations following short-term inhalation of $PM_{2.5}$ have also been reported, as well as increased BP, vulnerability to ischemia-associated arrhythmia and myocardial damage, effects which have been hypothesized to be the result of SNS activation and oxidative stress.

In conjunction with hemodynamic effects, both acute and long-term exposure studies have reported TRAP-related effects on vascular function. US cohort studies have found impaired vascular function (measured as a decrease in flow mediated dilation) associated with minor differences in residential TRAP levels, reporting effects similar in magnitude to those found in smokers.[87, 88] The results of controlled-inhalation studies have shown that similar—albeit transient—changes occur in endothelial reactivity in healthy adults following acute exposures to air pollution.[89, 90] Retinal arteriolar narrowing, a marker of resistance-vessel function and a predictor of hypertension, has also been associated with both long-term and

short-term exposure to air pollution, suggesting that inhaled pollutants can also induce changes in the microvasculature.[91,92]

*Cardiac rhythm and structure*

Cardiac electrical instability,[93] alterations in heart rate,[94] irregularities in myocardial and ventricular repolarization,[35] and reductions in heart rate variability (HRV), a marker of altered cardiac autonomic function,[36, 95] have also been associated with elevated air pollution levels. ECG evidence of myocardial ischemia following acute $PM_{2.5}$ exposure suggests that particle induced ischemia may predispose the heart to arrhythmia and cardiac death.[96] Epidemiological studies have detected alterations in heart-rate intervals in association with variations in ambient levels of air pollution, particularly in individuals with high levels of markers of oxidative stress and systemic inflammation.[95] However, pollutant-induced ventricular arrhythmia[93, 97] and atrial fibrillation[69, 98] have been predominantly detected in people with existing cardiac disease. In addition, the results of controlled-inhalation studies have shown no short-term risk of arrhythmia in healthy adults, suggesting that the association between pollution and the risk of cardiac rhythm disturbances is limited to highly susceptible individuals.[99]

Cardiac dysfunction and remodeling have been reported in studies of chronic exposure to air pollution in both humans and animals.[85] Right ventricular hypertrophy, a risk factor for HF,[100] has been tied to residential TRAP concentrations, and positively associated with residence time in neighborhoods with high pollution levels.[101, 102] Similar magnitude increases, comparable to the effect of smoking induced hypertrophy,[103] have also been reported in left ventricular mass.[102]

*Atherosclerosis*

As with cigarette smoke, inhaled pollutants contribute to multiple phases of atherosclerotic disease.[104] Minor differences in residential pollution levels have been associated with significant modifications in carotid intima-media thickness (IMT), a surrogate measure of atherosclerosis progression,[105-107] and coronary artery calcium,[108] a marker of coronary atherosclerosis, in population-based cohort studies. Both long and short-term exposures to urban air pollution also induce integral effects of atherogenesis, such as impaired vasoreactivity, protein and lipid oxidation, and inflammation. [87, 109]

Experimental research, which provides the bulk of the evidence linking air pollution with atherosclerosis, indicates that pollutant-induced inflammation and oxidative stress drive the atherogenic process. Exposures limited in duration to weeks or even days can increase atherogenic precursors, such as lipid peroxidation and HDL dysfunction.[110, 111] Compared with wild-type animals, mice that are genetically predisposed to atherosclerosis develop significantly more, higher-complexity plaques in the aortic root with chronic exposure to $PM_{2.5,}$[112] an effect that is enhanced by a Western (high-fat) diet.[113] Changes in plaque composition following exposure to pollutants, including heightened foam-cell formation, lipid content, and macrophage accumulation suggest that pollutants also affect the stability of atherosclerotic lesions, further contributing to the risk of rupture.[112, 114, 115]

## PROPOSED MECHANISMS UNDERLYING THE EFFECTS ON CVD

Despite substantial research on health outcomes related to air pollution, the physiological mechanisms that initiate these effects have not yet been ascertained. However, several groups of researchers have postulated two leading mechanistic frameworks.[7, 116-118] One hypothesis

proposes that inhaled particulate matter activates sensory receptors, triggering acute cardiovascular effects via a response of the autonomic nervous system (ANS). The second hypothesis proposes that exposure induces oxidative stress and inflammation, provoking endothelial activation and coagulation. Prolonged or repeated stimulation of these pathways is associated with the development of atherosclerosis, endothelial dysfunction, hypertension, and cardiac remodeling, and might be the underlying basis for the effects of chronic exposure.[119-121] These two hypotheses are not mutually exclusive, and it seems likely that these two mechanisms are both important and include shared molecular cascades.

The detection of rapid changes in blood pressure in controlled-exposure studies supports the hypothesis that that inhaled pollutants act via ANS dysregulation.[73] Inhaled particles might also interact with nociceptive or noradrenergic receptors to stimulate the sympathetic nervous system, raising circulating levels of vasoconstrictors, elevating blood pressure, and decreasing blood flow to the heart.[122] Neurogenic inflammation and the release of vasoactive mediators such as angiotensin II (AngII) and endothelin 1 (ET-1) can subsequently induce the expression of adhesion molecules, cytokines, and profibrotic mediators, and generate reactive oxygen species (ROS).[123] These effects can induce systemic inflammation, trigger plaque rupture, thrombosis and occlusion, or precipitate ischemia-associated arrhythmia and myocardial damage. ANS-mediated alterations in heart-rate parameters and atrial pressure can also trigger ventricular arrhythmia and atrial fibrillation, increasing the risks of HF or stroke.[95]

Systemic inflammation can also stem from a pulmonary response to injury induced within the lung.[124] Inhaled pollutants may initiate inflammatory or stress-mediated signaling cascades that can lead to systemic inflammation, oxidative stress, and tissue damage.[37, 125]

Alternatively, particles might translocate the alveolar epithelium, precipitating inflammation and oxidative stress directly via interaction with vascular and cardiac tissues. ROS production stimulated by pro-inflammatory mediators can potentiate inflammatory signaling, leading to positive feedback between oxidative stress and inflammation, and contributing to atherogenesis and endothelial injury. At the same time, inflammatory or oxidative activation of endothelial cells and smooth-muscle cells can promote vasoconstriction, platelet activation, and thrombosis, adding to the risk of plaque rupture.[126,37] Chronic, low-grade inflammation is associated with a number of CVD risk factors, and often precedes pathophysiological changes in autonomic tone, blood pressure, and endothelial function.[127] Although pollution-induced pulmonary inflammation has been consistently reported,[128] evidence regarding the nature of the systemic inflammatory response is more varied.[37, 95, 129]

Oxidative stress involves disruption of redox signaling and function, and is likely to be involved in multiple pollutant-induced pathways affecting cardiovascular risk. Inhaled electrophiles in particulate matter can generate oxidative stress directly, or indirectly via the secondary effects associated with an inflammatory response. ROS, when present in excess, can alter cardiac and vascular function through the disruption of important redox-sensitive signaling pathways, the depletion of vasodilators (predominantly NO) and antioxidants or the perturbation of cellular mechanisms such as repair, angiogenesis and lipid oxidation.[130-132] Although studies in humans are limited to the measurement of surrogate markers of oxidative damage, both occupational and population-based cohort studies have reported positive associations between exposure to particulate matter and markers of oxidation of protein, lipid, and DNA in blood and urine.[133, 134] Furthermore, pollution-induced oxidative stress may increase susceptibility to, or exacerbate the effects of, other environmental stressors. Stronger

effects of $PM_{2.5}$ have been reported among subjects with elevated markers of oxidative stress and inflammation, which may reflect a reduced response capacity or a synergistic effect.[95] Similarly, reductions in antioxidant capacity related to exposure to PM might increase susceptibility to—or exacerbate the effects of—other environmental or endogenous stressors.[35]

Epigenetic alterations induced by oxidative stress might also contribute to the effects of air pollution. Global and gene-specific hypomethylation patterns are independently associated with air-pollutant concentrations,[135, 136] heart disease and stroke mortality,[137] inflammation, and atherosclerosis.[138] A recent controlled inhalation study found both PM and PM-induced SBP increases were associated with decreased methylation, confirming findings from observational studies.[136]

## A REVISED *IN VITRO* MODEL FOR INHALATION TOXICOLOGY

Though epidemiologic studies provide strong evidence for the exposure-effect relationship, the cellular and molecular mechanisms are far from clear. A major limiting factor has been the lack of realistic *in vitro* models of inhalation exposure. *In vitro* toxicological techniques are well established, and are often considered as a basic starting point for research. Why then have air pollutant studies been so handicapped? Obstacles exist at multiple levels, from pollutant generation through exposure, which have limited both the use of *in vitro* methods and the usefulness of results from those that have been undertaken. Challengers questioning the validity of current methods have focused mainly on the form of the exposure and delivery methods.

Financial and technical hurdles were a driving force for the development of the prior methods in question. Traffic-related air pollution is a complex mixture of combustion generated pollutants; modeling this exposure in the laboratory requires large, cost-intensive facilities rarely available to researchers. In addition, the deposition of ultrafine (nanometer sized) particulate matter within the lung occurs via diffusion, a difficult and an inefficient process to replicate with cell cultures. Furthermore, cells are most easily and often cultured under submerged conditions. Due to these constraints, particles are often first collected onto a filter and then removed via chemical extraction. When required for an *in vitro* exposure the previously extracted particles are sonicated and resuspended in culture medium for dosing. The major criticism of this method involves the physical and chemical modification of the particles prior to exposure. Health effects are likely to be dependent on the particle's surface characteristics, surface chemistry, density, and size, which may regulate the mechanism of effect, the ability for translocation within the lung, and the cellular response.[139-141] Prior *in vitro* approaches have not been able to duplicate the properties of actual environmental nanoparticles. An *in vitro* study comparing the toxicity of particulate matter delivered in resuspension with particles deposited directly onto the culture found that only the direct exposure produced a significant inflammatory response in human lung cells; a sixteen-fold increase in the resuspension concentration was required to produce a detectable change in gene expression (*IL-8*), still below the initial response seen with direct deposition.[142] Gas chromatography/mass spectrometry analysis of the two exposure types confirmed that the particle collection and resuspension process is likely to modify toxicity. Another discrepancy is that toxicity rankings for constituents resuspended and applied to submerged cultures have

not aligned with toxicity results for the same material tested using intratracheal instillation *in vivo*.[143]

A second critique regards the route of exposure. As described above, the *in vitro* exposure is typically delivered as a single dose in resuspension to cells in a submersed culture. This does not reflect conditions *in vivo*, where exposure occurs via direct deposition at the air-liquid interface (ALI). This difference is also likely to influence the biological response, again drawing the criticism that findings may not represent the true effect of exposure.

The ability to accurately translate results from i*n vitro* to *in vivo* is essential in creating a useful model. The methods employed herein include the use of novel technology within an inhalation facility unique to UW, to create a model of exposure that address each of these concerns. This research takes advantage of the controlled exposure facility at the Northlake Laboratory, described below, which is currently optimized to deliver freshly generated, aged diesel exhaust (DE), the dominant source of urban traffic-derived $PM_{2.5}$.[144, 145] We use DE as a model for traffic-related $PM_{2.5}$ exposure (most particles in the facility are less than 100 nm in aerodynamic diameter). This fully equipped facility generates well characterized, stable and reproducible exhaust emissions and is currently used for both *in vivo* animal studies and controlled human exposures.

To create a more physiologically relevant model of nanoparticle deposition within the lung, the *in vitro* exposure must occur in a manner similar to *in vivo* inhalation. To accomplish this, I used a novel nanoparticle *in vitro* exposure device (NACIVT) developed by scientists at the Institute of Applied Physics at the University of Bern, and described in more detail below. This device incorporates a Krypton diffusion charger and a symmetric transfer

system to efficiently and evenly deposit nanoparticles onto cultured cells. In this device, cells can be exposed directly to the aerosol at the ALI, to particulates that have not been chemically or physically altered (beyond the application of a small charge). Though more complicated than submerged exposures, nanoparticle exposures at the ALI have been shown to produce fewer false negatives among a range of biological endpoints, providing a more conservative estimate of toxicity .[145] The chamber allows for simultaneous exposure of 24 cell culture inserts, allowing us to test multiple endpoints, with sufficient replicates, for each exposure.

In contrast to current methods, this model accurately estimates, and delivers, a repeatable experimental dose under physiological conditions. Research implies that there may be a wide range of susceptibility to exposure, and a significant degree of variability in exposure to the same concentration by individual. Rissler and colleagues reported doses of diesel exhaust in young healthy subjects ranged by twofold for the same concentration of diesel exhaust, with associations made between tidal volume and breathing frequency.[146] The ability to deliver a controlled and measured dose will improve our assessment of dose-response, a necessary component in predicting the health effects of exposure.

The *in vitro* experiments conducted here involve an assessment of the *in vitro* inflammatory response to nanoparticle deposition in human bronchial epithelial cells as well as a model of a secondary response to circulating mediators in human coronary artery cells. This study has been designed with exposure concentrations and outcome endpoints structured to parallel the human controlled study described in Chapter 2. Inflammation is one of the proposed mechanisms linking inhaled nanoparticles with adverse health outcomes. The airway epithelium has been shown to mediate pulmonary inflammation through the expression of genes that regulate cell signaling via the release of adhesion molecules and

inflammatory mediators. Based on the hypothesis that exposure to particulate matter acts via mechanisms within this pathway, I examined changes in chemokine, cytokine and permeability gene response patterns induced in normal human primary cells. The aim of the *in vitro* studies was to work in tandem with findings from the human controlled exposure to identify early markers of exposure and toxicity. Comparing *in vitro* biomarkers to those observed *in vivo* substantiates findings from both studies.

*Northlake Inhalation Facility*

The University of Washington's Northlake inhalation facility is one of only a few such facilities that exist in the US specifically designed to provide a controlled model of exposure to TRAP, both at ambient and occupational concentrations. $PM_{2.5}$, the dominant source of which is DE, is generated by a single-cylinder diesel engine (5.5 kW Yanmar model YDG5500EV-6EI, Yanmar America; Adairsville, GA) under load.

For the exposures described herein, a diesel engine, running at steady state prior to subject arrival, generated the exhaust. Load was maintained at 75% of rated capacity, using a load-adjusting load bank (Simplex, Springfield, IL), ultra low sulfur highway grade diesel fuel and Valvoline 15W-40 crankcase oil. Emissions were diluted with the air used for FA exposures: ambient air passed through a carbon matrix filter and HEPA filter (99.99% efficient). Final $PM_{2.5}$ concentrations were assessed in real-time using a tapered element oscillating microbalance (1400a $PM_{2.5}$, Rupprecht & Patashnick Co., Albany, NY), and adjusted continuously with a feedback control system based on nephelometry measurements to achieve a consistent concentration (200 or 300 $\mu g/m^3$) in the breathing zone (average 292 $\mu g/m^3$; standard deviation 25 $\mu g/m^3$). The 116 $m^3$ exposure room was maintained at a temperature of 20 to 21°C with 50% relative humidity. The facility's DE particle mass median diameter was 0.080 μm, with typical particle counts 2.8 x $10^3/cm^3$ for FA and 5.3 x $10^5/cm^3$ for DE exposures, based on one-minute averaged multistage impactor-collected samples. Nephelometers positioned within the room confirmed spatial uniformity of particle concentrations. For DE exposures, average concentrations of nitrogen dioxide were 35 ppb (approximately 1.5% of total NOx). Concentrations of CO averaged 0.30 ppm for FA and 0.80 ppm for DE. The exposure facility has been previously described in extensive detail.[147]

*Nano Aerosol Chamber for in vitro Toxicology (NACIVT)*

The NACIVT is a novel nanoparticle deposition chamber designed by scientists and engineers at the [FHNW] Institute of Applied Physics, University of Bern. The aerosol inlet is connected directly to the human exposure room at the Northlake controlled exposure facility (described above). Two mass- flow controllers pull a total aerosol flow of approximately 1.7 liters per minute (lpm). A Krypton unipolar diffusion charger gives particles within the aerosol a positive charge. The flow is then split: approximately 1.1 lpm is directed to an electrometer which measures the current generated by the charged particles and 0.6 lpm is brought by a symmetric transfer system to 24 wells containing Transwell inserts with cultured cells. A high voltage electrode below the wells creates an electric field which pulls the particles onto the cells. Electrostatic deposition increases the rate of deposition and allows for even distribution of the particles across the insert. Deposition efficiency increases inversely with the size of particles, with approximately 15% of 200 nm particles and 40% of particles <20 nm deposited. The developers of this system have done extensive testing to model deposition and show that exposure to the electric field, and to control air alone, do not adversely affect the cells.[148] The electrometer data is used to verify that the particle concentration is stable over the duration of the exposition, to compare concentrations between exposure days and, in conjunction with exposure monitors, to generally estimate concentration level. In our exposure facility, data from the electrometer aligned well with measurement of particle count (p-track) and to a lesser extent, black carbon (microaethalometer) as shown in Figure 1.6.

Prior to reaching the cells, the aerosol is heated and humidified to physiological levels (37° C and 85% relative humidity) via a 15cm long Goretex-tube in a heated water bath.

Humidity is measured before both and after the cells are exposure by two sensors in the chamber. Following the exposure the air is dried in a silicagel dryer, and passed through a HEPA filter. All functions of the NACIVT are controlled using Labview on a dedicated laptop, for constant monitoring and adjustment allowed for throughout each exposure.

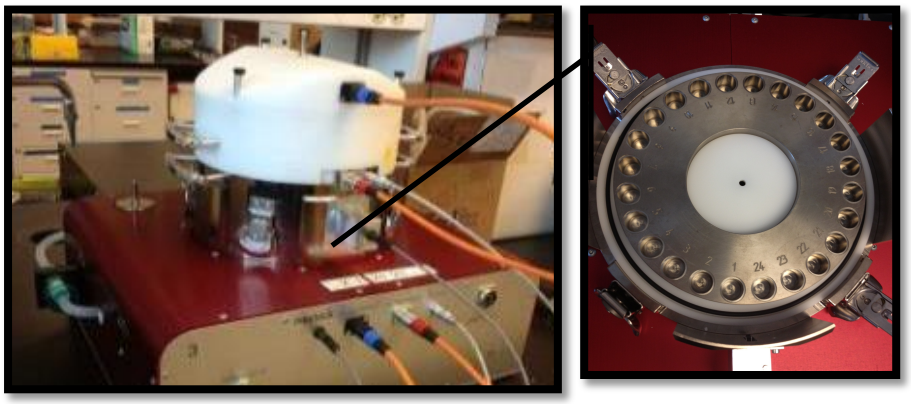

**Figure 1.5 Nano aerosol chamber for in vitro toxicology (NACIVT)**
Photos show the NACIVT with a close up of the 24 well titanium plate used for Transwell inserts.

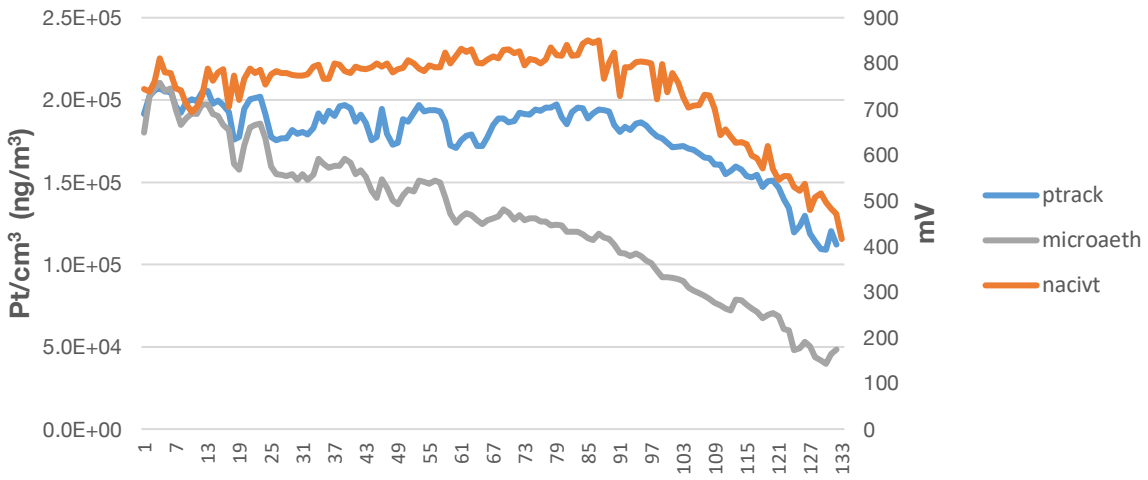


**Figure 1.6. NACIVT electrometer data vs. ptrack and microaethalometer.**
NACIVT electrometer data closely mirrors measures of particle count (ptrack) and, to a somewhat lesser extent, black carbon (microaethalometer) at Northlake controlled exposure facility.

## CHAPTER SUMMARY AND SPECIFIC AIMS

The overall aim of this translational research is to elucidate principal sequences and molecular markers in the pathogenesis of exposure-related cardiovascular effects. The studies in this proposal pair novel *in vitro* methods with parallel exposures and outcome measures in humans, integrating basic science with clinical measures to examine aspects of the vascular response to DE, a model traffic-related pollutant.[149]

My earlier findings demonstrate that exposure to DE results in a rapid, moderately sustained increase in SBP in young adults.[73] In susceptible individuals, a sudden rise in BP could induce multiple precursors to myocardial infarction, such as atherosclerotic plaque rupture and ischemia. The pilot studies I conducted prior to this research indicate that nociceptors, molecular sensors for environmental irritants and endogenous stimuli, play a role in this response, specifically the Transient Receptor Potential Vanilloid-1 (TRPV1), an ion channel shown to mediate circulating levels of vasodilators such as NO.[150] I hypothesized that inhaled particles interact with nociceptive receptors within the airway epithelium leading to the release of circulating vasoactive and inflammatory mediators, which in turn increase blood pressure and oxidative stress, raising the risk of cardiovascular events.

### Chapter 2

#### Rationale

The timing of the SBP response to DE inhalation I detected in previous work suggests that this response is mediated by the SNS. The $\alpha_1$-adrenoceptors are the predominant α-receptor located on vascular smooth muscle and activate smooth muscle contraction/vasoconstriction.

Genetic variation can be quite useful in unraveling specific questions regarding mechanisms and susceptibility. To examine variation in susceptibility via the nociceptor response, enrollment has been stratified *a priori* by genotype for the *TRPV1* SNP rs8065080, a highly novel protocol in clinical research.

**Aim 1. Investigate the role of the α-adrenergic receptor and nociceptor *TRPV1* in the acute response to inhaled DE in humans.**

**1a. Determine whether the effect of DE inhalation on blood pressure occurs via activation of the α-adrenergic receptor, using a pharmacological intervention.**

*Hypothesis:* DE exposure with placebo will be associated with a significant increase in SBP, confirming prior findings; this effect that will be significantly reduced by pretreatment with the $\alpha_1$-adrenergic blocker Terazosin.

**1b. Examine effect modification of the SBP response by *TRPV1*.**

*Hypothesis:* The effect of DE on SBP will be greater in subjects carrying the Val (C) allele for *TRPV1* rs8065080, indicative of lower channel activity.

## Chapter 3

### Rationale

Particulate-induced responses in the lung, an initial target of exposure, may play a causal role in the cardiovascular effects of exposure. Transcriptional regulation of inflammatory or nociceptive-related genes, or exposure-induced changes in barrier function, will help elucidate which mechanisms play a role in the pathway between exposure and outcome.

New *in vitro* methods that more accurately model exposure are needed to advance research in inhalation toxicology; data will provide information on hard-to-test mechanisms of the early response to exposure in humans and support the validation of advancing technology.

**Aim 2. Identify early cellular and molecular responses in the human airway preceding/initiating the systemic and cardiovascular effects of inhaled DE, using a novel electrostatic particle deposition approach at ALI.**

Primary normal human bronchial epithelial cells (NHBE) were exposed to DE and filtered air (FA) under conditions paralleling those in Aim 1.

*Hypothesis:* Alterations in gene expression, epithelial permeability and inflammatory mediator release *(e.g.* tight junction proteins *ZO-1, CDH1,* as well as genes investigated in Aim 1*)* are markers of an early response to DE.

**Aim 3. Using a translational *in vitro* model, investigate the secondary vascular response to acute DE exposure**.

Primary human coronary artery endothelial cells (HCAECs) were exposed to serum collected from human subjects pre and post exposure to DE and FA (in Aim 1) and to conditioned media collected from exposed & unexposed NHBE (in Aim 2).

*Hypotheses:* An initial response *in vivo* (i.e. release of circulating inflammatory mediators) will subsequently effect a secondary response in HCAECs, reflected by changes in gene expression related to adhesion, inflammation, and vascular remodeling.

**Aim 4. Investigate specific transcriptional effects induced by exposure to DE in circulating human leukocytes and effect modification by terazosin pretreatment (from Aim 1).**

*Hypothesis:* DE exposure will be associated with alterations in expression and protein levels of genes related to inflammation and oxidative stress *(e.g. MCP-1, CGRP, COX-2, GCLc)*. These effects will be reduced with terazosin prophylaxis.

## Chapter 4

### Rationale

The ratio of reduced (GSH) to oxidized (GSSG) glutathione is a commonly used marker of systemic antioxidant capacity and redox stress in humans.[151] A major detoxifying thiol, glutathione also acts to reduce peroxynitrites, superoxide and hydroxyl radicals, during which it is readily oxidized to its disulfide form.[152] A shift in the GSH/GSSG balance has been associated with altered cell signaling pathways and increased susceptibility to both acute and chronic disease.[153]

**Aim 5. Investigate the oxidative response to inhaled DE, based on changes in the major thiol antioxidant, glutathione, in human subjects and whether this response is blunted by antioxidant treatment prior to exposure.**

*Hypothesis:* Exposure to DE will deplete blood glutathione (GSH) levels, reflected in a decreased ratio of reduced to oxidized glutathione (GSH/GSSG); the DE-effect on GSH will be muted with antioxidant prophylaxis.

**5a. Determine whether the response to DE or antioxidant interaction is modified by the null polymorphism in the antioxidant response gene, *GSTm1*.**

*Hypothesis:* In the placebo arm, the DE-effect on blood GSH response will be greater among those with the *GSTm1* null genotype; this effect will be abrogated by antioxidant prophylaxis.

**5b. Examine exposure-related changes in markers of inflammation (plasma IL-6) and expression of antioxidant/stress response genes (*GCLc, HMOX-1, IL-6, TGFβ*).**

*Hypothesis:* Exposure to DE will increase the mRNA expression of antioxidant and stress response genes (*GCLc, HMOX-1, IL-6, IL-8, TGFβ*) in circulating leukocytes, and effect that will be blunted by antioxidant pretreatment and modified by *GSTm1* genotype.

**Chapter 2.** Systolic blood pressure response to inhaled diesel exhaust is eliminated by alpha adrenergic blockade and varies by *TRPV1* genotype in humans

## INTRODUCTION

Increased risk of cardiovascular disease is strongly associated with exposure to urban air pollution, specifically combustion-generated fine particulate matter ($PM_{2.5}$). While the evidence for the exposure-effect relationship is strong, questions remain regarding which pathway(s) govern these physiological responses. Experimental research suggests the involvement of several mechanisms, including a shift in autonomic balance, a systemic inflammatory response, and prolonged endothelial dysfunction.[7] Controlled exposure studies have demonstrated that inhaled diesel exhaust (DE), a validated model of traffic-related air pollution exposure, increases systolic blood pressure (SBP) in healthy adults, yet the mechanisms of this effect have yet to be ascertained. [149] [73, 154]

Investigating the theory that nociceptors, molecular sensors for environmental irritants and endogenous stimuli, also play a role in the BP response to DE, I first examined effect modification by genotype for the Transient Receptor Potential Channel Vanilloid-1(*TRPV1*) in a post-hoc analysis of earlier exposure studies. TRPV1 is a seven transmembrane cation channel activated by noxious stimuli, physiological temperatures over 42 °C, and acidic pH (<6.5).[155] These receptors are involved in the regulation of autonomic reflexes such as cough, as well as cardiovascular, respiratory and systemic responses to stimuli, including a number of inhaled irritants.[156] Found in sensory nerve fibers, epithelial cells, and the vascular endothelium, activated TRPV1 channels modulate a range of inflammatory processes and can

alter blood pressure through the regulation of vasoactive molecules such as nitric oxide (NO).[157]

*In vitro* studies have detected expression of *TRPV1* in a range of human cells types, including bronchial and alveolar epithelial cells[158-161], microvascular endothelial cells [159, 162], and circulating leukocytes[163], lymphocytes[164] and platelets[165, 166]. Cells from patients with refractory asthma have significantly increased *TRPV1* expression compared to healthy subjects[167] with TRPV1 sensitization involved in mucus hypersecretion and secretion of inflammatory cytokines in bronchial epithelial cells.[160, 168] Giving credence to the hypothesis that TRPV1 is involved in the response to inhaled pollutants, Yu and colleagues reported PM exposure induced mucin secretion was inhibited by the TRPV1 antagonist capsazapine.[169]

The role of TRPV1 in the cardiovascular system appears to be much more complex. In the vasculature, activation of the receptor predominately appears to be protective. In spontaneously hypertensive rats, a model of human hypertension, TRPV1 activation by the agonist capsaicin increased vasorelaxation via increased eNOS phosphorylation, increasing NO release and lowering blood pressure.[170] Capsaicin has also been shown to increase coronary blood flow in control mice, an effect that did not occur in a knock out (KO) model of mice lacking functional TRPV1.[171] Perhaps in line with that response, *in vitro* studies on atherosclerosis have shown upregulation of *TRPV1* mRNA following 24 hours exposure to an atheroprone blood flow model.[172] Furthermore, activation by capsaicin limited foam cell formation and induced autophagy *in vitro* [173], and dose-dependently reduced the accumulation of cholesterol storage and development of aortic atherosclerotic lesions in ApoE -/- mice fed a high fat diet.[174]

Genotype stratification of the SBP response to DE inhalation in my research indeed indicated possible effect modification by the *TRPV1* single nucleotide polymorphism (SNP) rs8065080, a missense mutation shown to alter channel activity (Figure 2.1).[150, 175]

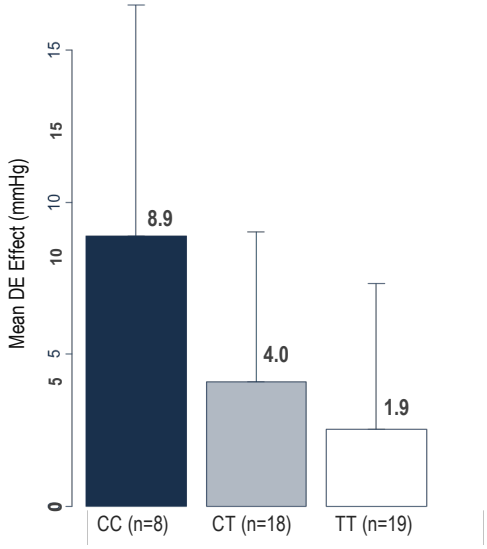


**Figure 2.1 Mean DE effect on SBP by *TRPV1* (at 30–90 min)**

In the current study I investigated the role of the α1-adrenergic receptor in the blood pressure response to DE inhalation, as a mode of action through which inhaled pollutants increase the risk of cardiovascular events. I hypothesized that inhaled particles interact with alpha-adrenergic and nociceptive receptors, leading to the release of circulating vasoactive and inflammatory mediators which, in turn, induce changes in blood pressure, raising the risk of cardiovascular events. Alternatively, particles may stimulate the secretions of endogenous ligands, such as catecholamines, that also regulate blood pressure. To examine whether the hemodynamic effect of exposure is modified by genotype for the nociceptor *TRPV1* Ile585Val, we genotype stratified enrollment *a priori*. The data we present here provide novel insight into the mechanism underlying the cardiovascular effects of exposure to urban air pollution, and describe a variation in response by *TRPV1* genotype.

## MATERIALS AND METHODS

Twenty adult volunteers participated in four exposure sessions conducted at the University of Washington's Northlake Controlled Exposure Facility. Subjects were healthy non-smokers,

with no history or evidence of hypertension, asthma, diabetes, hypercholesterolemia, cardiovascular illness, or other chronic medical condition, based on questionnaire, spirometry, fasting glucose and lipid panel, and electrocardiogram. Additional qualifications included body mass index (BMI) <30 kg/m$^2$, fasting blood sugar (glucose) <126 mg/dL, and blood pressure (BP) <130/85 mmHg. Age, sex, race/ethnicity, smoking, and medication use were self-reported. Urinary cotinine was measured in samples collected at the first visit to confirm non-smoking status using the CAS-COT kit (Innovacon, Inc.; San Diego, CA). Participants with cotinine levels greater than 200 ng/mL were excluded. Genotyping was done following screening and prior to enrollment.

Each subject was exposed on separate days to each condition, DE or FA, both with terazosin and matched placebo. Exposures were double-blind, crossover, and randomized to order, with sessions separated by a minimum three-week washout period to eliminate any potential carry-over effects. The same protocol was followed each session to limit any variation between sessions. Women were exposed only during the first two weeks of a menstrual cycle, and pregnancy was ruled out by a urine pregnancy test at the beginning of each session. Subjects were instructed to fast for at least eight hours prior to arrival at the research center.

The study protocol is outlined in Figure 2.2. Resting BP and heart rate (HR) measurements were taken upon arrival, during exposure (at 5, 30, 60, 90 and 110 minutes from exposure start), and 3, 5, 7 and 24 hours post exposure commencement, using an automated digital oscillometric monitor (Omron HEM-705CP, Vernon Hills, IL), with cuff placed on the left upper arm. Three BP readings were taken with the second and third used for analysis. Subjects were resting and seated during the exposure and post-exposure period.

Blood was drawn prior to exposure, during exposure and the 7 and 24 hour post time points for additional studies (presented in Chapter 3). Attempts to measure catecholamine levels are underway but remain challenging and will be reported elsewhere. Each subject received an identical defined composition meal between the three and five hour post exposure measurements. Subjects remained at the research center for a minimum of 9 hours after arrival and returned for follow-up measurements approximately 24 hours from the session start.

All researchers, nurses and technicians participating in the study were blinded to the treatment and exposure type, with the exception of the exposure engineer. To evaluate blinding adequacy, we asked subjects during each exposure to speculate what the exposure and treatment were for that session. We considered this perception of exposure in our analysis. All subjects provided written informed consent. The University of Washington Institutional Research Board, Human Subjects Division approved the study protocol and all consent forms.

**Exposure System**

A 5.5 kW Yanmar model YDG5500EV-6EI single-cylinder diesel engine, running at steady state prior to subject arrival, generated the DE (Yanmar America; Adairsville, GA). Load was maintained at 75% of rated capacity, using a load-adjusting load bank (Simplex, Springfield, IL), ultra low sulfur highway grade diesel fuel and Valvoline 15W-40 crankcase oil. Emissions were diluted with the air used for FA exposures: ambient air passed through a carbon matrix filter and HEPA filter (99.99% efficient). Final $PM_{2.5}$ concentrations were assessed in real-time using a tapered element oscillating microbalance (1400a $PM_{2.5}$,

Rupprecht & Patashnick Co., Albany, NY), and adjusted continuously with a feedback control system based on nephelometry measurements to achieve a consistent concentration of 300 $\mu g/m^3$ in the breathing zone (average 292 $\mu g/m^3$; standard deviation 25 $\mu g/m^3$). The 116 $m^3$ exposure room was maintained at a temperature of 20 to 21°C with 50% relative humidity. The facility's DE particle mass median diameter was 0.080 μm, with typical particle counts $2.8 \times 10^3$ per $cm^3$ for FA and $5.3 \times 10^5$ per $cm^3$ for DE exposures, based on one-minute averaged multistage impactor-collected samples. Nephelometers positioned within the room confirmed spatial uniformity of particle concentrations. For DE exposures, average concentrations of nitrogen dioxide were 35 ppb (approximately 1.5% of total NOx). Concentrations of carbon monoxide averaged 0.30 ppm for FA and 0.80 ppm for DE. The exposure facility has been previously described in extensive detail.[147]

**Treatment**

Terasozin hydrochloride is a selective alpha-1 adrenergic blocker used for the treatment of benign prostatic hyperplasia and hypertension. Subjects were administered 1 or 2 mg terazosin or matched placebo upon arrival, at approximately 7:30 am, at which time baseline BP and vital signs were taken. The initial dose (2 mg) was selected based on the expected reduction in BP and the minimal reporting of adverse events associated with the drug; this dose, however, was lowered to 1 mg for the last six subjects due to the number of subjects excluded at screening for dizziness. Individual subjects were administered the same dosage for all four exposures. Terasozin is rapidly absorbed and reaches peak plasma concentrations in 1 – 2 hours; exposures were started 2 hours after administration of the drug to coincide with maximum plasma levels.[176]

**Gene-Environment Interaction / Genotyping**

TRPV1 channels are expressed widely within the lung, heart and vasculature. To investigate the potential involvement of TRPV1 in the hemodynamic response to inhaled DE, we examined whether the BP response to DE varied by the common single nucleotide polymorphism I585V (rs8065080), the variant of which has been associated with lower channel activity. This polymorphism was selected *a priori*; study enrollment was stratified by genotype at screening to establish a balanced distribution of each genotype.

Blood for genotyping was collected at screening in a BD Vacutainer tube containing the preservative sodium citrate. DNA was isolated from blood using Qiagen's DNeasy kit (Valencia, CA) and genotyped for *TRPV1* I585V using the TaqMan™ SNP genotyping method with specific fluorogenic probes and primers designed by Applied Biosystems (Life Technologies; Waltham, MA). Specific probes were 3'-labeled with TAMRA quencher dye; wild type and variant probes were 5'-labeled with 6-FAM and VIC reporter dye, respectively. Sequencing reactions were performed and analyzed on a 7900 Fast Real-Time PCR System (Applied Biosystems).

**Statistical Analysis**

Statistical analysis was performed using Stata 10.0 statistical software (Stata Corp, College Station, TX). Descriptive analyses, paired *t*-tests, nonparametric median tests were used in initial analyses, with the $\chi^2$-test used for categorical variables. BP at each time point was first baseline-corrected by subtracting the same session's pre-exposure BP, giving change in BP measured at each recorded interval. The baseline-corrected value for the FA session was then

compared to the analogous value in the DE session for exposures with both placebo and terazosin, for a time-specific, participant-specific measure of effect. To estimate the effect at specific time points, the BP difference-in-differences (DD) was calculated for each participant $i$ and time point $t$ in minutes ($t = 0$ is the initiation of the exposure period):

$$DD_{i,t} \equiv y^{(DE)}_{i,t} - y^{(DE)}_{i,(-120)} - (y^{(FA)}_{i,t} - y^{(FA)}_{i,(-120)})$$

in which $y$ represents the BP measurement. At each time point, DD values from all participants were used for a raw estimate of the mean DE effect, with significance evaluated against the null hypothesis of no effect.

To pool data from different time points and allow for evaluation of potential confounders and the considerable individual variability in BP, SBP readings were analyzed in a hierarchical regression model. The model was adjusted for gender, *TRPV1* genotype, perception of exposure, and order of exposure.

## RESULTS

### Study Participants

Subject characteristics are included in Table 1. Among baseline measurements, only SBP varied by sex, with mean SBP in men 7.45 mmHg higher than that in women (109.9 mmHg vs. 102.5 mmHg, respectively, p=0.03). As *TRPV1* genotype was selected *a priori* and genotyping was done during screening, the distribution of subjects in our study is balanced and does not reflect the distribution in the general population. Baseline BP values did not vary by *TRPV1* genotype.

Compared with FA exposures, SBP increased 2.4 mmHg overall (p=0.009 vs. the two-sided hypothesis of no DE effect), with the peak effect detected 24 hours post exposure (6.8

mmHg; p=0.01; 1.4, 12.1), shown in Figure 2.3. The effect of DE on SBP did not differ by sex, order of exposure or perception. We did not find any significant change in diastolic blood pressure (DBP) or heart rate (HR) with DE inhalation at any time point (Figures 2.4 and 2.5 respectively).

Notably, terazosin prophylaxis completely abolished the effect of DE on SBP at all time points measured (-4.8; p=0.000 for the interaction DE x terazosin overall), an effect that persisted: as with the DE response, the greatest difference with treatment was measured at 24 hours (-10.75; p=0.005). While subjects administered the 2 mg dose had a greater decrease in SBP than those administered 1 mg, the difference in response was not significant (Figure 2.8. There was no difference in the terazosin response by sex, order of exposure or perception of exposure or treatment.

Interestingly, the DE effect on SBP was detected only in persons carrying the variant (C) *TRPV1* allele (Figures 2.6 and 2.7). When stratified by genotype (using a dominant allele model) SBP increased 4.1 mmHg (p=0.007; 95% CI: 1.1, 7.0) among those with the C allele versus a -2.7 mmHg (p=0.27; -7.6, 2.1) change among those homozygous for the T allele (6.8 mmHg difference; p=0.01; 95% CI: 1.4, 12.0). Terazosin treatment reduced SBP in all subjects, but the effect was only significant in those with the C allele at 7 hours (-7.4 mmHg; p=.04; .40, 14.3) and 24 hours (13.4 mmHg; p=0.002; -21.6, -5.1) post exposure.

## DISCUSSION

We present strong evidence for the role of the alpha adrenergic system in the SBP response to traffic-related air pollution, and replicate the findings that acute exposure to DE increases SBP in healthy adults. Furthermore, we report a variation in the SBP response by *TRPV1*

genotype, which suggests that nociceptors are involved in the hemodynamic response to inhaled DE.

The majority of controlled exposure studies in humans have detected increases in blood pressure following acute exposures to either DE or concentrated ambient particles (CAPs), though to varying extents.[76, 177, 178] Congruent with our findings, Mills and colleagues reported a non-significant increase in SBP and DBP (8 mmHg and 6 mmHg, respectively) in healthy men exposed to 300 $\mu g/m^3$ DE.[74] In a study of adults completing a two-hour walk along a Beijing roadway (86-140 $\mu g/m^3$ $PM_{2.5}$), Gong et al. found SBP was, on average, 7 mmHg lower when the participants wore PM-reducing masks.[179] In an analysis of nearly 200 exposure sessions, we previously found that, compared with FA, DE inhalation (at 200 $\mu g/m^3$) increased SBP in young adults within 60 minutes of the start of exposure. The timing of this supports an adrenergic response to exposure, which served as the basis for our study design.[73] Interestingly, in contrast to our earlier findings, here we report the greatest change in SBP 24 hours post exposure. This may be attributed to the higher exposure level (300 $\mu g/m^3$ $PM_{2.5}$) or to variation in the subject response. In either case, it is important to note that the mean effect of DE inhalation was not resolved within the timeframe of our study.

Consistent with our findings, epidemiological studies have reported increases in blood pressure associated with both long and short-term exposure to traffic-related air pollution, though null findings have also been reported.[78, 180-182,77, 79, 81] The ESCAPE study, encompassing 15 population-based cohorts across the UK and EU, found increases in BP and risk for prevalent hypertension associated both with traffic density and living within 100 m of a road.[78] Kirwa and colleagues reported similar findings in the U.S with a 9% higher prevalence of hypertension at 100 meters.[83] In an analysis of nearly 44,000 women

in the NIEHS Sister Study, a 10 $\mu g/m^3$ rise in $PM_{2.5}$ was associated with 1.4 mmHg higher SBP.[180] Of note, multiple population-based studies have found that TRAP-related BP effects appear stronger in unmedicated individuals, indicating that pollutants act through mechanisms common to other CVD-related risk factors, such as ANS dysregulation.[82, 83, 183]

The timing and bimodal pattern of the blood pressure response we detected points to both an immediate and secondary, or delayed, effect of exposure. As outlined by others preceding us, the effects of air pollution are likely to involve multiple pathways. It is possible that the initial response was mediated by the ANS, with secondary signaling (via TRPV1 or other pathways) inducing a secondary vascular response at 24 hours. Beyond vasodilation, TRPV1 activation reduces the production of free radicals, inhibits neutrophil infiltration, and limits inflammation via regulation of pro and anti-inflammatory cytokine release. The fact that blood pressure responses were blunted by terazosin treatment at early and late time points indicates that the blocking of the initial ANS response likely prevented any downstream signaling underlying the later rise in SBP. This is an important finding, as it demonstrates that an acute exposure induces effects that appear well beyond the window of exposure. It also provides support for the epidemiogical findings that persons not on anti-hypertensive medication show a greater response to traffic-related air pollution.

The sympathetic nervous system may be stimulated directly, via the vasoconstrictive effects of norepinephrine following $PM_{2.5}$ interaction with nociceptive or noradrenergic receptors in the lung, or indirectly as a secondary response to ANS activation, via catecholamine stimulation or alpha-1 vascular smooth muscle

receptors.[90, 122] Notably, we found that the SBP response to DE inhalation varied significantly when examined by genotype for the *TRPV1* SNP Ile585Val. Transient receptor potential (TRP) channels are family of cation channels involved in a broad range of physiological responses to endogenous and external stimuli. TRPV1 channels, expressed in sensory nerve and non-neuronal tissue, in particular appear to be involved in the regulation of blood pressure, via second messenger signaling and the release of neuropeptides such as the vasodilator calcitonin gene-related peptide (CGRP).[184, 185] In endothelial cells, TRPV1 agonism has been shown to activate eNOS/NO production, attenuate cytokine and chemokine release, and reduce the expression of adhesion molecules – essential factors in the potentiation of endothelial dysfunction and inflammation.[170, 186] Functional analysis of the SNP we selected demonstrates decreased channel activity with the genetic variant (C) allele;[175] as shown in Figures 2.4 and 2.5, SBP rose only participants with one or more C alleles when exposed to DE, indicating a sufficient protective response in TT homozygotes. To date, specifics on the functionality of this SNP in the vasculature have not been elucidated, however our results suggest a dominant rather than additive effect of genotype in this case. Our data show that terazosin reduced blood pressure in all subjects, while the SBP effect of DE inhalation occurred only in subjects with the *TRPV1* C allele. This indicates a TRPV1-ANS interaction in the response to DE - one which is significantly greater in TT homozygotes. Inflammatory mediators released during tissue damage have been shown to activate TRPV1, which may explain the differences in response by genotype at 24 hours in the placebo exposures.[187]

Recent experimental studies have demonstrated a relationship between nociceptor activation and pathophysiological responses to inhaled particles, though studies have not specifically focused on blood pressure.[188-191] In rats exposed to concentrated ambient particulate matter, Ghelfi et al found exposure mediated abnormalities in cardiac function were prevented by TRPV1 blockade.[189] Similarly, spontaneously hypertensive rats became sensitized to triggered arrhythmia 24 hours after one 4 hour exposure to DE, an effect mediated by activation of the sympathetic nervous system and by another member of the TRP family, TRPA1.[190] Physiologic-based modeling has demonstrated a possible direct interaction between TRP receptors and alpha adrenoreceptors, as well as with other G-protein coupled receptors which may underlie the effects on BP we report here.[192]

As in our previous controlled exposure studies, we did not find a significant increase in HR, suggesting the vasoconstrictive alpha-adrenergic response occurs without a corresponding beta-adrenergic modulation of HR.[193] We also did not see any exposure-induced change in DBP, an effect which has been reported in both controlled exposure and population-based studies.[177, 181, 182] To explain this discrepancy, we propose that diastolic function may be influenced by components of TRAP other than DE.[194]

Though our exposures do not represent the complete mixture of pollutants in urban cities, our facility produces DE at standardized, replicable composition and concentration levels, allowing for a highly-controlled crossover study. As our study employed human volunteers, we cannot account for all exposures and activities of subjects outside of each session, however the crossover design and adjustment for pre-

exposure BP enabled us to account for inter- and intra-individual variability and detect differences in response to exposure and treatment. We have no reason to believe that activities and exposures between sessions would be systematically different, and it is unlikely that any such factors would bias results toward those we observed.

The high level of DE used and the pharmaceutical intervention selected made subject blinding a concern, as perception of exposure or treatment could influence the sympathetic nervous system response. Subjects correctly identified the exposure better than expected by random chance (59 correct vs. 41% incorrect) but not the treatment (45% correct vs. 55% incorrect), as shown in Table 3. To address any potential effects of perception on our outcome measures, we adjusted for this in our statistical model.

Though our exposure concentrations far exceed average $PM_{2.5}$ concentrations in most U.S. cities, transient spikes in pollutant levels, and therefore exposure levels, are not uncommon. Worldwide, rapid urbanization and industrialization in countries such as China and India have produced peaks in ambient $PM_{2.5}$ levels two to three times those used in our model.[3] The effect we detected, though clinically insignificant in a healthy adult, may induce a variety of precursors to myocardial infarction in a susceptible person. It is also important to note that we are reporting a mean physiological response; individual responses varied considerably, and our findings likely underrepresent the risk in susceptible populations.

This highly controlled human exposure study provides important findings on the mechanisms underlying the SBP increase in healthy adults exposed to DE. The mitigation of DE effects with the alpha adrenergic blocker terazosin strengthens the hypothesis that the acute vascular response to inhaled pollutants occurs via activation of

the autonomic nervous system, and in our subject population, occurred only among persons carrying the *TRPV1* Ile585Vale C allele. The bimodal pattern of response further indicates that effects are both immediate and delayed; the prevention of the secondary effects by blockade of the initial response suggests that these effects are sequential. This evidence may be important in conceptualizing how short and long-term exposures can induce pathophysiological changes underlying clinical cardiovascular disease and outcomes.

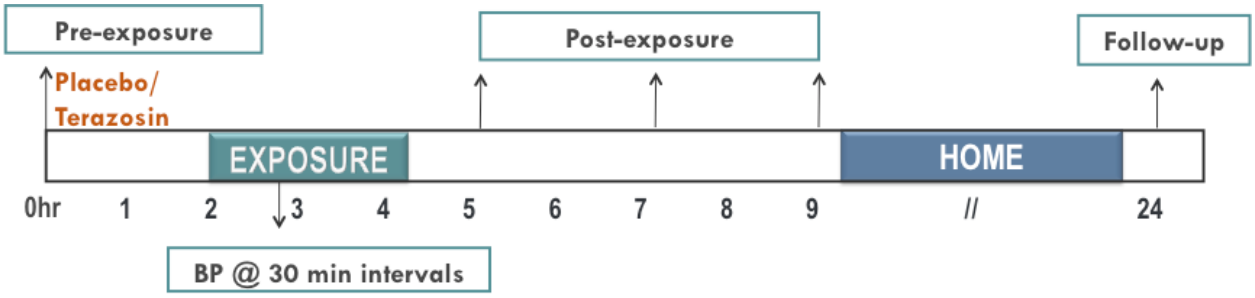


**Figure 2.2 Study protocol**

**Table 2.1 Study participant baseline characteristics.**
Numbers are reported as mean (± SD).

| Characteristic | Mean (± SD) |
|---|---|
| No. | 20 |
| Age, yrs | 28 ± 8 |
| Male, n (%) | 10 (50) |
| Caucasian, n (%) | 13 (65) |
| Asian, n (%) | 5 (25) |
| Body mass index | 24 ± 2.5 |
| Systolic Blood Pressure, mmHg | 106 ± 10.0 |
| Diastolic Blood Pressure, mmHg | 72 ± 6.7 |
| Heart Rate, bpm | 63 ± 9.7 |
| Total cholesterol, mg/dL | 160 ± 30 |
| LDL | 95 ± 23 |
| HDL | 50 ± 10 |
| Triglycerides | 76 ± 40 |

**Table 2.2 Distribution of participants by *TRPV1* genotype**

| | N (%) |
|---|---|
| TT | 6 (30) |
| CT | 8 (40) |
| CC | 6 (30) |

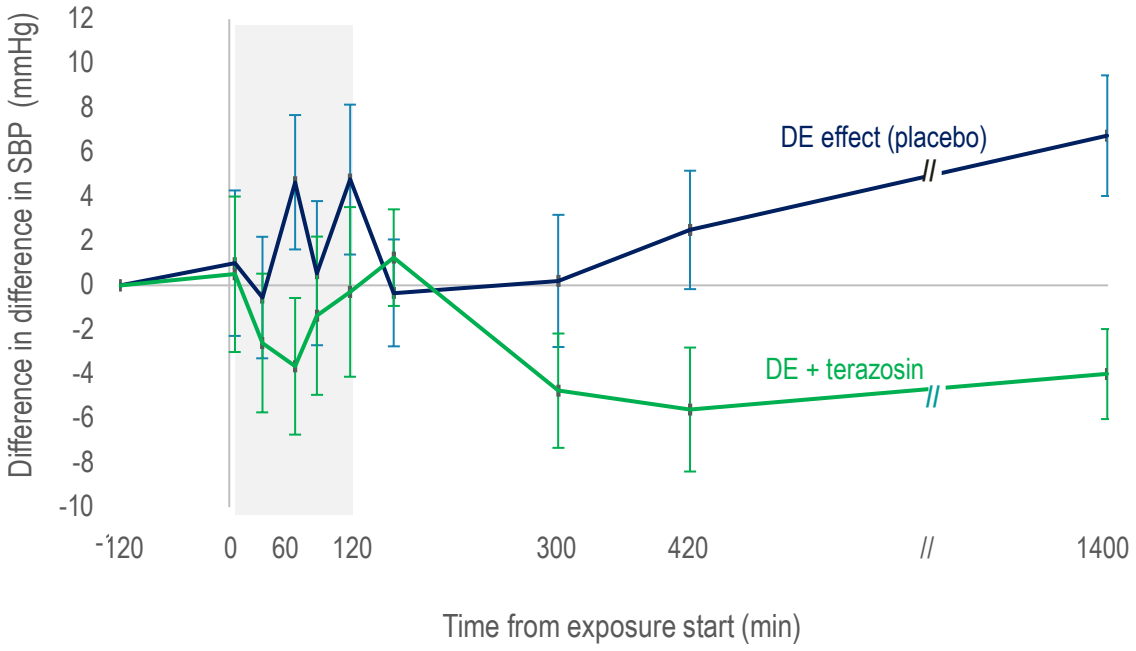


**Figure 2.3. DE Effect on SBP by treatment.** Mean within-subject difference in baseline adjusted SBP between DE and FA exposures, for placebo and terazosin sessions. The exposure session is highlighted in grey.

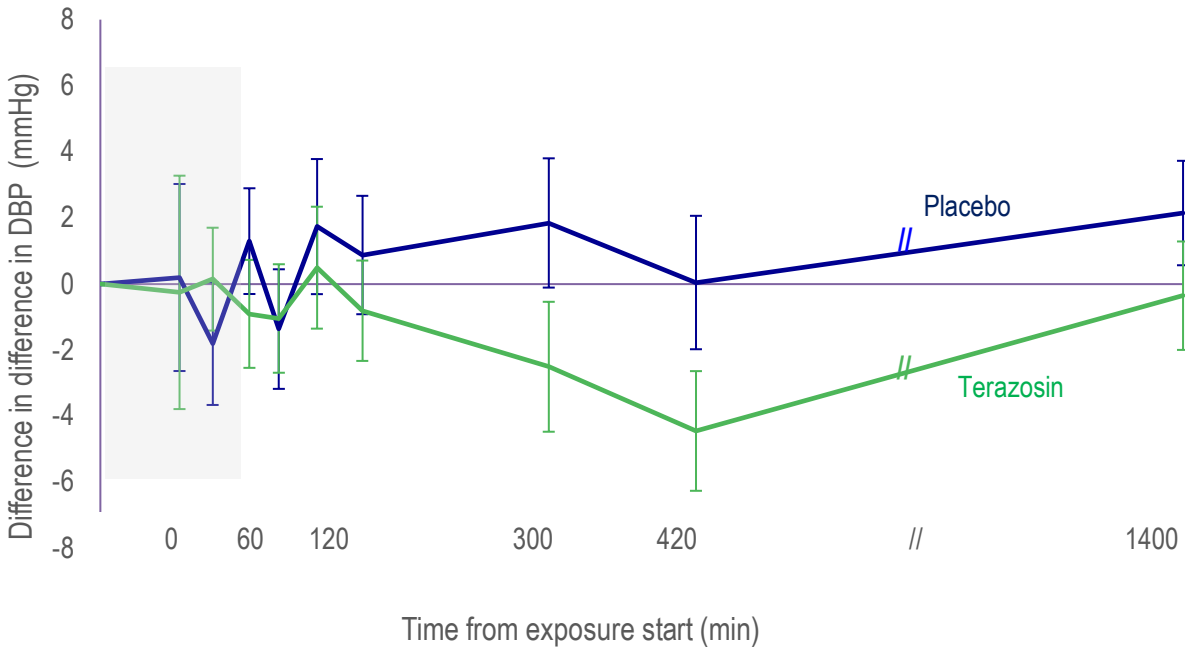


**Figure 2.4. DE Effect on DBP by treatment.** Mean within-subject difference in baseline adjusted SBP between DE and FA exposures, for placebo and terazosin sessions. The exposure session is highlighted in grey.

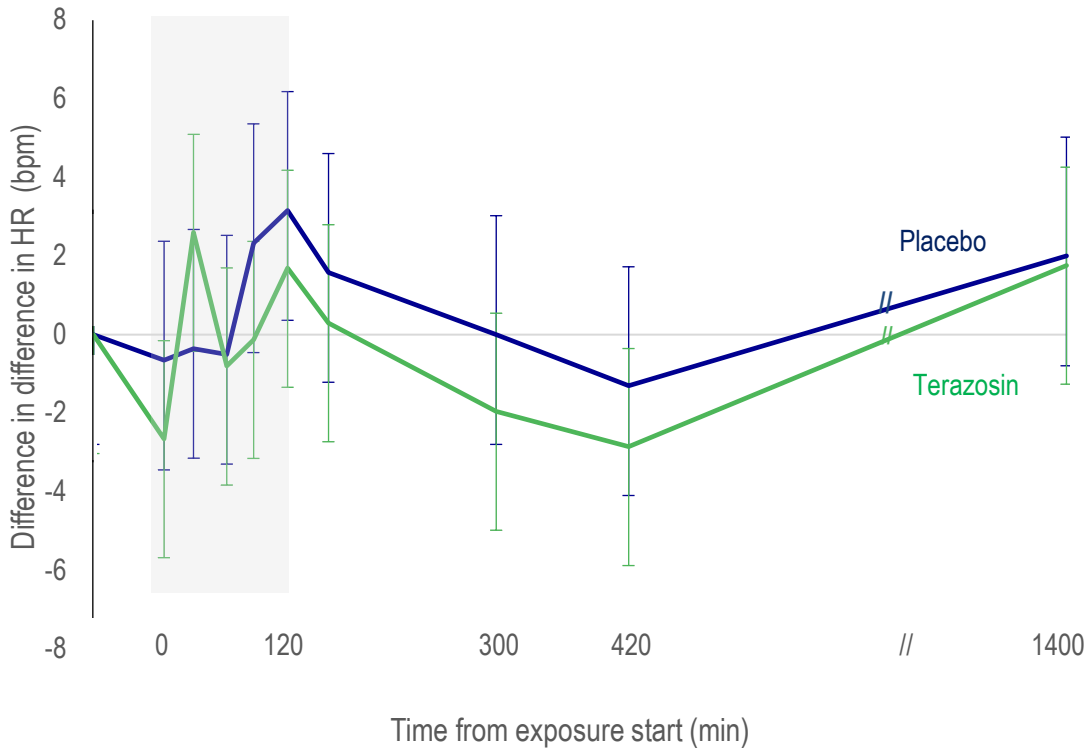


**Figure 2.5. DE Effect on HR by treatment.** Mean within-subject difference in baseline adjusted SBP between DE and FA exposures, for placebo and terazosin sessions. The exposure session is highlighted in grey.

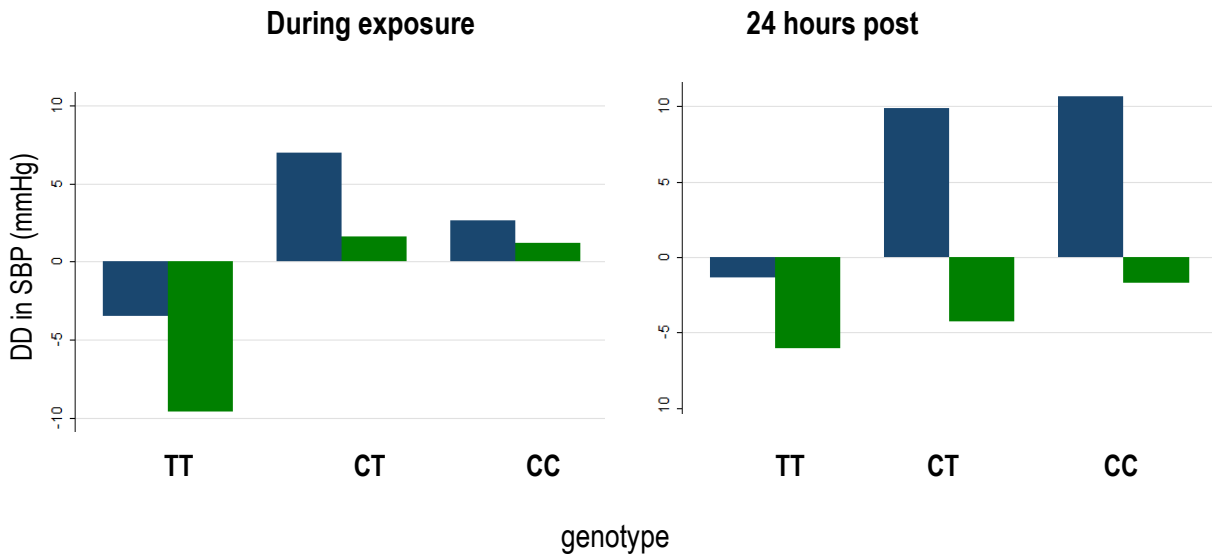


**Figure 2.6. Differential DE effect on SBP by TRPV1 genotype.** Mean difference in difference in SBP with DE exposure in subjects treated with placebo (blue) and terazosin (green) compared with FA control. Subjects with one or more C alleles show a significantly different response than TT homozygotes both during exposure (30-90 min) and 24 hours post exposure start.

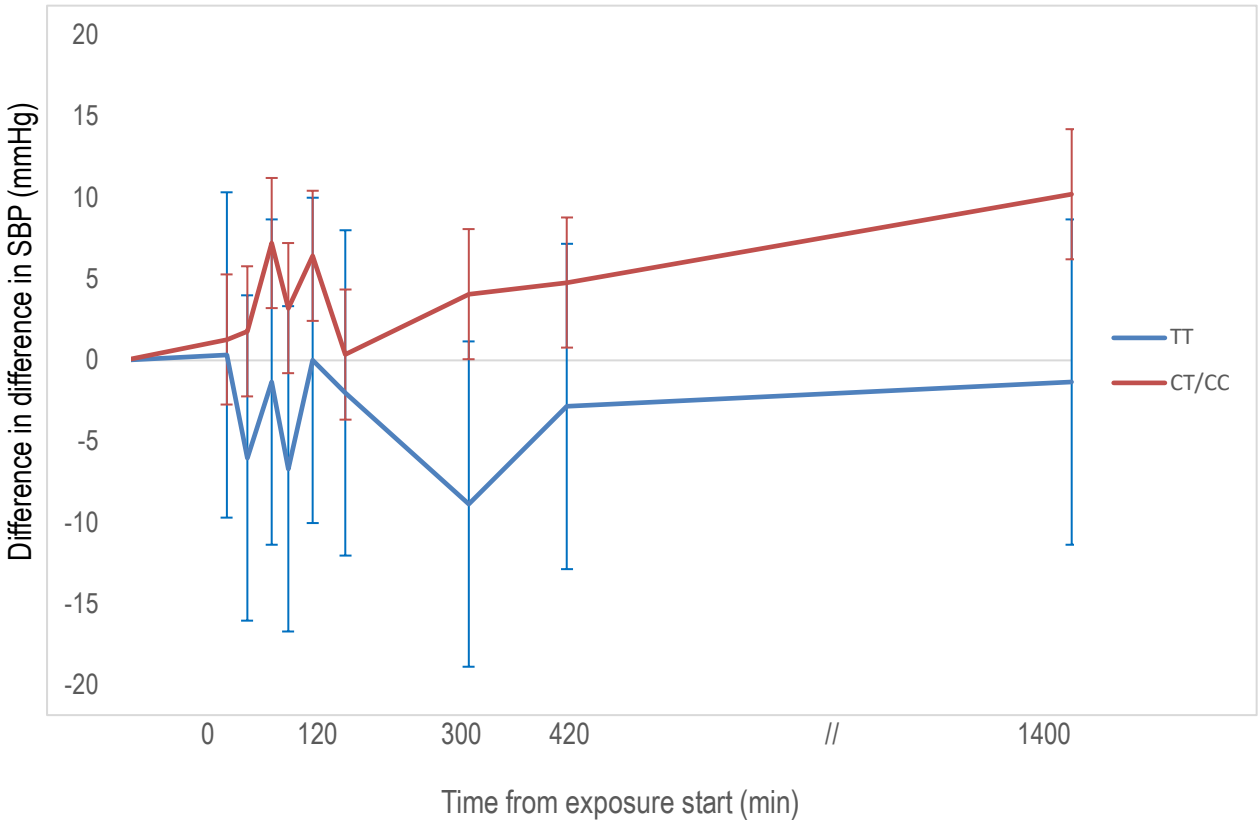


**Figure 2.7. Binary (dominant) model of main DE effect (placebo) on SBP by *TRPV1*.** Mean difference in difference in SBP with DE exposure in subjects treated with placebo at each time point measured, compared with FA control. Subjects with one or more C alleles show a different response than TT homozygotes at all time points.

**Table 2.3 Assessment of subject blinding by perception of exposure and treatment**

**Type of Exposure (DE/FA)**

| Guess | % |
|---|---|
| Correct | 59 |
| Incorrect | 41 |

**Treatment (terazosin/placebo)**

| Guess | % |
|---|---|
| Correct | 45 |
| Incorrect | 55 |

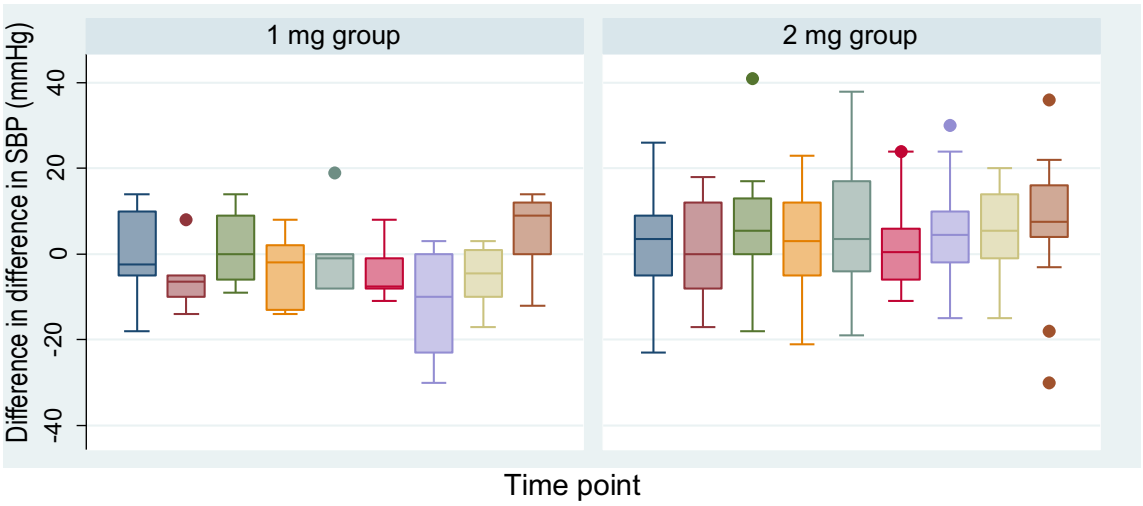


**Figure 2.8. Comparison of DE Effect by terazosin dose.** Though the higher dose elicited somewhat more variability in BP, there was no significant difference in effect of terazosin between the two groups. Graphs show mean SBP baseline adjusted change with DE (the DE effect) at each time point measured, compared to FA.

# **Chapter 3.** Diesel exhaust exposure alters expression of genes involved in inflammation and oxidative stress *in vitro*

## INTRODUCTION

Traffic-related air pollution is one of the most ubiquitous sources of inhaled particulate matter, and an established risk factor for respiratory and cardiovascular morbidity.[195] Research suggests that inhaled pollutants act via well-characterized pathways associated with coronary disease, augmenting processes underlying atherosclerotic plaque development, the triggering of ischemic events, the development of arrhythmias, and alterations in cardiac function or structure. Though such clinical outcomes have been well-substantiated in population-based studies, there remain gaps in knowledge regarding the pathophysiological mechanisms upstream of these effects. Experimental research suggests the process might involve several pathways, including disruptions in redox-mediated signaling, systemic inflammatory response mechanisms, and alterations in autonomic balance.[1]

Many effects of air pollution are believed to be indirect, induced in response to primary events initiated in the lung. Inhaled pollutants may trigger sensory receptors regulating autonomic control, induce local inflammation and, in turn, a systemic inflammatory response, or disrupt barrier function.[196, 197] Though animal models have provided useful information, findings have been inconsistent, and physiological responses appear to vary based on the exposure method, species and genotype/phenotype. Controlled human exposures have produced more congruent results, however, studies are limited in outcome measures and often challenged by variations in exposure concentrations, specifically when particles are sourced directly from roadways.

The first experiments in this chapter involve an assessment of the *in vitro* inflammatory response to DE-generated particle deposition in primary normal human bronchial epithelial cells (NHBE). Exposure concentrations and outcome endpoints were structured to parallel the human controlled exposure study described in Chapter 2. The second aim was developed to investigate whether or not initial cellular responses in particle-exposed epithelial cells can subsequently induce a response in cells outside of the lung via the release of soluble mediators. To test this, primary human coronary artery endothelial cells (HCAECs) were incubated with conditioned media from the NHBE studies and with serum sampled from humans (a subset of subjects described in Chapter 2) exposed to FA and DE under the same conditions. In addition, we investigated the role of the alpha-adrenergic system in the transcriptional response, by examining changes in human plasma and in HCAECs exposed to serum sampled from subjects treated with terazosin, an alpha-1 adrenergic blocker, prior to each exposure. Based on the hypothesis that inhaled particulate matter acts via mechanisms associated with oxidative stress and inflammation, we examined chemokine, cytokine and permeability gene response patterns induced in the human cells and blood following exposure to DE.

## MATERIALS & METHODS

### *Aerosol Exposure System*

A 5.5 kW Yanmar model YDG5500EV-6EI single-cylinder diesel engine, running at steady state prior to subject arrival, generated the DE (Yanmar America; Adairsville, GA). Load was maintained at 75% of rated capacity, using a load-adjusting load bank (Simplex, Springfield, IL), ultra low sulfur highway grade diesel fuel and Valvoline 15W-40 crankcase oil. Emissions were diluted with the air used for FA exposures: ambient air passed through a

carbon matrix filter and HEPA filter (99.99% efficient). Final $PM_{2.5}$ concentrations were assessed in real-time using a tapered element oscillating microbalance (1400a $PM_{2.5}$, Rupprecht & Patashnick Co., Albany, NY), and adjusted continuously with a feedback control system based on nephelometry measurements to achieve a consistent concentration of 300 $\mu g/m^3$ in the breathing zone (average 292 $\mu g/m^3$; standard deviation 25 $\mu g/m^3$). The 116 $m^3$ exposure room was maintained at a temperature of 20 to 21°C with 50% relative humidity. The facility's DE particle mass median diameter was 0.080 μm, with typical particle counts 2.8 x $10^3$ per $cm^3$ for FA and 5.3 x $10^4$ per $cm^3$ for DE exposures, based on one-minute averaged multistage impactor-collected samples. Nephelometers positioned within the room confirmed spatial uniformity of particle concentrations. For DE exposures, average concentrations of nitrogen dioxide were 35 ppb (approximately 1.5% of total NOx). Concentrations of carbon monoxide averaged 0.30 ppm for FA and 0.80 ppm for DE. The exposure facility has been previously described in extensive detail.[147]

*In vitro exposure system*

The Nano Aerosol Chamber for In Vitro Toxicology (NACIVT) is a novel nanoparticle deposition chamber that significantly increases the deposition of ultrafine particles sampled from an aerosol, under physiological conditions of the lung. The aerosol inlet is connected directly to the human exposure room at the Northlake controlled exposure facility described above. Two mass- flow controllers pull a total aerosol flow of approximately 1.7 lpm. A Krypton unipolar diffusion charger positively charges particles within the aerosol. The flow is then split: 1.1 lpm flows to an electrometer which measures the current generated by the charged particles and 0.6 lpm is directed to 24 wells containing normal human bronchial epithelial cells cultured on Transwell inserts. A high voltage electrode below the wells creates

an electric field, which pulls the particles onto the cells and increases the rate of deposition of the smallest particles to 40%, making this method both rapid and efficient. The electrometer data is used to verify that the particle concentration is stable over the duration of the exposition, to compare concentrations between exposure days and, in conjunction with exposure monitors, to generally estimate concentration level.

Prior to reaching the cells, the aerosol is heated and humidified to physiological levels (37 C and 85% relative humidity) via a 15cm long Gore-Tex-tube in a heated water bath. Humidity is controlled by changing the temperature of the water bath, and is measured, both before and after the cells are exposed, by two sensors in the chamber. Following the exposure the air is dried in a silica gel dryer, and passed through a HEPA filter. All functions of the NACIVT are controlled using Labview on a dedicated laptop, for constant monitoring and adjustment allowed for throughout each exposure. The system has been fully characterized and tested, and is described in detail here.[148]

*In vitro aerosol exposures*

Primary normal human bronchial epithelial cells (NHBE) were used for the aerosol exposures, and were provided courtesy of Dr. Teal Hallstrand at the University of Washington Center for Lung Biology. Cells were harvested from the tracheal section of a healthy lung during a lung transplant and cryopreserved until use. Cells (at passage 2) were plated on Corning Costar polyester Transwell inserts (6.5 mm2, 0.4 μm pore size) at a density of 1 x $10^5$ cells/$cm^2$ in 0.15 ml initial culturing medium on the apical side and 0.5 ml in the basolateral compartment. Initial culturing medium was BEGM basal medium (Lonza; Allendale, NJ) supplemented with 1 μl of 0.1mM all-trans-Retinoic acid (Sigma Aldrich; St. Louis, MO) and 0.4 μl of 25 μg/ml rhEGF (VWR; Radnor, PA) per ml of basal medium. Medium was replaced in the

basolateral compartment only at 24 hours and then in both apical and basolateral compartments every 48 hours until cells were confluent, approximately 7-10 days after initiation. At this point, the apical medium was removed and the basolateral medium was replaced with differentiation medium (BEGM with 1 µl of 0.1mM all-trans-Retinoic acid and .002 µl of 25 µg/ml rhEGF per ml base medium). Medium was changed every 48 hours until cells were fully differentiated, approximately 21 days later. Tight junction formation was confirmed with transepithelial electrical resistance (TEER) measurements.

Prior to exposure, the medium in the basolateral compartment was changed and supplemented with 10mM HEPES buffer. The cells were transferred to the NACIVT running at steady state (37° C, 85% relative humidity) in the UW Northlake Controlled Inhalation Facility for a 60 minute exposure to FA or DE (300 µg/m$^3$) drawn from the human exposure room. Cells were exposed to DE or FA in triplicate or quadruplicate, with four independent sessions conducted for each exposure condition. Concentrations of 300 µg/m$^3$ DE for a 60 minute exposure were calculated to correspond to a dose of 112 ng per insert, based on a mean particle diameter of 80 nm and a 25% deposition rate (range 15 – 40% for 200 nm – 20 nm particles, respectively).[146, 148]

Following the exposure, cells were returned to the incubator for 3 or 24 hours. Cells were examined using light microscopy to ensure cells retained normal morphology. Prior to RNA extraction, medium from the exposed cells ("conditioned media") was removed and stored at –80 C for exposures to coronary artery endothelial cells (described below).

*Human subject selection*

Twenty adult volunteers participated in four exposure sessions conducted at the University of Washington's Northlake Controlled Exposure Facility. Subjects were healthy non-smokers, with no history or evidence of hypertension, asthma, diabetes, hypercholesterolemia, cardiovascular illness, or other chronic medical condition, based on questionnaire, spirometry, fasting glucose and lipid panel, and electrocardiogram. Subjects with body mass index (BMI) >30 kg/m$^2$, fasting blood sugar (glucose) >126 mg/dL, and blood pressure (BP) >130/85 mmHg were also excluded. Age, sex, race/ethnicity, smoking, and medication use were self-reported. Urinary cotinine was measured in samples collected at the first visit to confirm non-smoking status using the CAS-COT kit (Innovacon, Inc.; San Diego, CA). Participants with cotinine levels greater than 200 ng/mL were excluded.

*Terazosin treatment*

Terasozin hydrochloride is a selective alpha-1 adrenergic blocker used for the treatment of benign prostatic hyperplasia and hypertension. Subjects were administered 1 or 2 mg terazosin or matched placebo upon arrival, at approximately 7:30 am, with the baseline blood draw immediately following. The initial dose (2 mg) was selected based on medical dosing guides and the minimal reporting of adverse events associated with the drug; this dose, however, was lowered to 1 mg for the last six subjects due to the number of subjects excluded at screening for dizziness. Individual subjects were administered the same dosage for all four exposures. Terasozin is rapidly absorbed and reaches peak plasma concentrations in 1 – 2 hours; exposures were started 2 hours after administration of the drug to coincide with maximum plasma levels.[176]

*Human exposures*

Each subject was exposed on separate days to each condition, DE + placebo, FA + placebo, DE + terazosin and FA + terazosin. Exposures were double-blind, crossover, and randomized to order, with sessions separated by a minimum three-week washout period to eliminate any potential carry-over effects. The same protocol was followed each session to limit any variation between sessions. Women were exposed only during the first two weeks of a menstrual cycle, and pregnancy was ruled out by a urine pregnancy test at the beginning of each session. Subjects were instructed to fast for at least eight hours prior to arrival at the research center. Each subject received an identical defined composition meal at each session, approximately one hour after the exposure. Subjects remained at the research center for a minimum of 9 hours after arrival and returned for follow-up measurements approximately 24 hours from the session start. All researchers, nurses and technicians participating in the study were blinded to the treatment and exposure type, with the exception of the exposure engineer.

*In vitro exposures to human serum and conditioned media*

Primary coronary artery endothelial cells (HCAEC), purchased from Lonza, were used for the serum exposures. Cells were cultured per the manufacturer's instructions, and plated at 5 x $10^3$ cells/$cm^2$ using epithelial growth medium supplemented with human Epidermal Growth Factor (hEGF), Vascular Endothelial Growth Factor (VEGF), R3-Insulin-like Growth Factor-1 (R3-IGF-1), Ascorbic Acid, Hydrocortisone, human Fibroblast Growth Factor-Beta (hFGF-β), Heparin, Fetal Bovine Serum (FBS), and Gentamicin/Amphotericin-B (GA) (EGM-2 Bulletkit; Lonza) in 25 $cm^2$ flasks. Medium was changed every other day and cells were

passed at approximately 75% confluence. For exposures, cells were plated in 24-well plates at 1 x $10^4$ cells/$cm^2$ and grown to confluence.

HCAECs were exposed to conditioned media from aerosol exposures (FA and DE) or to human serum, drawn pre and post exposure to DE or FA, diluted in fresh media (1:3) for 24 hours at which point RNA was extracted for analysis. Serum was used from nine subjects randomly selected from the human subject pool. At time of selection, no data were available on subjects beyond demographic and screening data. Experiments were done in duplicate, with exposures competed for each human subject (n=9) under each condition (n=4; DE+placebo, FA+placebo, DE+terazosin, FA+terazosin) and independent exposures (n=4 each, FA and DE) for each NACIVT condition.

*Fluidigm analysis of gene expression*

For human samples, blood was collected pre, during, and post (5 and 22 hours) exposure in a BD Vacutainer CPT glass molecular diagnostics tube containing density gradient polymer gel and sodium citrate. Cells were pelleted by centrifugation and resuspended in TRIzol for RNA extraction. RNA was isolated from peripheral blood mononuclear cells (PBMCs) using an RNeasy kit (Qiagen; Valencia, CA); complementary DNA was synthesized with Oligo-dT primers and reverse transcriptase (Qiagen), using the same total RNA concentration for each sample. For *in vitro* exposures, cells were lysed and RNA extracted using a miRNeasy kit (Qiagen). Complementary DNA was synthesized using the same method and materials used for human samples, described above, to limit any technical variation.

Each sample was multiplexed using the Fluidigm-based qPCR assay, on a 96.96 Dynamic Array Integrated Fluidic Circuit BioMark HD System (Fluidigm, San Francisco,

CA). An internal control for each gene was spiked in at various dilutions to generate a standard curve to estimate threshold cycle C(t). Samples were run in duplicate or triplicate. Data were normalized to the reference gene *GAPDH*, selected from four reference genes tested (*GAPDH, β-actin, TATA-box protein and peptidylprolyl isomerase A*). Data were collected using the Fluidigm BioMark Data Collection Software and analyzed using Fluidigm Real-Time PCR Analysis Software 4.1.2.

*Immunohistochemistry*

Protein analysis is preferable to measuring mRNA, as the latter may be regulated post transcription, however, the ability of particles to bind cytokines in culture media complicates (or potentially invalidates) the use of standard assays to assess alterations in protein levels.[143] To complement the gene expression findings reported herein, we therefore used immunohistochemistry analysis of the NACIVT exposed samples. ZO-1 was selected based on changes detected in mRNA levels and due to its importance in maintaining cell barrier function. Disruption of this protein may demonstrate cell injury and suggest a means by which inhaled pollutants cause injury in pulmonary tissue, induce local inflammatory reactions and, possibly, translocate into the systemic circulation.

Primary normal human bronchial epithelial cells (NHBE) were cultured to differentiation at ALI and exposed to both DE and FA using the NACIVT exposure system, as described above. Following exposure, cells were incubated for 24 hours, after which they were frozen in Tissue-Tek OCT and stored at -80$^{o}$C. Prior to staining, tissue was sectioned (10 μm) at -17$^{o}$C and mounted onto VWR glass Superfrost Plus micro-slides. Sections were fixed with 4% paraformaldehyde for 15 minutes and washed with PBS (3 times at 5

min/wash), then blocked for 60 min with 5% normal goat serum and 0.3% Triton X-100 in PBS. Samples were stained with rabbit anti-ZO-1 primary antibody (1:50 = 2 μg/ml) in a dilution buffer (comprised of 1% BSA and 0.3% Triton X-100 in PBS) and incubated overnight at 4ºC. After washing in PBS (3 times at 5 min/wash), specimens were incubated in Alexa Fluor 488 Conjugate anti-rabbit IgG secondary antibody (2 drops/ml) in dilution buffer for one hour at room temperature in the dark. Nuclei were labeled with Hoechst (4 μg/ml) and slides cover slipped with Prolong Gold Antifade Reagent. Primary and secondary antibodies were purchased from Invitrogen (California). Images were captured using a Nikon Labophot-2 microscope (Nikon, Inc.; New York) and Nuance 3.0 imaging software (PerkinElmer, Inc.; Massachusetts). Fluorescence intensities were analyzed using MetaMorph image analysis software (Molecular Devices, California).

**Statistical Analysis**

For the PBMC and serum *in vitro* studies, data were converted to $\log_2$ values and analyzed using a mixed effects model with subject and session treated as random effects, with the pre-treatment time point set as baseline. Using the FA control (placebo) samples as baseline, the interaction terms are comparisons between a given exposure/treatment/time and the baseline (e.g., the coefficient DE_Tera:time1 tests (DE Terazosin time 1 - DE Control time 0) - (FA Control time 1 - FA Control time 0). There are nine such interaction terms per gene. The likelihood ratio test was used to test if any interaction terms were significant; for this we compare the full model (with interaction terms) against a reduced model (without), which is a 9 degree of freedom Chi-square test. Data for the NACIVT exposures were analyzed in a similar manner, minus subject and treatment terms. A robust regression was used for most

comparisons to mitigate the impact of outliers.

## RESULTS

Using our *in vitro* model of inhalation, we found that NHBE cells exposed to DE showed significantly increased mRNA expression in nine genes (*GCLc, HIF1α, ICAM1, COX2, ZO-1, MMP2, IL-6, CCL2* and *SLPI,* all $p<0.03$), when compared to FA at 24 hours post exposure (Table 3.5, Figure 3.2) We did not detect similar differences at 3 hours (Table 3.4). In addition, we detected significant changes in expression in HCAECs treated with conditioned media from the NHBE studies, sampled at both 3 and 24 hours post exposure: two genes (*MT3, OCLN*) were notable in the 3 hour post-exposure samples (Table 3.6; Figure 3.3) and seven (*eNOS, COX2, CCL5, IL-6, OCLN, GCLc, TRPV1*) from samples taken 24 hours post (Table 3.7). Interestingly, most of the significant genes show a consistent down regulation in cells incubated with conditioned media sampled 3 hours post DE and increased expression in cells incubated with media samples 24 hours post (Figure 3.3)

We did not detect any changes in gene expression in HCAECs treated with serum from human subjects (Table 3.8, Figure 3.4). Similarly, there was no evidence that DE exposure affects the expression of genes in human PBMC, during or within 24 hours of exposure (Table 3.9). Treatment with terazosin did not elicit measureable effects on mRNA, with or without exposure to DE. *TRPA1* was not detected in any samples.

Immunohistochemistry studies were consistent with our findings for gene expression, with higher fluorescence in tissue exposed to DE (Figure 3.5). These findings were not statistically significant, due to the small number of samples, however serve as proof of concept/pilot studies for future expanded testing.

## DISCUSSION

Our findings demonstrate that an acute 60 minute exposure to DE induces significant changes in the expression of inflammatory, oxidative stress and barrier-related gene expression in primary human lung cells, which further elicits a secondary response in coronary artery cells. These findings substantiate evidence reported in epidemiology research and provide support for new in vitro models in inhalation toxicology.

To examine how DE modulates genes in the airway we employed NHBE cells exposed to aerosol under physiological conditions. We selected NHBE cells, as they are an early and primary target of inhaled pollutants; the airway epithelium serves as both an essential barrier and as a modulator of the response to toxicants via the production of antioxidants, protease inhibitors and inflammatory and immune mediators.[198] While inhaled particulate matter is predominately cleared by mucocilliary transport or phagocytosis, activation of the epithelium and airway leukocytes can induce the release of cytokines, chemokines and other proteins associated with an inflammatory response. Furthermore, the smallest particles, known as ultrafine particulate matter, are not efficiently cleared from the lung and are believed to have greater potential for both translocation and interaction with cellular proteins and other mediators.

Both population-based and controlled human studies have reported adverse respiratory outcomes associated with exposure to $PM_{2.5}$, including increased airway hyper-responsiveness, inflammation, reduced lung function and exacerbations of asthma.[199-201] Exposure to DE in particular has been shown to induce an oxidative stress response and neutrophilic airway inflammation, pathways involved in systemic inflammation and the development of disease.[113, 202-204] Here we report that NHBE cells exposed to DE showed

significantly increased expression in genes associated with oxidative stress (*GCLc, HIF1α*), inflammation *(CCL2, COX2, MMP2, IL-6,* and *SLPI),* adhesion *(ICAM1*) and barrier function *(ZO-1*), 24 hours post exposure.

$PM_{2.5}$ may induce oxidative stress directly, via reactive electron donors, or indirectly, as a secondary effect of inflammatory signaling or target cell interactions (i.e. altered mitochondrial function or calcium homeostasis).[125] [205, 206] Under the hierarchical model of oxidative stress, an abundance of reactive oxygen species (ROS) initially stimulate protective responses, e.g. activation of the transcription factor Nrf2, which regulates expression of anti-oxidant response genes such as *GCLc* (Tier 1).[207] *GCLc* is the catalytic subunit of glutamate cysteine ligase (GCL), which catalyzes the rate-limiting step of the key detoxifying peptide, glutathione (GSH). *GCLc* induction occurs via a number of mechanisms and plays an important role in GSH maintenance and the response to oxidative stress.[208] Our lab has shown a drop in circulating GSH in humans following a two hour exposure to controlled DE, indicating induction of this redox-sensitive protective response. Previous studies in mouse endothelial cells exposed to DE have reported similar increases in *GCLc* expression.[209]

Further stress (Tier 2) activates signaling cascades such as the mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF-κB) pathways, which mediate the expression of cytokines, chemokines and adhesion proteins. In line with our findings, ROS-induced nuclear localization of NF-κB increases the transcription of *HIF1α*.[210] *HIF1α* transcription signaling plays a role in the regulation of over 500 genes, with predominately protective effects in terms of lung injury.[211]

In contrast to this, we saw a significant upregulation of the monocyte chemotactic cytokine *CCL2,* which is implicated in the pathogenesis of allergic airway inflammation and

asthma, and is regulated by a variety of mediators, including *TNFα*, *HIF1α* and NF-κB (Baay-Guzman 2012).[212] CCL2 is pivotal in the recruitment of monocytes, memory T-cells, and dendritic cells to sites of tissue injury.[213] Over expression of *CCL2* has also been associated with higher IL-6 levels, activation of the epithelium and with the development of a fibrotic phenotype.[214, 215] In line with this, we report significant increases in the major inflammatory genes *IL-6* and *COX-2* following exposure to DE.

Interestingly, we also found a significant increase in the expression of *SLPI*. *SLPI* is a serine protease inhibitor broadly expressed by epithelial cells that reduces tissue damage (via inhibition of neutrophil elastase and wide variety of other proteases) and modulates the transcription of proinflammatory genes via inhibition of NF-κB.[216] Though the molecular mechanisms underlying transcriptional changes remain unclear, expression appears to be primarily upregulated by proinflammatory mediators.[217, 218] Tobacco smoke, another inhaled toxicant, has been shown to increase mRNA levels both *in vitro* and *in vivo*.[219]

We also detected significant changes in genes related to barrier function and repair, specifically *MMP2* and *ZO-1*. MMPs regulate cytokine and chemokine activity as well as endothelial function; MMP2 specifically cleaves the transmembrane tight junction protein occludin, leading to increased cell permeability.[313] While the mechanisms are not well characterized, human and experimental studies have demonstrated that *MMP2* expression is highly upregulated in human and experimental studies of pulmonary fibrosis, and presumed to act via disruption of basement membranes.[220, 221] In murine studies of gasoline exhaust exposure, Lund and colleagues reported significant increases in *MMP2*, an effect which was modified by antioxidant supplementation, suggesting a role of ROS in the response.[222]

Though we did not detect any change in the expression of *OCLN*, we did find a significant upregulation of the important scaffold protein *ZO-1*. These findings appear to be consistent at the protein level, suggested by our preliminary findings using immunohistochemical staining. Disruption of the epithelial junctions potentiates the risk of both particle translocation and further cellular damage, and can alter signaling pathways involved in differentiation and repair. For example, ZO-1 functions as a feedback regulator of gene expression, most notably of epithelial proliferation and cell density. Recent work in a murine model found that activation of *HIF1α* during lung injury promoted the resolution of epithelial permeability, which may also underscore the upregulation we report here.

Both short and long-term exposures to $PM_{2.5}$ have been associated with the onset of acute ischemic coronary events.[20, 60] To test the hypothesis that DE exposure induces the release of circulating mediators in the lung that, in turn, affect other organs such as the heart and vascular system, we exposed primary HCAECs to conditioned medium taken from the NHBE cells described above. Our findings support this theory and provide important insight into the timing of such effects. We saw upregulation in only two genes following exposure to media sampled three hours post exposure to DE, but a much stronger effect in cells exposed to conditioned media sampled 24 hours post exposure. This is congruent with our findings in NHBE cells, where all significant changes in gene expression were detected 24 hours post exposure. While we cannot rule out additional effects that occurred within this time period, these findings may inform future research on the timing of molecular changes in DE studies.

HCAECS exposed to NHBE conditioned media also demonstrated upregulated expression in genes involved in the antioxidant (*GCLc, HIFa*) and inflammatory response pathways (*COX-2, IL-6, CCL5*), suggesting that mediators released following the initial

exposure continue to induce defensive mechanisms within cells not directly exposed. Although the underlying mechanisms have not been elucidated, there is evidence indicating that inhaled PM causes oxidative injury to the endothelium, leading to inflammation, endothelial cell dysfunction and the advancement of coronary and cardiovascular disease. Unfortunately we do not know which specific components of the conditioned medium spurred the responses we report herein.

Interestingly, we detected significant upregulation in *eNOS* in HCAECs. Under normal conditions, eNOS regulates the production of vascular nitric oxide (NO), an important vasodilator that inhibits platelet activation and the development of atherosclerosis.[223] Under conditions of oxidative stress, however, eNOS can dysfunction (eNOS uncoupling), leading to the production of superoxide instead of NO. This dysfunction is a key mechanism in conditions such as hypertension and atherosclerosis, as it both reduces levels of protective NO and further exacerbates the oxidative environment.[224] Controlled human exposures have reported impaired endothelium dependent and independent vascular reactivity following acute exposure to DE, effects attributed to the hypothesis that $PM_{2.5}$ elicits vessel constriction through oxidative stress induced eNOS uncoupling.[74, 90, 204, 225] While experimental research has demonstrated that such uncoupling occurs, the implications of our findings in isolation are difficult to interpret.[122, 226] We also report an increase in the mRNA expression of *TRPV1*. TRPV1 is an ion channel and sensor of a number of chemical and physical irritants involved in tissue injury, inflammation and vascular function.[155] Though research on the role of TRPV1 in the cardiovascular system still remains limited, *in vitro* and animal studies have shown that TRPV1 channels directly regulate myocardial blood flow via eNOS-NO-

dependent vasodilation and ameliorate myocardial damage by reducing the release of inflammatory cytokines and inhibiting neutrophil infiltration.[171, 227, 228]

HCAEC show significant upregulation in expression of the tight junction protein *OCLN*, when exposed to conditioned media sampled at both 3 and 24 hrs post DE. In contrast to this, coronary and cardiovascular events associated with barrier dysfunction, including stroke, hypoxia and inflammatory disease, are associated with *OCLN* downregulation.[229] While higher expression levels generally correlate with enhanced barrier properties, *OCLN* overexpression has been shown to increase sensitivity to cytokines and decrease the barrier for large solutes.[230]

Surprisingly, we did not detect any changes in gene expression when testing the serum of subjects exposed to DE or in serum exposures in HCAECs. This perhaps reflects the more sophisticated response to exposure *in vivo*, which involves a wide range of protective mechanisms, however, other similarly designed studies have detected transcriptional changes in both PMBCs and cell culture.[231] Alternatively, circulating markers of exposure may not reflect damage or effects occurring at the tissue level. For example, circulating levels of SLPI are approximately 1000 times lower than cellular levels in the lung, supporting the premise of location-focused expression. It is also important to note that our exposures were conducted in young healthy adults. Effects may not be apparent in the limited biomarkers available in clinical research; this is why *in vitro* findings under consistent controlled conditions can provide especially constructive data.

The use of primary cells makes our model highly translatable, as differentiated airway epithelial cells show a transcriptional profile that is highly conserved when compared to samples *in vivo* (bronchial brushings).[232] Furthermore, prior studies have shown fully

differentiated NHBE cells to be more resistant to the effects of air pollutants than cell lines or other model cultures, indicating that our model is likely more conservative than most. [233, 234] While our cells were sourced from one donor, research on the use of primary lung cells has demonstrated a high level of consistency in transcriptional profiles between donors, as well as among cells sampled from the trachea and bronchial regions, suggesting that biological replicates do not play the significant role they may in other studies (Pezzulo 2010).[232]In addition, our selection of coronary artery endothelial cells makes this data directly relevant to the pathogenesis of coronary atherosclerosis and coronary heart disease, as endothelial cells from different vascular origins can differ in gene expression profiles.

It should be noted that our study used DE generated by an engine running at 75% load, mirroring conditions on a moving thoroughfare. Studies on emissions have reported that the inhaled dose by particle number is two times higher for idling conditions than transient driving conditions, due to a higher proportion of the particles in nucleation mode.[146] Models of human lung deposition report that the fraction of inhaled particles deposited in the lung ranges from 10% for larger particles ( >500 nm) to near 90% for the smallest particles (<10 nm), suggesting that exposure and effects in urban cities or during high traffic commutes are likely greater than our findings suggest.[235] There is also a wide variability in the deposited dose (over twofold among healthy adults) and biological response among subjects; our studies employed healthy human cells and therefore do not encompass the effects in more susceptible populations.[141, 146, 236]

Our data reveal transcriptional changes in primary human cells that may underlie the pathophysiological effects associated with exposure to common ambient air pollutants. Within 24 hours of exposure to DE, mRNA levels were significantly altered in multiple genes

involved in the regulation of inflammatory processes implicated in the development of disease. More importantly, our data provide evidence that effects elicited in the airway epithelium can induce secondary effects in coronary artery endothelial cells, suggesting that the epithelium may play an initial role in inflammatory processes and barrier dysfunction outside of the lung.

## TABLES AND FIGURES

### Table 3.1. Experimental setup for NACIVT exposures

| Experimental Variable | Number of Variables | Level of Variation |
|---|---|---|
| Primary Normal Human Bronchial Epithelial Cells (NHBE) | 1 | Donor 1 |
| Exposure Types | 2 | DE & FA Control |
| Exposure Level | 1 | 300 ug/$m^3$ |
| Experimental Repetitions | 4 | Cells exposed to each exposure level (2) four separate times |
| Experimental Replicates | 2 - 3 | 3 for gene expression (GE);<br>2 for IHC |
| Time points | 2 | 3 hrs post exposure (GE)<br>24 hrs post exposure (GE & IHC) |
| Cellular Markers | 16 | See Table 3.3 |

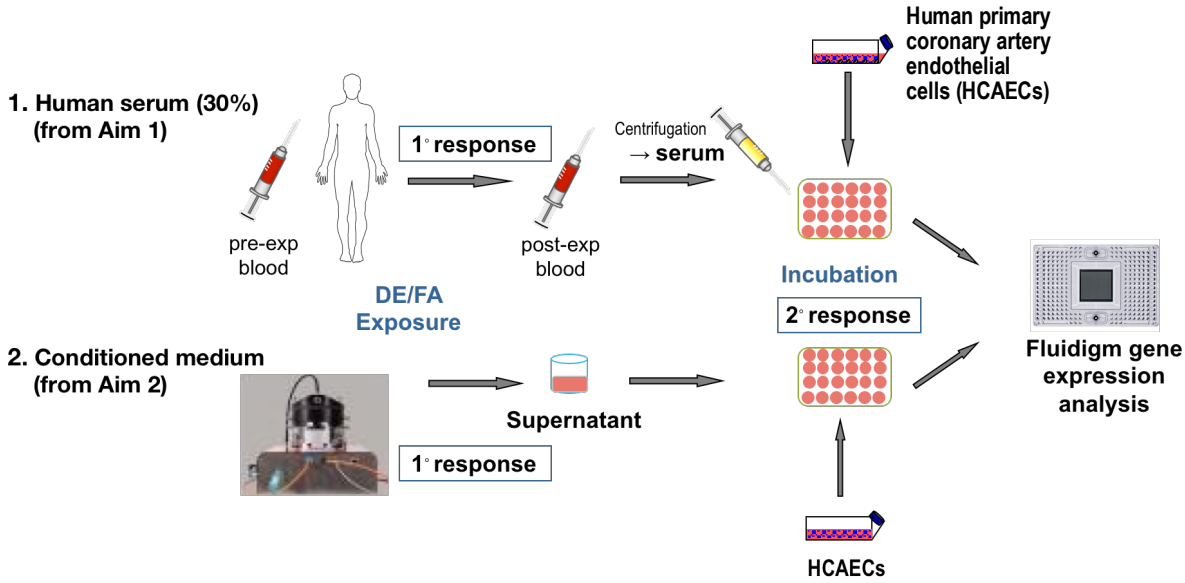


**Figure 3.1 Overview of protocol to assess secondary response to soluble mediators.** HCAECs were incubated with serum from human subjects or conditioned media from NHBE cells exposed to FA and DE under the same conditions.

**Table 3.2 Human exposure study participant baseline characteristics.**
Numbers are reported as mean (± SD).

| Characteristic | Mean (± SD) |
|---|---|
| No. | 20 |
| Age, yrs | 28 ± 8 |
| Male, n (%) | 10 (50) |
| Caucasian, n (%) | 13 (65) |
| Asian, n (%) | 5 (25) |
| Body mass index | 24 ± 2.5 |
| Systolic Blood Pressure, mmHg | 106 ± 10.0 |
| Diastolic Blood Pressure, mmHg | 72 ± 6.7 |
| Heart Rate, bpm | 63 ± 9.7 |
| Total cholesterol, mg/dL | 160 ± 30 |
| LDL | 95 ± 23 |
| HDL | 50 ± 10 |
| Triglycerides | 76 ± 40 |

**Table 3.3 Genes selected for analysis by function**

| | |
|---|---|
| **Ion Channel/Vascular** | |
| *CALCA* | Calcitonin related polypeptide alpha |
| *CALM1* | Calmodulin-1 |
| *ICAM-1* | Intracellular Adhesion Molecule |
| *TRPA1* | Transient Receptor Potential Cation Channel, Ankyrin 1 |
| *TRPV1* | Transient Receptor Potential Cation Channel, Vanilloid 1 |
| **Inflammatory Cascade** | |
| *COX2* | Prostaglandin-Endoperoxide Synthase 2 (PTGS2) |
| *CCL2* | Chemokine (CC motif) Ligand 2 (MCP-1) |
| *CCL5* | Chemokine (CC motif) Ligand 5 |
| *IL-1β* | Interleukin 1 beta |
| *IL-6* | Interleukin 6 |
| *MMP2* | Matrix Metallopeptidase 2 |
| *SLPI* | Secretory Leucocyte Protease Inhibitor |
| **Oxidative Stress** | |
| *GCLc* | Glutamate-Cysteine Ligase, catalytic unit |
| *eNOS* | Nitric Oxide Synthase 3 (NOS3) |
| *HIF-1α* | Hypoxia Inducible Factor 1, alpha subunit |
| *MT3* | Metallothionein 3 |
| **Barrier Function** | |
| *OCLN* | Occludin |
| *ZO-1* | Zona occludens 1 (TJP1) |
| **Reference genes** | |
| *ACTB* | Beta actin |
| *GAPDH* | Glyceraldehyde 3-phosphate dehydrogenase |
| *PPIA* | Peptidylprolyl isomerase A |
| *TBP* | TATA-binding protein |

**Table 3.4. Test for differential expression between DE and FA 3 hrs post exposure in NHBE cells.** Adjusted p-values are false discovery rates (FDR), which estimate the proportion of false positives in a set of significant results.

| Gene | Fold Chg (log) | Avg Expression | Adj. p-value |
|---|---|---|---|
| *GCLc* | 0.924 | -3.266 | 0.176 |
| *CCL5* | 1.476 | -9.887 | 0.176 |
| *eNOS* | 1.154 | -14.015 | 0.176 |
| *ICAM1* | 0.891 | -8.707 | 0.241 |
| *COX2* | 0.824 | -6.222 | 0.241 |
| *SLPI* | 0.268 | 2.163 | 0.491 |
| *ZO1* | 0.365 | -5.703 | 0.872 |
| *CALCA* | 0.526 | -14.064 | 0.930 |
| *MMP2* | 0.425 | -7.230 | 0.963 |
| *TRPV1* | 0.160 | -10.374 | 0.963 |
| *HIF1a* | 0.076 | -3.763 | 0.963 |
| *CCL2* | 0.207 | -10.532 | 0.963 |
| *IL6* | 0.230 | -9.698 | 0.963 |
| *CALM1* | 0.034 | -5.065 | 0.963 |
| *IL1b* | 0.034 | -5.065 | 0.963 |
| *OCLN* | -0.059 | -6.043 | 0.963 |
| *MT3* | -0.032 | -7.335 | 0.963 |

**Table 3.5. Test for differential expression between DE and FA 24hrs post exposure in NHBE cells.** There are 9 genes with an FDR < 0.03 (bold); all show increased expression with DE exposure compared to FA.

| Gene | Fold Chg (log) | Avg Expression | adj. p-value |
|---|---|---|---|
| *GCLc* | 2.890 | -3.266 | **0.000** |
| *HIF1a* | 1.297 | -3.763 | **0.000** |
| *ICAM1* | 2.847 | -8.707 | **0.000** |
| *COX2* | 2.652 | -6.222 | **0.000** |
| *ZO1* | 1.627 | -5.703 | **0.005** |
| *MMP2* | 2.826 | -7.230 | **0.005** |
| *IL6* | 3.673 | -9.698 | **0.011** |
| *CCL2* | 2.878 | -10.532 | **0.012** |
| *SLPI* | 0.604 | 2.163 | **0.024** |
| *CCL5* | 1.353 | -9.887 | 0.163 |
| *TRPV1* | 0.888 | -10.374 | 0.163 |
| *MT3* | -1.273 | -7.335 | 0.182 |
| *CALCA* | -0.937 | -14.064 | 0.322 |
| *CALM1* | 0.287 | -5.065 | 0.411 |
| *IL1b* | 0.287 | -5.065 | 0.411 |
| *eNOS* | -0.121 | -14.015 | 0.862 |
| *OCLN* | 0.172 | -6.043 | 0.848 |

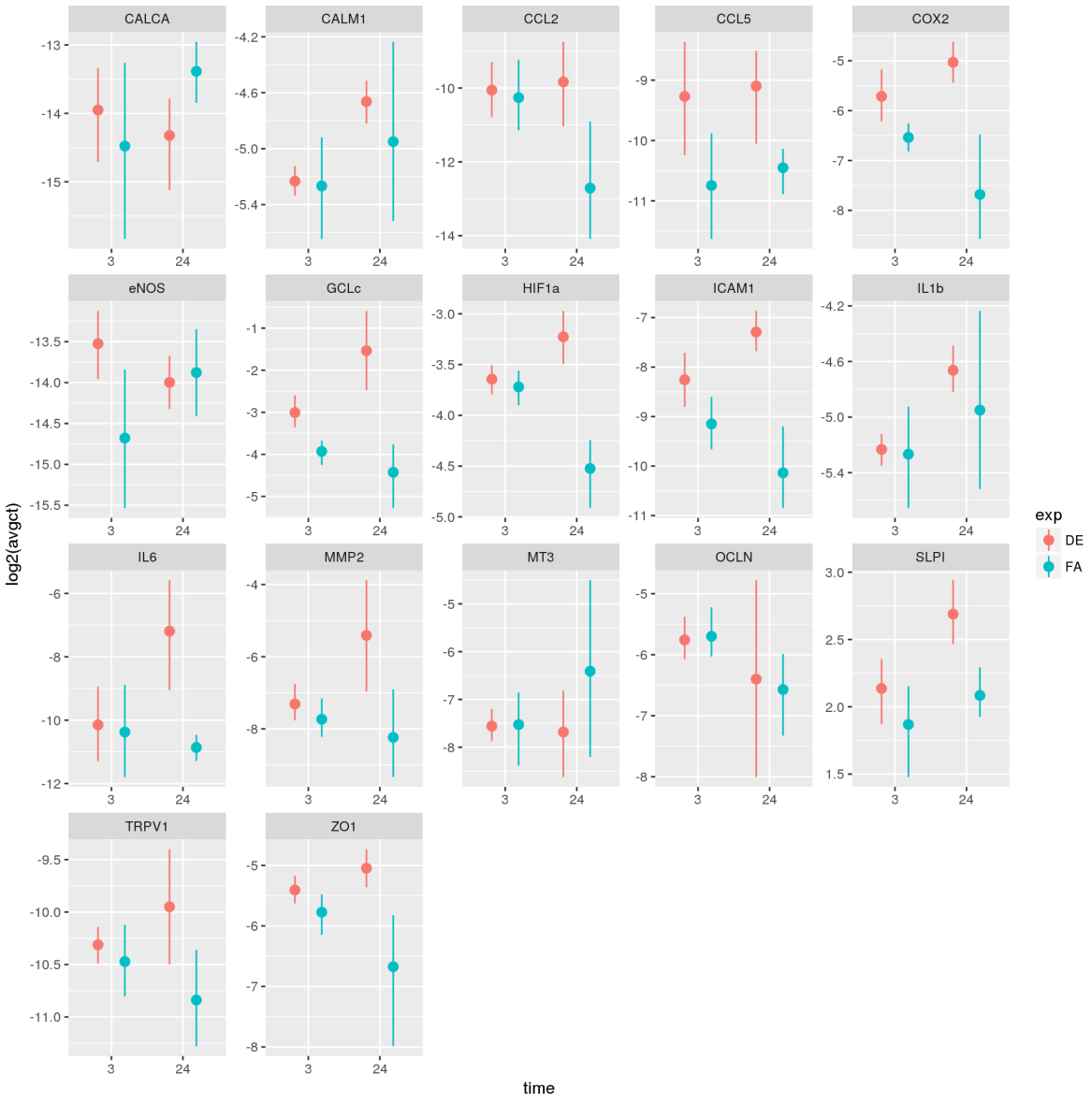


**Figure 3.2 Mean expression levels by gene 3 and 24 hours post exposure to diesel exhaust (DE) and filtered air (FA) in NHBE cells.**

**Table 3.6. Change in mRNA expression in HCAECs treated with conditioned medium sampled 3 hrs post DE exposure.** There are two genes with an FDR < 0.06 (an estimated maximum of 6% false positives).

| Gene | logFC | Avg Expression | Adj. p-value |
|---|---|---|---|
| *MT3* | -1.459 | -10.254 | **0.056** |
| *OCLN* | -1.103 | -10.524 | **0.056** |
| *CCL2* | -0.529 | -5.530 | 0.192 |
| *IL1b* | -2.555 | -80.822 | 0.216 |
| *eNOS* | -0.456 | -5.898 | 0.216 |
| *GCLc* | -0.498 | -7.792 | 0.315 |
| *ZO1* | -0.347 | -6.962 | 0.331 |
| *TRPV1* | -0.687 | -14.052 | 0.331 |
| *HIF1a* | -0.321 | -4.838 | 0.421 |
| *CALM1* | -0.198 | -6.536 | 0.610 |
| *MMP2* | 0.233 | -5.517 | 0.794 |
| *IL6* | -0.120 | -9.991 | 0.882 |
| *ICAM1* | 0.323 | -8.634 | 0.882 |
| *CCL5* | -0.093 | -12.414 | 0.884 |
| *COX2* | -0.092 | -8.219 | 0.884 |
| *CALCA* | -0.006 | -16.584 | 0.993 |

**Table 3.7. Change in mRNA expression in HCAECs treated with conditioned medium sampled 24 hrs post DE exposure.** Adjusted p-values are false discovery rates, which estimate the proportion of false positives in a set of significant results. Seven genes are significantly upregulated (with an estimated maximum of 9% false positives).

| Gene | logFC | Avg Expression | Adj. p-value |
|---|---|---|---|
| *eNOS* | 0.660 | -5.898 | **0.084** |
| *COX2* | 1.336 | -8.219 | **0.084** |
| *CCL5* | 1.162 | -12.414 | **0.084** |
| *IL6* | 0.952 | -9.991 | **0.084** |
| *OCLN* | 0.793 | -10.524 | **0.086** |
| *GCLc* | 0.735 | -7.792 | **0.086** |
| *TRPV1* | 1.088 | -14.052 | **0.088** |
| *HIF1a* | 0.578 | -4.838 | 0.102 |
| *ZO1* | 0.301 | -6.962 | 0.381 |
| *CALCA* | 0.891 | -16.584 | 0.418 |
| *ICAM1* | 0.859 | -8.634 | 0.418 |
| *CCL2* | 0.260 | -5.530 | 0.490 |
| *MT3* | 0.423 | -10.254 | 0.490 |
| *MMP2* | 0.210 | -5.517 | 0.679 |
| *IL1b* | -0.295 | -80.822 | 0.849 |
| *CALM1* | 0.049 | -6.536 | 0.849 |

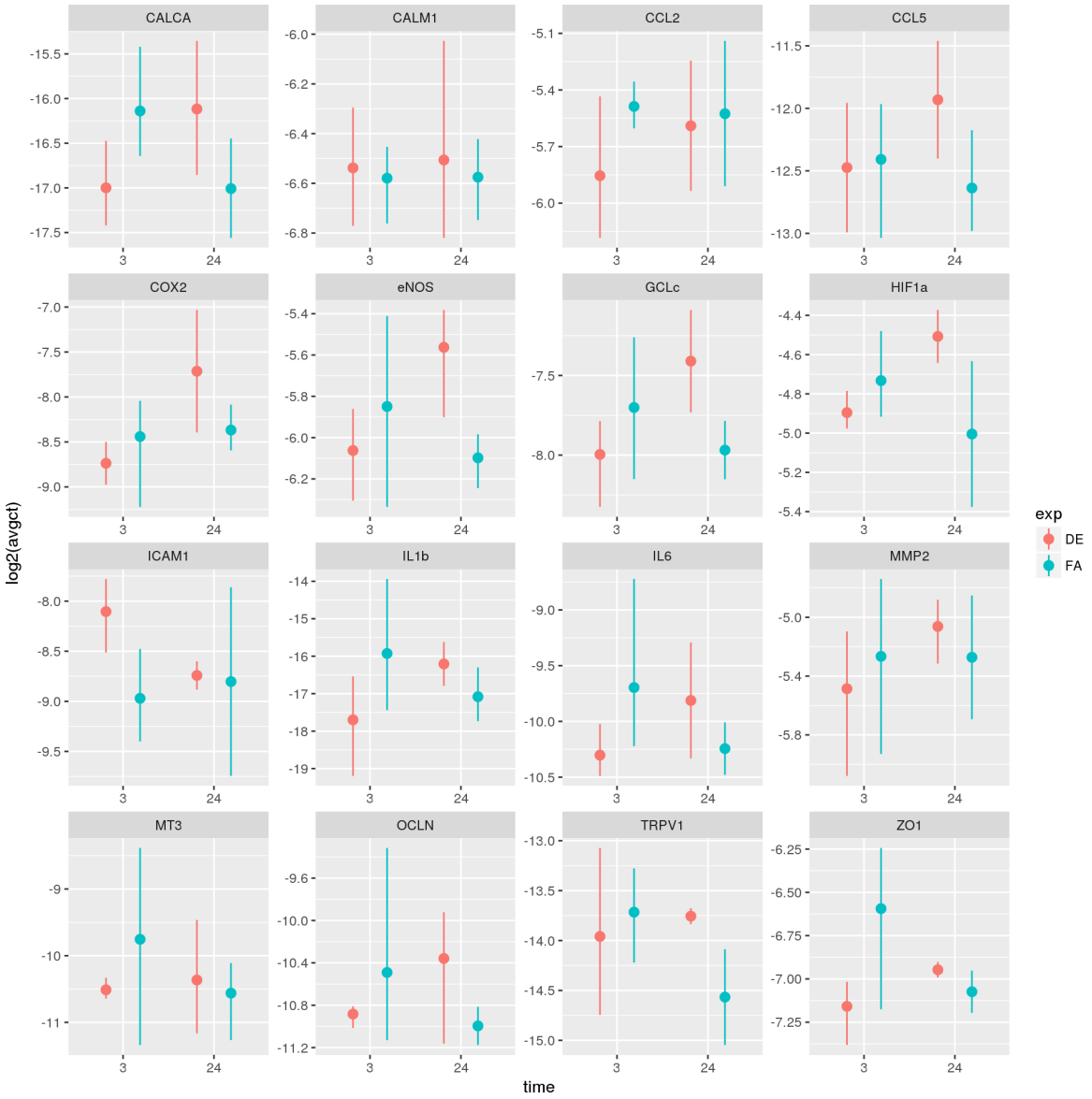


**Figure 3.3. Mean expression change in HCAECs treated with conditioned media from NHBE cells exposed to DE and FA.** Time represents incubation time post exposure in NHBE cells.

**Table 3.8. Test for interaction between exposure and terazosin treatment in HCAEC exposed to serum collected from human subjects.** No significant changes were detected.

| Gene | Fold Chg (log) | Avg Expression | Adj. p-value |
|---|---|---|---|
| *MT3* | 1.18 | -11.38 | 0.34 |
| *CALM1* | 0.34 | -6.54 | 0.56 |
| *IL1b* | 0.34 | -6.54 | 0.56 |
| *OCLN* | 0.42 | -38.49 | 0.80 |
| *eNOS* | 0.29 | -33.34 | 0.80 |
| *COX2* | 0.31 | -21.08 | 0.80 |
| *GCLc* | 0.23 | -35.53 | 0.80 |
| *MMP2* | 0.20 | -19.07 | 0.80 |
| *IL6* | -0.18 | -37.48 | 0.80 |
| *CCL5* | -0.18 | -37.48 | 0.80 |
| *ZO1* | 0.18 | -34.58 | 0.80 |
| *ICAM1* | 0.17 | -34.74 | 0.80 |
| *TRPV1* | -0.24 | -55.23 | 0.80 |
| *CALCA* | -0.07 | -70.39 | 0.88 |
| *HIF1a* | 0.08 | -18.92 | 0.88 |
| *CCL2* | -0.00 | -5.57 | 1.00 |

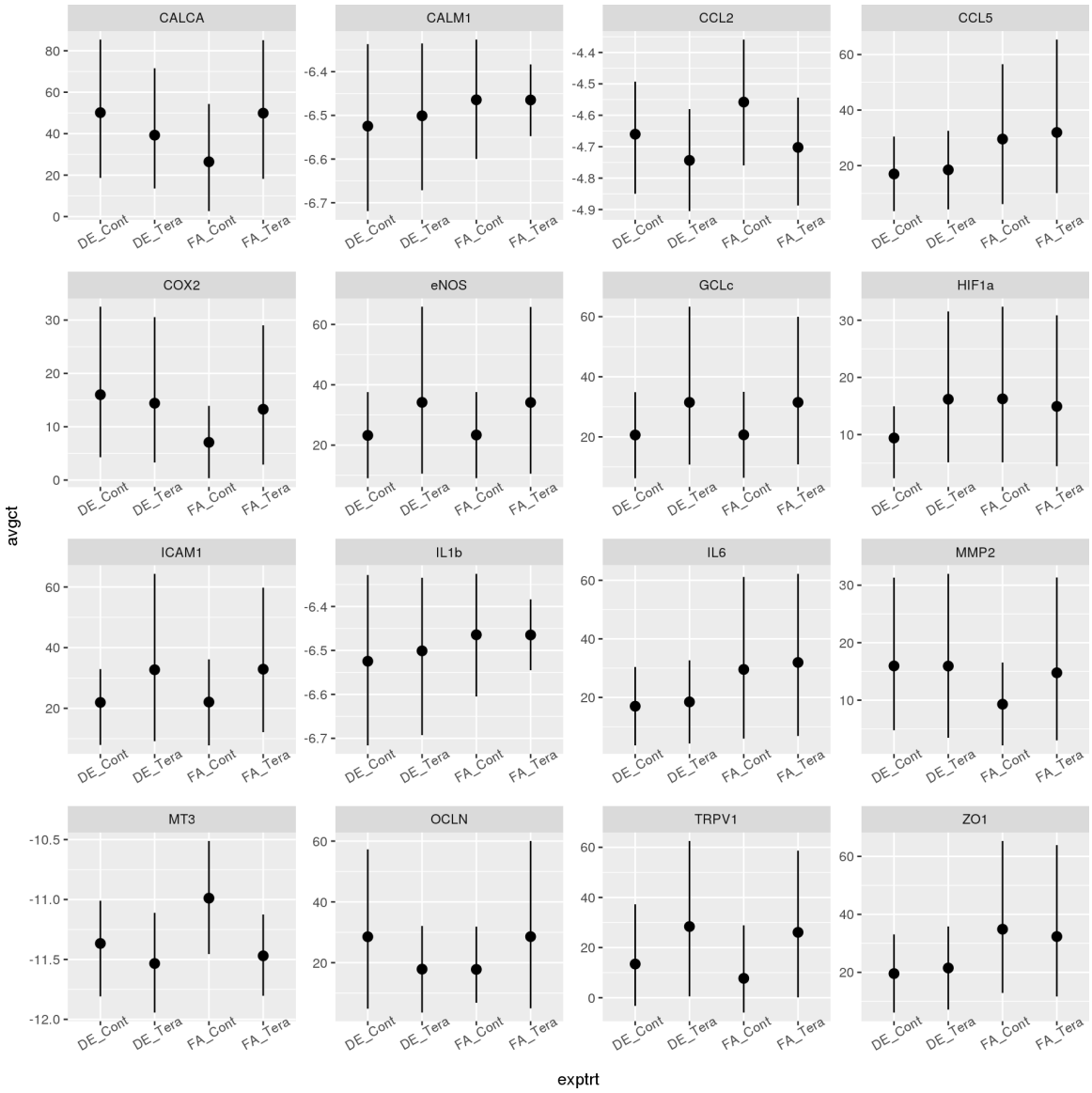


**Figure 3.4. Mean expression in serum exposed HCAEC for exposure (DE/FA) and treatment [placebo (control)/terazosin] controlling for subject specific differences.**

**Table 3.9. Test for interaction between exposure (DE/FA) and treatment (terazosin/placebo) in plasma collected from human subjects**. Likelihood ratio tests (LRT) and p-values for the interaction terms for each gene. Compares the full model (interaction terms) against a reduced model (no interactions terms); there is no evidence of effect on expression by exposure or treatment.

| Gene | LRT | p-value |
|---|---|---|
| *CALM1* | 5.21 | 0.816 |
| *CCL2* | 11.21 | 0.262 |
| *CCL5* | 4.33 | 0.888 |
| *CDH1* | 8.49 | 0.485 |
| *COX2* | 8.70 | 0.465 |
| *eNOS* | 4.93 | 0.840 |
| *GCLc* | 8.83 | 0.453 |
| *HIF1a* | 8.97 | 0.440 |
| *ICAM1* | 5.70 | 0.769 |
| *IL1b* | 9.13 | 0.425 |
| *IL6* | 12.83 | 0.171 |
| *MMP2* | 8.75 | 0.461 |
| *MT3* | 6.74 | 0.665 |
| *OCLN* | 13.27 | 0.151 |
| *SLPI* | 10.47 | 0.314 |
| *TRPV1* | 6.31 | 0.709 |

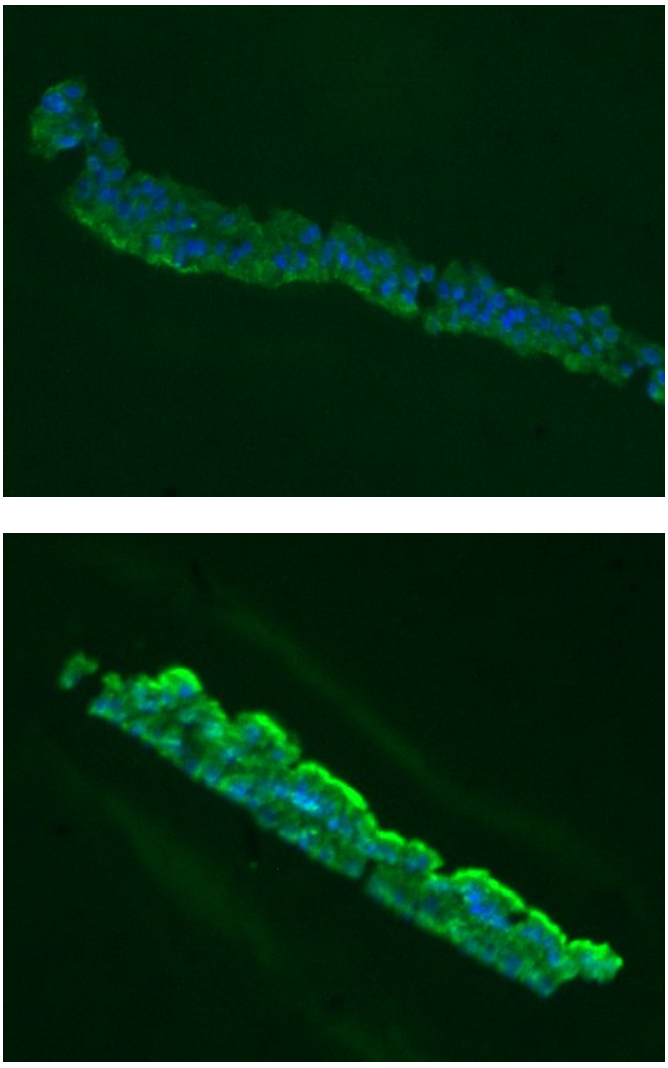

**Figure 3.5. ZO-1 in NHBE cells following exposure to DE (bottom) compared to FA (top).**

# **Chapter 4.** Acute exposure to traffic-related air pollution alters antioxidant status in healthy adults

## INTRODUCTION

Exposure to urban air pollution is associated with a range of adverse health effects, most notably increased cardiovascular disease and mortality.[7] Air quality is of particular concern in densely populated urban centers, where commercial transport and traffic congestion can drive pollutant levels well above national standards. Vehicle exhaust emissions predominately generate fine particulate matter ($PM_{2.5}$), a complex composite of organic and inorganic particles, metals, acids and other reactive species small enough to be inhaled into the deepest part of the lung. From there, respirable particles may initiate a number of pathophysiological pathways, including sensory receptor activation of the autonomic nervous system and induction of a pulmonary and systemic inflammatory response. [7, 144] Oxidative stress, an established risk factor for a number of inflammatory, cardiovascular and age-related conditions, plays an integral role in each of these pathways.[237]

Evidence suggests that inhaled pollutants precipitate these effects via multiple pathways involving oxidative stress. Previous controlled exposure studies have demonstrated strong associations between inhaled pollutants and markers of pulmonary oxidative stress,[201, 202, 238, 239] yet few have directly examined circulating markers of an oxidative response. Postulating that oxidative stress is a mechanism underlying the pathophysiological effects of $PM_{2.5}$, we investigated the effect of an acute exposure to diesel exhaust (DE), the dominant source of urban $PM_{2.5}$, on the measurement of reduced to oxidized glutathione (GSH/GSSG and redox potential, $E_h$), in healthy adults. We also investigated whether pre-treatment with an

antioxidant cocktail modified the effect of inhaled DE on antioxidant capacity, and examined exposure-related changes in antioxidant/stress response leukocyte gene expression (*GCLc, HMOX-1, IL-6, TGFβ*) and plasma IL-6 levels.

## MATERIALS AND METHODS

Nineteen healthy adults participated in four exposure sessions in a randomized, crossover design. Qualification for enrollment included body mass index <30 kg/m$^2$, and no evidence or history of hypertension (blood pressure <130/85 mmHg), asthma, diabetes, hypercholesterolemia, cardiovascular illness, or other chronic medical condition, based on spirometry, fasting glucose, lipid panel, electrocardiogram and questionnaire. Age, sex, race/ethnicity, smoking, and medication use were self-reported. In addition, subjects were required to be nonsmokers and not regularly exposed to second-hand smoke. Non-smoking status was confirmed by urinary cotinine (CAS-COT kit, Innovacon, Inc.; San Diego, CA) in samples collected prior to exposure. One subject was excluded from the analysis based on a positive cotinine screening (>200 ng/mL). Women were scheduled for exposures only during the first two weeks of a menstrual cycle, and pregnancy was ruled out prior to each exposure with a urine pregnancy test.

To test the hypothesis that antioxidant prophylaxis may attenuate the oxidative effects of DE, subjects were given oral N-acetylcysteine (NAC), a cysteine precursor in GSH synthesis, and ascorbate (vitamin C) prior to exposure in a crossover design. The treatment consisted of 500 mg ascorbate or matched placebo every 12 hours for seven days prior to exposure, and 600 mg NAC or matched placebo every 12 hours one day prior to exposure. Subjects were administered an additional 1000 mg ascorbate and 600 mg NAC (or placebo) upon arrival at each session, approximately one hour before exposure commencement. The

antioxidant and matched placebo were prepared by the Investigational Drug Service at the University of Washington Medical Center. Subjects were instructed not to consume supplemental vitamins or vitamin C-fortified food and drink the week prior to exposure. Plasma ascorbate, from blood drawn prior to each exposure session, was used to confirm compliance with antioxidant treatment.

**Exposure System**

The complete exposure system has been previously described in detail.[147] In brief, DE was generated using a 2002 model turbocharged direct-injection 5.9-L Cummins B-series engine in a 100 kW generator set, running at steady state prior to the exposure (6BT5.9G6; Cummins, Inc., Columbus, IN). Load was maintained at 75% of rated capacity, using a load-adjusting load bank (Simplex, Springfield, IL), no. 2 undyed on-highway low sulfur diesel fuel and Valvoline 15W-40 crankcase oil (Lexington, KY). Carbon matrix and HEPA filtered (99.99% efficient) ambient air was used for filtered air (FA) sessions and for DE dilution. Emissions were diluted in two phases to achieve a 200 $\mu g/m^3$ $PM_{2.5}$ concentration in the 116 $m^3$ exposure room breathing zone (average 205.4 $\mu g/m^3$; SD 5.4 $\mu g/m^3$). $PM_{2.5}$ concentrations were assessed in real-time throughout each exposure using a tapered element oscillating microbalance (1400a $PM_{2.5}$, Rupprecht & Patashnick Co., Albany, NY) and were continuously adjusted, based on nephelometry measurements, with a feedback control system. The exposure room was maintained at a temperature of 20 to 21°C with 50% relative humidity. Based on multistage impactor-collected samples, the typical particle count was 2.8 x $10^3$ for FA and 5.3 x $10^5$ per $cm^3$ for DE exposures, based on one-minute averages; DE particle mass median diameter was 0.080 μm. Nephelometers positioned within the exposure

room were used to confirm spatial uniformity of particle concentrations. Average nitrogen dioxide concentrations during DE exposures were 35 ppb (approximately 1.5% of total $NO_x$). Concentrations of carbon monoxide averaged 0.30 ppm for FA and 0.80 ppm for DE.

**Exposure Sessions**

Each subject was exposed to DE and FA, on separate days, both with and without antioxidant pretreatment, in a placebo-controlled four-way crossover design, randomized to order of the four conditions. To reduce carry-over effect, a minimum 2-week washout period separated each session. Researchers followed set and scheduled protocols to limit any heterogeneity between sessions. Subjects were instructed to fast for 10 hours prior to exposure. Upon arrival at the University of Washington's Clinical Research Center (CRC), vitals were recorded, an intravenous catheter was placed in the subject's left arm and pre-exposure (baseline) blood was collected. Exposures began at approximately 9:00 am. Blood was collected 90 minutes into the exposure (during exposure), and again five hours post exposure. Following exposure, the subject received an identical defined composition meal (to limit any short-term dietary effects) and rested at the CRC for at least 6 hours until release. Subjects returned to the CRC the next morning, approximately 24 hours after baseline measurements were taken, for a final assessment and blood draw.

All subjects, researchers and technicians (other than the exposure engineer) were blinded to the exposure condition. Subject blinding was evaluated by asking the subject to estimate the level of DE (as high, medium, or none) during each exposure. The Human Subjects Division of the University of Washington approved subject consent forms and all study protocols.

**Ascorbate assay**

Blood was collected pre-exposure in a BD Vacutainer tube containing sodium heparin and centrifuged to separate plasma. 50 µl of meta phosphoric acid/dithioreitol (MPA/DTT) was added to 500 µl plasma. In this assay, ascorbic acid is oxidized to dehydroascorbic acid by ascorbic acid oxidase and dehydroascorbate is converted to the quinoxolone derivative by a fast reaction with 0-phenylenediamine at pH 6.5. Absorbance (340 nm) of the product is directly proportional to the ascorbic acid concentration. The assay was performed using an Olympus AU400 chemistry immuno analyzer (Center Valley, PA). Reagents were purchased from Sigma-Aldrich (St. Louis, MO).

**Glutathione assay**

Blood was collected pre-exposure and 5 hours-post exposure commencement into a BD Vacutainer tube containing sodium heparin. Concentrations of total glutathione ($GSH_T$) and oxidized (GSSG) glutathione in whole blood were determined via enzymatic reaction with Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid [DTNB]) and spectrophotometric quantification at 412 nm using a Microplate Assay for GSH/GSSG (Oxford Biomedical Research; Oxford, MI). To prevent oxidation of GSH, samples for GSSG quantification were treated with a thiol-scavenging reagent prior to freezing at -80˚C. Concentrations were determined based on an eight-point standard curve for $GSH_T$ and GSSG. GSH/GSSG was calculated as $GSH_T - 2GSSG / GSSG$. The redox state values (Eh) were calculated using the Nernst equation: $E_h = E_o + RT/nF \ln [GSSG]/[GSH]^2$, where $E_o$ is the standard potential for the redox couple, R is the gas constant, T is the absolute temperature, n is the number of

electrons transferred (2), and F is Faraday's constant. The standard potential $E_o$ for the GSH/GSSG couple used was −264 mV for pH 7.4 (Jones 2002).

**Genotyping**

Effect modification by genotype for glutathione S-transferase mu 1 (*GSTM1*) was also included in our analysis, as the null polymorphism has been associated with decreased detoxification capacity and increased susceptibility to the effects of air pollution in other research.[240] Blood was collected at screening in a BD Vacutainer tube containing the preservative sodium citrate. DNA was isolated from blood using Qiagen's DNeasy kit (Valencia, CA) and genotyped for *GSTM1* (deletion/no deletion) using a multiplex PCR, with ß-globin as a positive control.[241] Specific probes were 3'-labeled with TAMRA quencher dye; wild type and variant probes were 5'-labeled with 6-FAM and VIC reporter dye, respectively. Sequencing reactions were performed and analyzed on a 7900 Fast Real-Time PCR System (Applied Biosystems).

**Gene expression**

Blood was collected pre, during, and post (5 and 22 hours) exposure in a BD Vacutainer CPT glass molecular diagnostics tube containing density gradient polymer gel and sodium citrate. Cells were pelleted by centrifugation and resuspended in TRIzol for RNA extraction. RNA was isolated from peripheral blood mononuclear cells (PMBCs) using an RNeasy kit (Qiagen); complementary DNA was synthesized with Oligo-dT primers and reverse transcriptase (Qiagen), using the same total RNA concentration for each sample. Expression levels were measured by quantitative real-time PCR (RT-qPCR) using Taqman primers and

the ABI 7900 Sequence Detection System (Applied Biosystems). Samples were run in duplicate or triplicate. The threshold cycle C(t) was calculated using a standard curve; expression was normalized to the reference gene β-actin.

**Plasma IL-6**

Plasma IL-6 was assayed using the Quantikine High Sensitivity Human IL-6 enzyme linked immunosorbent assay (ELISA) kit (R&D Systems; Minneapolis, MN). Briefly, 100 μL of standard and sample were incubated on a 96-well microplate for two hours, followed by washing and two hours incubation with IL-6 conjugate. Substrate solution was added to the samples and incubated for one hour, followed by 30 minutes incubation of amplifier solution. Plates were read at 490 nm with the SpectraMax Plus (Molecular Devices; Sunnyville, CA) immediately after addition of stop buffer. Plasma IL-6 concentrations were log transformed for statistical analysis.

**Statistical Analysis**

Data were analyzed using a multivariate mixed model to assess the effect of DE exposure, antioxidant supplementation and an exposure x antioxidant interaction on outcome measures, by subject. Outcome values were baseline corrected by subtracting the same session's pre-exposure measurement; the baseline corrected change for DE exposure was then compared with the analogous value in the FA exposure, to give a subject-specific measure of effect. Antioxidant vs. placebo values were evaluated similarly. The model was fit via linear mixed effects to estimate the mean effect, accounting for random variations in session-specific baselines by individual.

Effect modification was examined by *GSTm1* genotype and pre-exposure GSH/GSSG. Descriptive analyses, paired *t*-tests and linear regression were used in the initial analyses. For expression analysis, we adjusted using the Benjamini and Hochberg method[242] for False Discovery Rate to account for multiple comparisons. Statistical significance was considered at $\alpha = 0.05$. Results are reported as subject-specific change, or mean ± SE, unless otherwise noted. All statistical analyses were completed using Stata/IC v 12.1 (StataCorp, College Station, TX).

## RESULTS

Subject characteristics are summarized in Table 4. Frequency of the *GSTM1* null genotype (47%) in our study was similar to population distributions described in the literature, which range from 23-62% among studies conducted in the U.S.[243, 244] Pre-exposure plasma ascorbate levels were significantly increased with antioxidant supplementation (0.9 ± .05 vs 1.4 ± .08 respectively, $p< 0.000$), confirming subject compliance with treatment (Figure 4.5). Mean pre-exposure GSH/GSSG ratios were similar to those reported in other studies of healthy adults and did not differ by sex. Pre-exposure GSH/GSSG was, on average, increased by antioxidant treatment, however the effect was not significant (143; 95% CI: -56, 343; p=0.16). Redox potential did not differ by treatment at baseline or following exposure to FA.

The mean effect of DE exposure (measured as change from baseline) on blood GSH/GSSG is shown in Figure 4.1. Compared with FA, GSH/GSSG was significantly decreased with exposure to DE (-317; 95% CI: -527, -106; p=0.003). This effect was not mediated by antioxidant supplementation; (40; 95% CI: -170, 252; p=0.71) and when examined separately, the DE-related change in GSH/GSSG was similar in both placebo and

antioxidant groups (-337 and -296, respectively). *GSTM1* genotype did not modify the effect of DE, with or without antioxidant pre-treatment. When examining the change using the redox potential (Figure 4.2), again, DE exposure elicits an increase in values, demonstrating a more oxidized state following inhalation (5.5; 95% CI: -.36, 11.4; p=0.06), but no difference in effect between treatment groups (-0.2; 95% CI: -6.1, 5.7; p=0.95). Though not quite statistically significant, the effect modeled as redox potential clearly mirrors data shown as GSH/GSSG.

In addition, we examined DE-related changes in the expression of select antioxidant, inflammatory, and stress response genes (*GCLc, IL-6, TGFβ, HMOX-1*) and plasma IL-6 concentrations. Compared to FA exposures, mRNA expression of *IL-6* was significantly increased over four fold (0.67, p=0.01, Figure 2) five hours post DE exposure start in placebo exposures. In contrast to our findings for GSH/GSSG, there is some suggestion that antioxidant pretreatment ameliorated the DE effect as there was no significant change in expression following DE exposures with antioxidant (0.16, p=0.58), however the interaction was not significant. There were no significant differences in *IL-6* expression detected at other time points measured in treated or placebo groups. While changes in plasma IL-6 protein levels mirrored the change in mRNA expression in the placebo group, the effect detected did not reach statistical significance. DE exposure was not associated with expression changes in *GCLc, HMOX-1, or TGFβ* at any time points examined.

As with prior experiments in our lab[245], subject blinding to exposure type was effective; only three subjects identified the correct exposure more than twice.

## DISCUSSION

In healthy adults, an acute exposure to DE significantly decreased the ratio of reduced to oxidized glutathione in whole blood, and increased circulating PBMC gene expression of the pro-inflammatory cytokine *IL-6*, in the period following exposure. Antioxidant pretreatment did not attenuate the DE-effect on GSH/GSSG, however it notably reduced the exposure-related increase in *IL-6* expression. These findings support the hypothesis that oxidative stress is one mechanism underlying the pathophysiological effects of exposure to traffic-related air pollution, and suggest that acute exposures may reduce the capacity of an essential cellular defense system.

Oxidative stress occurs when reactive oxidants, most commonly oxygen and nitrogen species (herein referred to as ROS), exceed the antioxidant capacity of the cell.[246] $PM_{2.5}$ may induce oxidative stress directly, via reactive electron donors, or indirectly, as a secondary effect of inflammatory signaling or target cell interactions (i.e. altered mitochondrial function).[125 205, 206] Under conditions of surplus, ROS can induce alterations in redox-sensitive functions and signaling, leading to the disruption of normal physiological responses.[247, 248] For example, an excess of superoxide can severely diminish levels of the vasodilator nitric oxide (NO), an important regulator of blood pressure, endothelial homeostasis, coagulation, and adhesion. In addition, ROS can induce the release of vasoactive (e.g. angiotensin II) and pro-inflammatory mediators, circulating factors known to promote cardiovascular risk-related mechanisms underlying hypertension, cardiac hypertrophy, and atherosclerosis.[130,249, 250]

Previous controlled exposure studies have found that inhaled pollutants induce pulmonary oxidative stress, [201, 202, 238, 251] however, few have examined markers of a systemic response. We previously reported differential regulation of stress response genes in healthy

subjects exposed to 200 μg/m$^3$ DE, but found no change in urinary markers of oxidative stress among subjects with metabolic syndrome exposed to the same conditions.[252] Multiple population and occupational cohort studies have reported associations linking inhaled PM with markers of protein, lipid and DNA oxidation,[134, 253-255] outcomes that also have been observed in controlled exposures[256] and, more consistently, in cellular and animal research.[112, 257-261] Animal studies on exposure to fine and ultrafine PM have specifically detected enhanced GSH oxidation, as well as protein and endothelial nitric oxide synthase (eNOS) S-glutathionylation, which inhibits vascular bioavailability NO.[262, 263] Controlled DE inhalation studies have also reported subclinical effects attributed to oxidative stress, including endothelial dysfunction, blood pressure elevation, and reduced NO bioavailability,[73, 90, 131] outcomes which have been independently correlated with GSH levels in both healthy adults and in smokers.[264]

The ratio of reduced to oxidized glutathione is an accepted biomarker of systemic antioxidant capacity and redox status in humans.[151] A shift in the GSH/GSSG balance has been associated with altered cell signaling pathways and increased susceptibility to both acute and chronic disease, including the development and progression of atherosclerosis.[153 265] Our results are in line with recent findings that women living in areas with higher residential air pollutants had low blood GSH levels;[266] however unlike chronic GSH depletion, which is associated with sustained oxidative stress, transient drops in the thiol are believed to reflect a strong antioxidant defence.[151, 267] The DE-effect we observed indicates that inhaled $PM_{2.5}$ directly or indirectly induces an oxidative response, precipitating the fall in GSH/GSSG. Importantly, this finding suggests that DE exposure may influence or exacerbate the effects of other endogenous or exogenous compounds detoxified by GSH.

The GSH/GSSG redox potential has also been used to demonstrate oxidative stress associated with disease. Our findings show that GSH/GSSG redox is increased over 5 mV following DE inhalation, confirming changes in levels of oxidation reflected in the ratios above. Research has reported that GSH/GSSG redox is oxidized by more than 30 mV in association with type 2 diabetes and 9 mV – in line with our estimates – in smokers compared to nonsmokers. [314, 315]

Though antioxidant treatment (or free-radical scavengers) have been shown to attenuate PM-related insults experimentally,[257, 268] findings in human studies have been far from conclusive. In a controlled exposure to concentrated ambient PM, Brook and colleagues found exposure-related increases in blood pressure were unchanged by pretreatment with 2000 mg vitamin C, in line with our results.[269] Other studies have shown dietary antioxidant intake to be inversely associated with systolic blood pressure[270] and with urinary biomarkers of oxidative stress[271] in persons exposed to higher residential air pollutants, but not at levels sufficient to ameliorate all effects attributable to exposure.

While higher baseline dietary antioxidant levels are strongly correlated with better health,[272-274] clinical studies have generally been unable to reproduce these effects using antioxidant supplementation.[275-278] Proposed reasons for this disparity have included insufficient dosing and/or method of delivery, non-specific targeting of the antioxidant, and differences in baseline antioxidant capacity. The latter proposes that supplementation is effective only for those with low or reduced baseline capacity, a theory noted specifically for NAC, and one that may explain our findings.[279, 280] NAC contributes to intracellular levels of GSH via free reduced cysteine following cleavage of the acetyl group; its antioxidant properties have therefore been attributed to increased GSH capacity. For this reason it has

been proposed that, barring GSH depletion, NAC has limited benefit.[279, 281] Baseline GSH/GSSG for subjects in our study was well within healthy levels, and likely sufficient to handle a short-term exposure to DE. Other clinical studies similarly have found ascorbic acid and NAC supplementation confer no benefit in the absence of disease, which may underscore the minor effect of treatment in our study population.[282] Indeed, in our laboratory, antioxidant supplementation did not prevent—and appeared to enhance—the vasoconstrictive effects of DE.[225] The effectiveness of supplementation in this study may also have been limited by the route of administration; intestinal N-deacetylation and first-pass metabolism diminish the bioavailability of oral NAC.[283] Pre-exposure GSH/GSSG following antioxidant supplementation did not differ significantly from those in the placebo arm.

It is likely that the effectiveness of supplementation depends both on the baseline capacity of endogenous defenses and the amount or duration of the oxidative insult. Subjects with existing health conditions, reduced antioxidant levels, or otherwise compromised detoxification capacity may have a more pronounced response to both exposure and to supplementation.

We did detect a DE-related increase in leukocyte expression of the proinflammatory cytokine *IL-6*. IL-6 plays a fundamental role in immune regulation, hematopoiesis, and the acute phase response,[284] and chronically elevated levels have been identified as a marker of increased cardiovascular risk.[285] Furthermore, experimental research has demonstrated that PM-induced IL-6 release promotes a prothrombotic state, in conjunction with ROS and adenylyl cyclase.[286] Interestingly, our data suggest antioxidant supplementation may modify the DE-induced increase in expression of *IL-6*, as stratification by treatment shows mean exposure-related changes were over four fold lower with antioxidant pre-treatment than with

placebo. While we detected a similar pattern in plasma IL-6 levels in the placebo group, the effect was not statistically significant. This may have been due to our sample collection time, approximately 16 hours later. Alternatively, changes in gene expression induced by DE may reflect a response, which, in healthy adults, is not sufficient to induce large alterations in protein production. Positive associations with short-term $PM_{2.5}$ levels have been most commonly reported in studies of vulnerable subgroups, such as the elderly and those with comorbid conditions.[287-290]

We also did not detect differences in the response to DE exposure by *GSTM1* genotype. *GSTM1* deletion has been associated with increased susceptibility to the effects of long-term air pollution exposure: studies have reported greater risk of lung cancer,[291] reduced lung function,[292 293] insulin resistance,[294] inflammation,[37] and alterations in heart rate variability.[295] However, with the exception of ozone,[296] short-term and controlled exposure studies have more commonly reported outcomes to be independent of *GSTM1* genotype, in line with our results.[297-299] Interestingly, Bhattacharjee and colleagues recently found *GSTM2* overexpression compensated for *GSTM1* deletion, noting similar levels of plasma GST enzymatic activity in both positive and null individuals.[300] We too detected no difference in baseline GSH/GSSG by *GSTM1* genotype, with or without antioxidant supplementation. This compensatory mechanism may explain the discrepancy between controlled inhalation and epidemiological studies: a healthy adult may be able to adequately manage an intermittent acute exposure, irrespective of this deletion. Alternatively, it is possible that we would find a different result with a larger sample size, or that the increased risk detected in other studies involved additional gene-gene or gene-environment interactions not present in our study population, or not examined in this analysis.[301-303] While our findings suggest that inhaled

pollutants reduce antioxidant capacity, it is likely that exposure provokes other pathophysiologic mechanisms in addition to or associated with oxidative stress.

Though our experimental model is designed to replicate exposure to traffic-related air pollution, the exposures do not reflect the complexity and variability of pollutants in urban air. Our facility does, however, produce consistent, replicable exposures to the most common traffic-related air pollutant, at a standardized composition and concentration. Concentrations used in our controlled exposures are above those commonly experienced in the U.S., however, average $PM_{2.5}$ levels in many major cities worldwide regularly exceed such concentrations.[304] It is also important to note that our findings reflect only an acute response, and not the effects of long-term, repetitive exposures. Furthermore, the decrease in GSH/GSSG we report represents the mean response to DE inhalation in young, healthy adults; individual responses within our subject pool varied considerably, and our findings are likely to significantly underrepresent the risk of exposure in susceptible populations.

This controlled inhalation study demonstrates that an acute exposure to DE is associated with a reduction in the GSH/GSSG ratio consistent with shift in balance toward an oxidized state, along with an increase in *IL-6* expression in healthy adults. These findings may help clarify how exposure to traffic-related air pollution can promote or exacerbate the development of disease.

**Table 4. Characteristics of study participants**
All values reported are mean (± SE) unless otherwise noted.

| Characteristic | |
|---|---|
| No. | 19 |
| Age, yrs | 29 ± 9 |
| Male, n (%) | 14 (74) |
| Caucasian, n (%) | 16 (84) |
| Body mass index | 23 ± 2.2 |
| SBP, mmHg | 115 ± 13 |
| DBP, mmHg | 70 ± 9.7 |
| Heart Rate, bpm | 65 ± 9.0 |
| Total cholesterol, mg/dL | 163 ± 30 |
| LDL | 101 ± 20 |
| HDL | 46 ± 11 |
| Triglycerides | 79 ± 46 |
| Glucose, mg/dL | 90 ± 4.6 |
| Ascorbate, mg/dL | .90 ± .05 |
| *GSTM1* + | 10 (53%) |

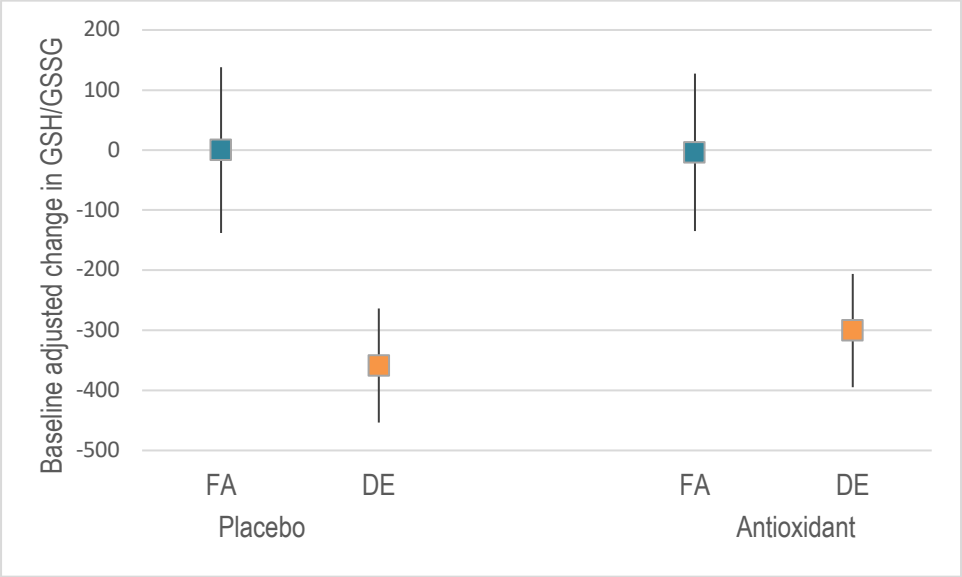


**Figure 4.1. Mean Change in GSH/GSSG from Baseline by Exposure and Antioxidant Treatment.** Baseline adjusted difference in GSH/GSSG following exposure to diesel exhaust (DE) and filtered air (FA). Mean subject specific effect relative to FA control, shown for exposures with placebo and with antioxidant pretreatment. Error bars represent SE. * p=0.003 compared with FA control.

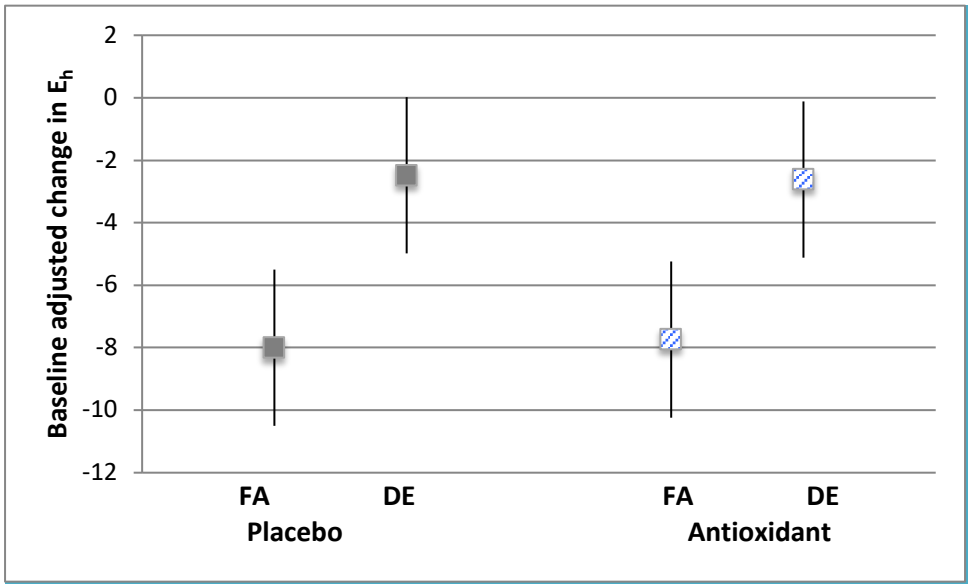


**Figure 4.2. Mean Change in Redox Potential from Baseline by Exposure and Antioxidant Treatment.** Baseline adjusted difference in $E_h$ following exposure to diesel exhaust (DE) and filtered air (FA). Error bars represent SE.

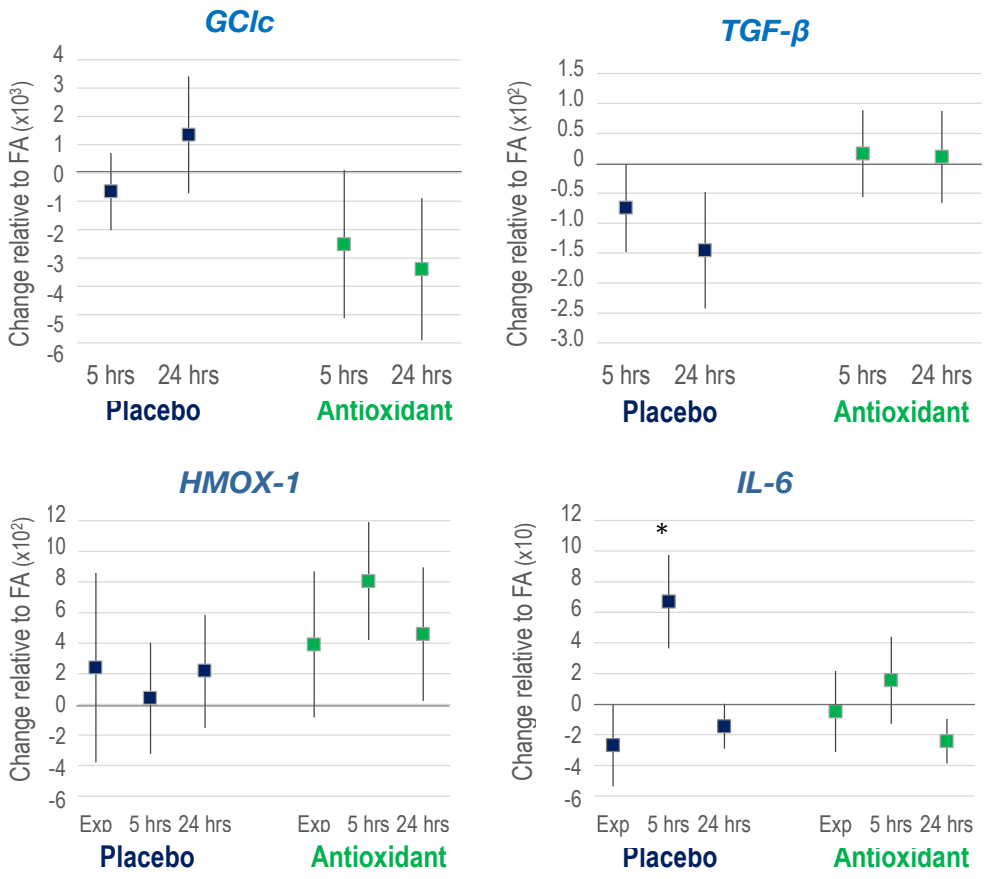


**Figure 4.3. Mean Diesel Exhaust Effect on Changes in Gene Expression.**
Mean difference between change (from pre-exposure) in normalized leukocyte mRNA expression with DE compared with FA control for exposures with antioxidant (hatched) pre-treatment and without (placebo, solid) for each time point measured (exp=during exposure; 5 = 5 hrs post; 24 = 24 hours post exposure start). Error bars represent SE. * p=0.02 compared with FA control.

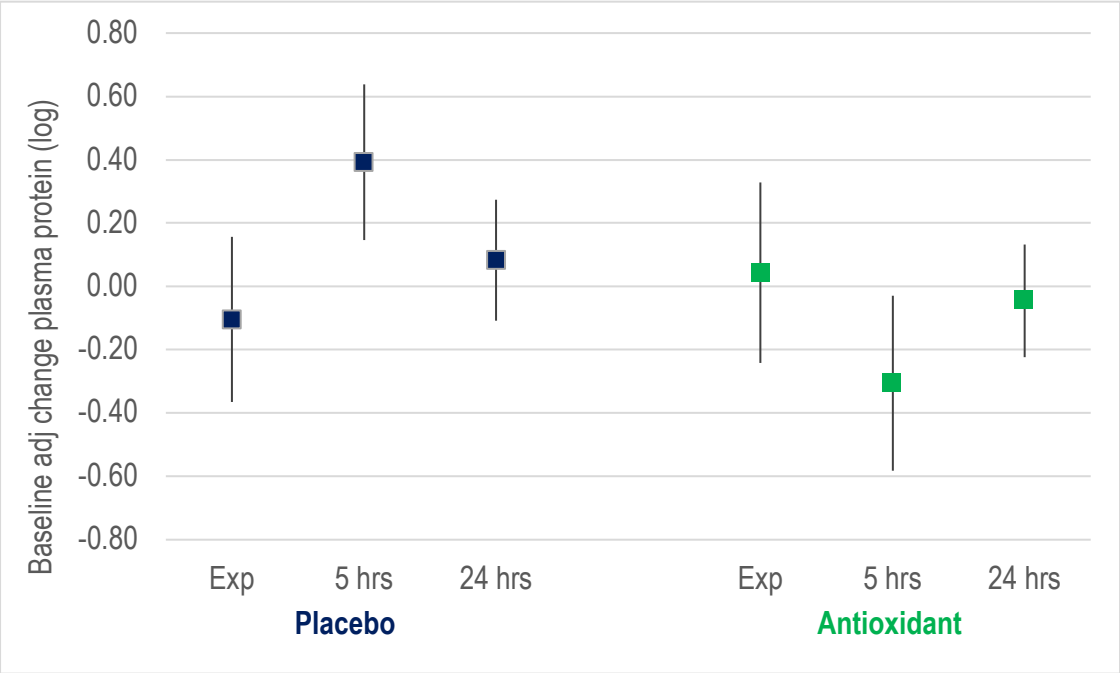


**Figure 4.4. Mean Diesel Exhaust Effect on Changes in IL-6 protein levels.**
Mean difference between change (from pre-exposure) in plasma IL-6 protein with DE exposure compared with FA control, with placebo and antioxidant pre-treatment for each time point measured (exp=during exposure; 5 = 5 hrs post; 24 = 24 hours post exposure start). Error bars represent SE.

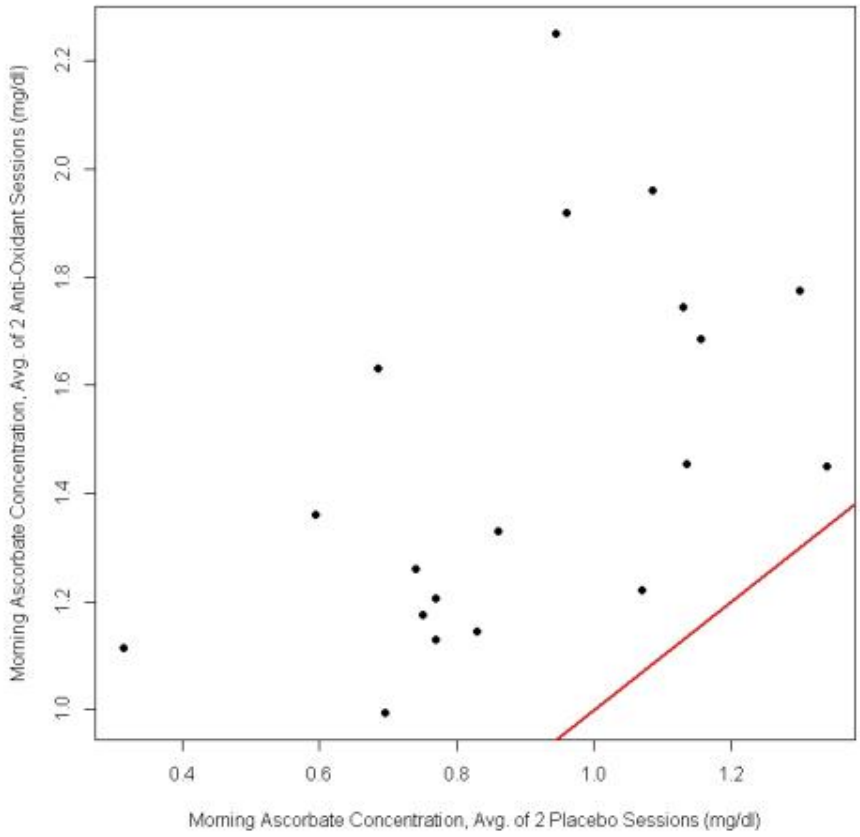


**Figure 4.5. Subject compliance with antioxidant treatment.** Compared to placebo, pre-exposure plasma ascorbate levels were significantly greater with AO treatment ($p < 0.000$) compared with placebo confirming subject compliance.

# **Chapter 5.** Significance and Conclusions

## Summary of results

Inhaled environmental pollutants are a major contributor to human health problems. In addition to respiratory diseases such as asthma and COPD, airborne toxicants contribute to range of non-pulmonary pathologies, including cardiovascular, developmental and neurological conditions. My long term objective is to contribute to the elucidation of mechanisms by which inhaled pollutants act on the cardiovascular system, and potentially identify new markers of susceptibility. The complementary studies in this dissertation provide evidence for a number of new and notable findings on the health effects of inhaled DE.

First, we present evidence for the role of the alpha adrenergic system in the SBP response to traffic-related air pollution. The bimodal pattern of response detected indicates that effects are both immediate and delayed; the prevention of secondary effects by blockade of the initial response suggests that these effects are sequential. Based on findings in Aims 2 and 3, it could be hypothesized that augmentation of the ANS response to exposure prevented the activation of pathways involved in oxidative stress and inflammation, which in turn contribute to the subsequent rise in SBP at 24 hours. Furthermore, we report variation in the SBP response by *TRPV1* genotype, which suggests that this nociceptor is involved in the hemodynamic response to inhaled DE; in our subject population, the DE-induced increase in SBP occurred only among persons carrying the *TRPV1* Ile585Val C allele, a potentially notable finding in terms of population-based susceptibility. Future work should be done to further explore this potential susceptibility; the association we report does not necessarily imply that this variant has a pathogenic (or the common variant protective) effect, but merely

that it is a place to start to begin additional work. Cardiovascular disease, in general, is a complex pathogenesis which involves both environmental and genetic influences.

We then investigated primary and secondary transcriptional responses to the same controlled exposure *in vitro* and *ex vivo*. Using a novel electrostatic deposition chamber and physiological conditions of the lung, we detected significant changes in the expression of inflammatory, oxidative stress and barrier-related gene expression in primary human lung cells, 24 hours after a one hour exposure to DE. Perhaps more importantly, our data provide evidence that effects elicited in the airway epithelium can induce secondary effects in primary coronary artery endothelial cells, suggesting that the epithelium may play an initial role in inflammatory processes and barrier dysfunction. In contrast to these findings, we did not detect any significant changes in blood sampled from human subjects exposed in the first study; this finding may suggest a robust *in vivo* response or indicate that gene expression within circulating PBMCs is not a strong biomarker for the early effects of exposure. Alternatively, current studies underway by Dr. Michael Rosenfeld and colleagues indicate that the inflammatory effects induced by circulating mediators may only be evident following adhesion. We do not know what mediators in the conditioned media were responsible for the effects seen in HCAECs. Answering this question is an exciting avenue for future work that could involve both proteomic and metabolomic studies. In addition, more extensive studies using antagonists (e.g. of TRPV1, NfKB) could help elucidate the driving mechanisms or sequence of transcriptional activation. Nanoparticle interaction in protein assays challenge our ability to examine the translational response in airway cells, but additional work using IHC could advance our understanding beyond gene expression. While these studies are limited by the nature of *in vitro* work –the lack of a full physiological response system – the

use of primary human cells and improved exposure conditions increases the likelihood that our findings represent real effects.

Lastly, we report that in healthy adults, an acute exposure to DE significantly decreased the ratio of GSH/GSSG, and increased circulating leukocyte gene expression of the pro-inflammatory cytokine *IL-6*. Antioxidant pretreatment did not attenuate the DE-effect on GSH/GSSG, however it notably reduced the exposure-related increase in *IL-6* expression. In line with findings from Aims 2 and 3, these data support the hypothesis that oxidative stress is one mechanism underlying the pathophysiological effects of exposure to traffic-related air pollution, and suggest that acute exposures may reduce the capacity of an essential cellular defense system. Additional testing of a second marker of the oxidative response is planned, and will strengthen the data reported here if findings are similar. Future work on how to mitigate this response, and to better understand the response of persons with already depleted antioxidant levels could be extremely valuable at the population level.

The studies in this dissertation integrate basic science with clinical measures to examine aspects of the cardiovascular response in humans exposed to traffic-related air pollution. Our findings substantiate evidence reported in epidemiology research and provide support for new *in vitro* models in inhalation toxicology. Our findings only represent the effect of acute exposures and therefore may not be translatable to long-term effects, however, it is likely that similar mechanisms are involved in both types of exposure. This evidence, from highly controlled human exposure studies, may be important in conceptualizing how exposure to traffic-related air pollution can induce pathophysiological changes underlying clinical cardiovascular disease and outcomes. Our results may also support future regulation

to limit ubiquitous exposure and inform additional research on susceptibility and preventative treatments.

**Benefits of improved *in vitro* modeling**

One aim of this research was the use of an improved *in vitro* model of inhalation toxicity. By employing a realistic exposure scenario and an accurate estimate of the exposure dose, this system increases our ability to identify effects that are currently difficult to detect (early in the response pathway) or obscured by variability in human and animal studies. Researchers have also noted the need to establish models that are capable of cost-effective, high-throughput testing; this is highly relevant considering the prevalence and complexity of cardiovascular disease and number of potential gene-environment interactions. Our model addresses this need for one of the most challenging routes of exposure. The data presented would have been strengthened by additional exposures at different doses or for different exposures periods (demonstrating, perhaps, a dose-response curve). Repeated exposures would also be informative. Though this model does not reflect chronic exposures or replicate all traffic-related pollutant mixtures, the effects detected may be important in conceptualizing how inhaled pollutants can affect the development of cardiovascular disease. It also provides a framework for better understanding how *in vitro* responses are related to outcomes detected *in vivo*.

New *in vitro* techniques that can link a response to inhaled pollutants in human cells with experimental, clinical or population-based findings can provide significant opportunities for future research. Methods we employed can be used with different cell types, genotypes or disease states to investigate any number of endpoints, exposures, or signaling pathways,

providing a broad range of opportunities. Furthermore, *in vitro* results can inform and improve the design of animal and human exposure studies, and ideally reduce the number needed to improve health outcomes.

**How small changes in air quality can have large impacts**

Although the absolute increased cardiovascular risk of daily exposure to higher levels of air pollutants can be considered low (a few percent increase per 10μg/$m^3$), the unavoidable and ubiquitous nature of exposure contributes to a large risk at the population level. When the magnitude of risk and prevalence of exposure are accounted for, exposure to traffic-related pollution ranks above physical exertion, coffee, and alcohol in a comparative risk assessment of triggers of MI.[61] In China, only diet, high blood pressure, and tobacco use rank above ambient air pollution as risk factors for healthy life-years lost.[305]

Studies designed to quantify the benefits of improved air quality at the population level have found the effects on health and productivity were notable. Adjusting for demographic and socioeconomic variables, and proxy indicators for the prevalence of cigarette smoking, one study found that for each 10 μg/$m^3$ decrease in $PM_{2.5}$ levels (between the late 1970s and early 2000s), mean life-expectancy in the US increased by 0.61 years.[306] Similarly, a follow-up analysis of a large-cohort study found that the risks of total and cardiovascular mortality were reduced by 27% and 31%, respectively, for a 10 μg/$m^3$ decrease in $PM_{2.5}$ levels.[13]

Although policy-based interventions for pollution control often take decades to implement, short-term pollution-control measures enacted during the 2008 Beijing Olympics enabled researchers to assess the health effects associated with rapidly changing levels of

exposure. Restrictions on traffic and industrial activity led to 13–60% reductions in the levels of air pollutants relative to pre-restriction levels, an effect which quickly reversed when restrictions were lifted. These rapid changes in exposure levels were associated with similar effects on biomarkers of inflammation, oxidative stress, and thrombosis in healthy adults.[124, 128, 307, 308]

The health benefits associated with improvements in air quality have been notable in the US since the enactment of the Clean Air Act, [311] and further improvements are projected even in areas meeting current standards. Data suggest that the curve describing the $PM_{2.5}$ dose–response relationship is steeper at lower concentrations of pollutants than at higher concentrations.[71, 309, 310] The EPA estimates that the Clean Air Act prevented over 160,000 deaths and 130,000 cases of MI in 2010, estimates that are projected to reach 230,000 and 200,000, respectively, in 2020.[312] In highly polluted countries, improvements in air quality could result in even greater health benefits in the near future.[305]

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