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# Mechanisms of augmented coronary artery constriction following exposure to diesel exhaust

Tom Cherng

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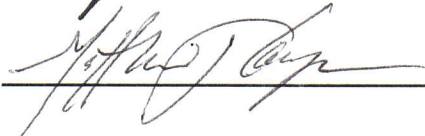
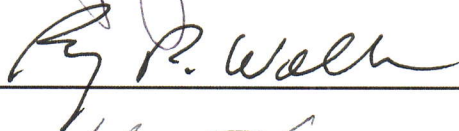
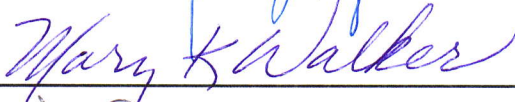
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*Approved by the Dissertation Committee:*



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MECHANISMS OF AUGMENTED CORONARY  
ARTERY CONSTRICTION FOLLOWING EXPOSURE  
TO DIESEL EXHAUST

**by**

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B.S. Biochemistry and Cell Biology, University of  
California, San Diego, 2003

DISSERTATION

Submitted in Partial Fulfillment of the  
Requirements for the Degree of

Doctor of Philosophy  
Biomedical Sciences

The University of New Mexico  
Albuquerque, New Mexico

July, 2010

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**ABSTRACT**

Numerous epidemiology studies demonstrate that acute increases in air pollutants correlate with an increase in cardiovascular disease-related mortality. The pollutant diesel exhaust (DE) has been shown to impair both flow-mediated and agonist-induced dilation of the brachial artery, used as a surrogate for coronary artery function. It is speculated that enhanced sensitivity to the endogenous vasoconstrictor ET-1 impairs cardiac blood flow and contributes to the immediate onset of myocardial ischemia and infarction in humans following DE exposure. In addition, impaired endothelium-dependent dilation can be improved with the restoration of nitric oxide (NO) synthase (NOS) activity. We therefore sought to determine the mechanism by which inhalation of DE impairs coronary artery function by assessing responses to ET-1 and to the endothelium-dependent vasodilator acetylcholine (ACh) in arteries from rats exposed to DE compared to responses in arteries from rats exposed to filtered air. Given that DE is a source of reactive oxygen species (ROS) we hypothesized that inhaled DE generates ROS which uncouples NOS-dependent dilation to augment coronary artery constriction. We observed augmented vasoconstrictor sensitivity to ET-1 and blunted vasodilator response to ACh

in coronary arteries following DE exposure. Interestingly, these alterations in vascular reactivity appear to result not only from the loss of NO, but also from a gain in NOS-derived constrictors. Furthermore, basal activity of NOS was not altered by DE exposure. Elevated ROS are known to oxidize and deplete tetrahydrobiopterin (BH<sub>4</sub>) a necessary cofactor that prevents the uncoupling of NOS. ROS scavenging or BH<sub>4</sub> supplementation prevented the generation of superoxide in isolated arteries as did NOS inhibition. These treatments also restored dilation to ACh. Therefore, acute inhalation of DE appears to deplete bioavailable BH<sub>4</sub>, uncouple NOS and lead to NOS-dependent superoxide generation. The increased oxidative stress likely scavenges and decreases synthesis of NO leading to endothelial dysfunction which may contribute to the acute coronary events initiated by air pollution.

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## CHAPTER 1. INTRODUCTION

### *Air Pollution Causes Cardiovascular Disease*

Epidemiological studies have linked air pollution to adverse health effects and increased cardiovascular morbidity and mortality. Short term exposures exacerbate cardiovascular and respiratory diseases and increase the incidence of acute myocardial infarctions (74). Chronic exposures increase the risk of mortality from ischemic heart disease and heart failure (21). The severity of these trends correlate with the ambient concentration of fine and ultrafine particulates (diameter  $<2.5\mu\text{m}$  and  $<100\text{nm}$ , respectively). This is thought to be related to the small size of the ultrafine particles, which allows them to deposit in all areas of the respiratory tract, including the deep lung. After deposition, soluble components or even the particles themselves may translocate across cellular barriers into the bloodstream reaching target organs such as the heart (68). One study suggests that the biphasic increases in mortality rates following air pollution exposure are associated with the immediate effects of fine particles and a delayed effect of the ultrafine particles (40).

Exposure to particulates is of great concern for public health because retention of certain particles can last up to 70 days in rodents and 700 days in humans (24), which may promote sustained effects long after exposure. The World Health Organization estimates 800,000 deaths worldwide are related to inhalation of particulate matter (PM) (55). According to the Environmental Protection Agency, fine particulates and the associated gaseous pollutants contribute to 15,000 premature deaths each year in the United States (95). The most susceptible populations to the effects of air pollution are the elderly, infants and those with preexisting cardiovascular diseases (77).



Elevated levels of particulate air pollution correlate with increases in cardiovascular mortality and hospital admissions for cardiovascular diseases (81-83). Importantly, particulate pollution rarely occurs alone since emission-source PM is generated in concert with nitrogen dioxide, carbon monoxide, and volatile organic compounds. Additionally, combustion-derived toxicants are added to an ambient background of other solid and gaseous pollutants. Increased exposure to the gaseous components of diesel and gasoline engine exhausts (94) also associates with an increase in cardiac arrhythmias (20; 73; 79) and studies have shown that exposure to air pollutants increases heart rate (HR) and decreases HR variability (HRV) (78), risk factors for sudden cardiac failure.

#### *Particulate Matter Deposition and Systemic Translocation*

The biological effects of airborne particles depend largely on the site of deposition. Based on the predictive mathematical model by the International Commission on Radiological Protection, most particles that enter the respiratory tract deposit in the nasopharyngeal region. Particles less than 100 nm travel deeper into the lungs, reaching the tracheobronchial and alveolar regions (68). Deposited particles in the upper regions of the airway are effectively cleared by the actions of the mucociliary escalator. Cilia lining the surface of the respiratory tract move debris to the pharynx where it is swallowed (24; 68). Phagocytosis of particulates by alveolar macrophages is the predominate mechanism of clearance in the alveolar region. These particle-laden macrophages then migrate to the mucociliary escalator for clearance. However, lung lavage studies have shown macrophages are inefficient in the clearing of ultrafine particles, recovering approximately 20% of particles less than 80 nm (46; 66; 67).

Deposited particles can be leached of hydrophilic elements which are soluble in both intracellular and extracellular compartments. Absorption, diffusion or protein binding of these elements permits movement into the systemic circulation. In addition to absorption of leached elements, epithelial cells may take up intact ultrafine particles through endocytosis or transcytosis (8) enabling additional systemic translocation. Ultrafine particles may also move between epithelial cells to reach interstitial sites permitting access to the lymphatic system and possibly the circulation, although the movement from the lymphatics to the circulation is hydrodynamically unfavorable (67). Translocation of particles and their components into the systemic circulation would allow direct interaction with the distal cardiovascular system, providing a potential mechanism for air pollutants to initiate cardiovascular events, along with the direct actions of soluble gases such as CO and NO (71; 101).

#### *Diesel Exhaust has Significant Cardiovascular Effects*

The worldwide use of diesel engines in both road and non-road machinery and vehicles contributes substantially to current air pollution and health problems. DE is composed of particulate and gaseous phases, both exerting unique adverse health effects. The more studied particulates are composed of a carbon core with adsorbed sulfates, nitrates, organic compounds, and metals (94). Diesel engines emit 30 to 100 times more particulates than gasoline engines with up to 80% of DE particulates ultrafine in size. In the US Mid-Atlantic region, approximately 20% of particulates in urban air arise from diesel combustion (94). In 2000, Pope showed that a  $10 \mu\text{g PM}/\text{m}^3$  increase was associated with greater risk of cardiovascular mortality; interestingly, PM exposures did not associate with increased risk of respiratory mortality (77). Following elevated PM

exposure, there was a PM association with both an immediate (2 hours) and delayed (2 days) risk of myocardial infarction (19) suggesting there are multiple mechanisms of air pollution-related deaths.

Animal models also exhibit cardiovascular responses to DE and its components that are similar to those reported in humans. In spontaneously hypertensive rats instrumented to record electrocardiograms, DE exposure elevated HR and prolonged PQ intervals, two markers of ventricular arrhythmias (13). Modest DE exposure also decreased HRV, a measure of autonomic control, in both control and chronic heart failure rats but caused ventricular proarrhythmias only in rats with chronic heart failure (5) suggesting pre-existing cardiovascular disease increases susceptibility. Men with coronary artery disease were also found to develop a greater electrocardiographic ST depression during exercise with DE exposure, indicative of greater myocardial ischemia. Furthermore, an elderly population without preexisting cardiovascular conditions exhibited a reduction in HRV with acute exposure to air pollutants (1) that was absent in young healthy volunteers (18). These findings suggest an imbalance in parasympathetic and sympathetic regulation of the electrical control of the heart may be a risk factor for cardiac arrhythmias and sudden cardiac death.

In addition to hemodynamic changes, inhalation of DE in healthy human volunteers enhanced resting vascular tone and diminished agonist-mediated vasodilatation of the brachial artery in manner persistent at least up to 24 h post exposure (58; 72; 93). Infusion of endothelin (ET)-1 after this exposure elicited vasoconstriction only in the DE exposed group. Although plasma ET-1 levels were not altered with DE exposure, ET<sub>B</sub> receptor-mediated dilation was impaired suggesting a shift to a more

constrictive and less dilatory state (52). Similarly, aortic rings from mice exposed to DE particles (DEP) had impaired endothelium-dependent vasodilation to ACh (35). The ET-1 constrictor response was found to be enhanced following DE inhalation in various vascular beds (12; 45), suggesting the pollutant has global cardiovascular effects. Thus it is clear that DE adversely affects cardiovascular health and increases the incidence of cardiac events, but little is known about the mechanism underlying these effects.

#### *Coronary Artery Biology*

Contributing to less than 1% of the total body mass, the heart utilizes approximately 5% of resting cardiac output and the coronary arteries provide oxygenated blood to the heart tissues. Unlike skeletal muscles, the heart is metabolically very active at rest, consuming up to 20 times more oxygen per mass than skeletal muscle for energy production. To meet the demand for oxygen consumption, 70-80% of the delivered oxygen is extracted from the blood. Therefore increases in oxygen demand above resting conditions (i.e. exercise) must be primarily facilitated by increasing coronary blood flow.

Epicardial coronary arteries are low resistance conduit arteries and contribute little to the regulation of cardiac blood flow. The coronary microcirculation is responsible for the majority of resistance and blood flow regulation. Endothelium-dependent dilation primarily regulates vascular tone in coronary arterioles, 40 – 200  $\mu\text{m}$  in diameter (50). The inherent ability of vascular smooth muscle to respond to changes in intraluminal pressure, myogenic tone, is the primary regulator of tone in the microvessels with increases in pressure causing vasoconstriction and falls in pressure leading to relaxation of the vascular smooth muscle (48). The smallest of coronary arterioles are regulated predominately by metabolic activity (49) whereby increases in metabolic demand cause

dilation of these smallest arteries to decrease pressure upstream. Myogenic dilation of the upstream microvessels will further increase flow through the arterioles (41). This mechanism of coronary vasculature autoregulation ensures blood flow matches cardiac tissue metabolic demands and buffers it from the primary regulators of the systemic circulation, sympathetic nervous system activity and circulating hormone agonists. This is beneficial for the heart which must be oxygenated throughout systole to provide the nutrients for the active myocardial contraction.

Many vasoactive agents produced in the endothelium modulate coronary vascular tone in the coronary artery bed. Pharmacological inhibition of endothelium-derived nitric oxide (NO) has been reported to reduce coronary blood flow in isolated, perfused rat hearts. This inhibition can be competitively reversed with L-arginine supplementation, a substrate for NO generation, suggesting this vasodilator is involved in the regulation of basal tone (9). Endogenous endothelium-derived vasoconstrictors such as ET-1 also regulate vasomotor tone. Infusion of an ET<sub>A</sub> receptor antagonist in the left coronary artery dilated distal coronary arteries with a corresponding increase in coronary blood flow (56).

Autonomic innervation provides an additional mechanism of coronary vascular regulation. Sympathetic activation causes constriction of the microvessel mediated by  $\alpha_1$ -adrenergic receptors without affecting coronary arteriole and epicardial artery tone. Hypoperfusion of the coronary circulation revealed  $\alpha_2$ -adrenergic receptor mediated constriction that is absent under basal, normoxic conditions (14). Vasodilation of the coronary artery from the activation of  $\beta$ -adrenergic receptors modulates the  $\alpha$ -adrenergic receptor mediated constriction. Both  $\beta_1$ - and  $\beta_2$ -adrenergic receptors are found in the

coronary vasculature with greater expression of  $\beta_1$ -adrenergic receptors in larger and  $\beta_2$ -adrenergic receptors in small, resistance arteries (63; 96). The heterogeneous distribution of adrenergic receptors throughout the coronary vasculature results in differential responses to autonomic nervous system activation to protect the larger arteries from constriction during systole which would limit delivery of nutrients to the myocardial muscle.

Therefore, dysregulation of endothelium-derived vasoactive agents or increases in sympathetic outflow can compromise control of coronary artery tone and blood flow. The loss of endothelium-derived dilators would have the biggest impact in the small arteries where a large portion of vascular resistance to flow is generated. It was this size of artery that we studied. In these arteries, an imbalance toward a constricted state would reduce oxygen delivery downstream to the small arterioles and a severe imbalance would lead to prolonged ischemia of all downstream tissues with subsequent necrosis of the myocardium resulting in myocardial infarction.

#### *Coronary dysfunction*

It was estimated in 2006 that more than 17.5 million people have coronary artery disease (CAD), the single leading cause of death for both men and women in the United States (4; 105). CAD is the buildup of plaque in the coronary arteries thereby occluding the vessel and limiting blood flow to cardiac tissues. Long term exposure to concentrated ambient particles ( $\sim 15 \mu\text{g PM}/\text{m}^3$ ) have been demonstrated to accelerate atherosclerotic plaque development in apoE<sup>-/-</sup> mice and impair aortic vascular function (87). Decreased oxygen delivery in the face of various physiological challenges that demand an increase in cardiac workload will lead to cardiac ischemia, angina and eventual myocardial

infarction. The increase in vascular resistance in hypertension can exacerbate the effects of CAD by increasing the workload of the heart. Angiotensin-converting enzymes inhibitors are commonly used for the treatment of hypertension and have been suggested to improve CAD patient outcome by decreasing blood pressure (25; 26).

Myocardial infarction from insufficient oxygen delivery to cardiac tissues leads to myocyte necrosis and initiates localized inflammatory responses at the infarct zone. This initiates a cascade of events leading to the remodeling of ventricles, most notably left ventricular hypertrophy (88). The change in ventricular structure is associated with loss of function from decreased elasticity and compression of coronary arteries (32). Therefore CAD may accelerate the progression of heart failure. In support, heart failure patients with CAD with a history of coronary artery bypass graft surgery have decreased mortality rates from heart failure (31).

#### *Endothelial Cell Function*

The single layer of endothelial cells lining the luminal side of the microvascular system was classically thought to function as a physical barrier separating the blood from the surrounding tissues. Since the 1980s, however, when Furchgott and Zawadzki demonstrated that the endothelium is also a source of vasoactive agents (30), numerous studies have shown the importance of the endothelium in regulating platelet aggregation, leukocyte adherence, vascular smooth muscle proliferation and several other functions (3; 47; 65). Release of various endothelium-derived vasoactive agents such as NO, prostacyclin, endothelium-derived hyperpolarizing factors and endothelin-1 help regulate vascular smooth muscle tone to maintain cardiovascular homeostasis.

### *Nitric Oxide*

All three isoforms of NOS--neuronal, inducible and endothelial--have been identified in the vascular system. Neuronal (nNOS) and endothelial (eNOS) isoforms are low output enzymes mainly expressed in neurons and endothelium, respectively. The inducible (iNOS) form of NOS can be found in almost any cell type after certain insults and, when activated, is a high output NO generating enzyme. All three isoforms of NOS are similarly homodimers with each monomer containing nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) binding sites on the C-terminal reductase domain (figure 1). The oxygenase domain has binding sites for heme, (6R)-5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>) and the substrate L-arginine that are linked to the reductase domain by a calmodulin binding domain. Stable NOS dimers are formed in the presence of heme and Zn, allowing interaction between the reductase and oxygenase domains of opposite monomers (2; 29; 62).

Functional eNOS requires sufficient amounts of the substrate L-arginine and the cofactor BH<sub>4</sub> for the production of NO. BH<sub>4</sub> is thought to stabilize the physical and/or electrochemical coupling of the NOS dimer and is generated from GTP through a *de novo* pathway mediated by GTP cyclohydrolase or recycled from the oxidized form of BH<sub>4</sub>, 7,8-dihydrobiopterin (BH<sub>2</sub>), by dihydrofolate reductase (86). Depletion of BH<sub>4</sub> or increases in BH<sub>2</sub> electrochemically uncouple eNOS, resulting in the generation of superoxide anion radical (O<sub>2</sub><sup>•-</sup>) rather than NO (15; 86). In a cascade fashion, peroxynitrite (ONOO<sup>-</sup>) production from the reaction of O<sub>2</sub><sup>•-</sup> and NO not only depletes bioavailable NO but can also oxidize BH<sub>4</sub> to catalyze further eNOS uncoupling.



### Coupled NOS

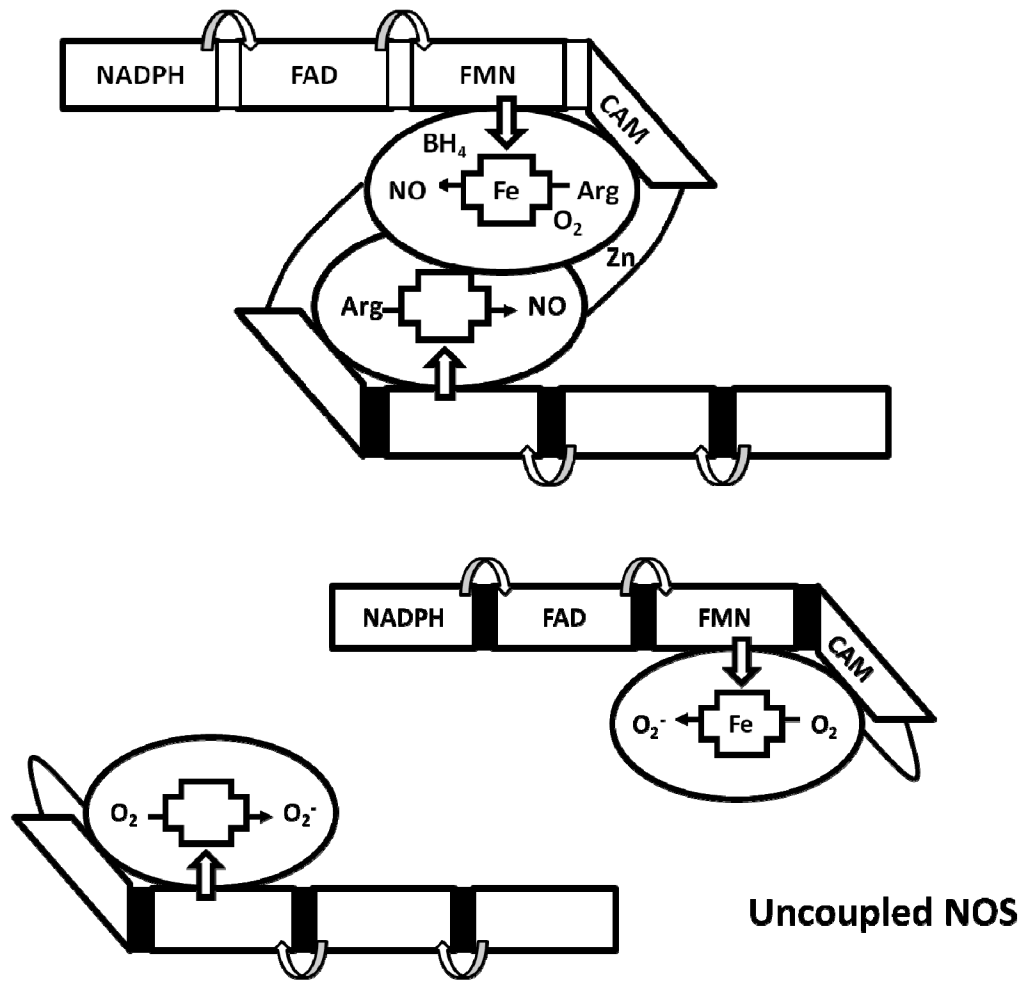


Figure 1: NOS dependent synthesis of NO and O<sub>2</sub><sup>-</sup>. Nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), calmodulin binding domain (CAM), L-arginine (Arg), tetrahydrobiopterin (BH<sub>4</sub>). Adapted from Munzel *et al.* Arterioscler Thromb Vasc Biol. 2005

The generation of NO and L-citrulline from L-arginine and O<sub>2</sub> starts with flavin-mediated NADPH electron transfer to the heme center, activating O<sub>2</sub>. Two separate oxidation steps are required to generate NO and L-citrulline from the intermediate N $\omega$ -hydroxy-L-arginine. Both intracellular calcium levels and phosphorylation states modulate eNOS activity. Increases in intracellular calcium allow binding of calmodulin to its domain in eNOS to facilitate efficient NO synthesis (84). Additionally, eNOS activity can be enhanced by phosphorylation at S1179 by calmodulin kinase II or inhibited by PKC phosphorylation of T497.

NO diffuses to the nearby vascular smooth muscle cells and other tissues where its primary functions are mediated by binding the heme iron of soluble guanylate cyclase. The heterodimeric soluble guanylate cyclase (1 $\alpha$  and 1 $\beta$  subunit) catalyzes the production of cyclic GMP (cGMP) from GTP to have varying effects depending on cell type. Stimulation of soluble guanylate cyclase can lead to phosphorylation of the inositol triphosphate receptor by cGMP-dependent kinase to inhibit Ca<sup>2+</sup> efflux into the cytosol from the sarcoplasmic reticulum. Additionally, cGMP-dependent kinase-dependent phosphorylation increases the open probability of the large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels (BK<sub>Ca</sub>), reducing Ca<sup>2+</sup> influx by hyperpolarizing the smooth muscle cell (62). The reduction in smooth muscle intracellular calcium in turn decreases contraction allowing vasodilation.

### *Cyclooxygenase*

Although encoded by distinct genes, the two isoforms of cyclooxygenase (COX) are structurally and functionally similar (22). COX catalyzes the conversion of arachidonic acid liberated from cellular phospholipids by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) to

prostaglandin  $H_2$  (PGH)<sub>2</sub>, by action of specific synthases or isomerases, is further converted to the prostanoids PGD<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2</sub>, PGI<sub>2</sub> (prostacyclin), or TXA<sub>2</sub> (thromboxane A<sub>2</sub>) (97). COX-1 is constitutively expressed in most mammalian cell types. COX-3, sometimes known as COX-1b or COX-1 variant, is a splice variant of COX-1 where intron one is retained, causing a reduced enzymatic activity compared to COX-1 or COX-2. COX-2 expression is regulated and induced by endogenous stimuli such as cytokines and growth factors. COX-2 is undetectable in most cell types except rodent brain and kidneys, where it is constitutively expressed (22; 44; 97).

Regulation of the two COX isoforms is quite different. The inducibility of COX-2 is attributed to the various response elements in the promoter and enhancer regions. Namely, the cAMP response element in the promoter region is an essential regulation site of COX-2 responding to both cAMP-dependent and independent factors (i.e. c-fos, c-jun homo-/heteromers) (44). Although classically considered an inducible isoform, recent studies show the involvement of constitutive COX-2 in various organ system functions such as renal, brain and nervous system (22).

The rate limiting step in prostanoid production is the COX enzyme oxidation of arachidonate and under normal physiological conditions is dependent on COX-1. In the vascular endothelium, the vasodilator PGI<sub>2</sub> is the predominant prostanoid product, up to eight fold higher than other prostanoids (22; 64). PGI<sub>2</sub> binds to its G-protein (Gs) coupled receptor (IP) on the vascular smooth muscle to increase cAMP leading to vasodilation. IP receptor activation also has been shown to inhibit proliferation of vascular smooth muscle cells. Altered expression of VEGF and Zn finger transcription factors, along with 81

other genes was detected by microarray analysis following IP receptor stimulation of human pulmonary artery vascular smooth muscle cells with iloprost, a PGI<sub>2</sub> analog (64).

#### *Endothelium-Derived Hyperpolarizing Factors*

The liberation of arachidonic acid from cellular phospholipids by PLA<sub>2</sub> also provides substrate for the cytochrome P450 to produce epoxyeicosatrienoic acids (EETs). Four regioisomeric EETs (14,15-, 11,12-, 8,9-, and 5,6-EETs) are synthesized by the endothelium. However 14,15- and 11,12-EETs are the major metabolites. Vascular smooth muscle cells can metabolize arachidonic acid but to a lesser degree, and production of EETs was not detected in bovine coronary artery smooth muscle cells (11).

EETs have been described as endothelium-derived hyperpolarizing factors, vasodilator compounds that do not including NO and prostacyclin and which activate potassium channels to hyperpolarize and therefore relax vascular smooth muscle cells. Concentration-dependent arachidonic acid-mediated dilation of bovine coronary artery was abolished with endothelium removal, suggesting that arachidonate is converted in the endothelium to one or more vasoactive products. In the presence of COX inhibition, cytochrome P450 inhibition blocked arachidonate-mediated dilation completely. Furthermore, the dilatory effects of exogenous EETs were inhibited by elevating extracellular K<sup>+</sup> or by blocking calcium activated K<sup>+</sup> channels (K<sub>Ca</sub>). When smooth muscle membrane potential was measured following 11,12-EET application, an iberiotoxin-sensitive hyperpolarization was observed, suggesting that EETs mediate relaxation by activating large conductance K<sub>Ca</sub> (BK<sub>Ca</sub>) to hyperpolarize the smooth muscle and decrease Ca<sup>2+</sup> influx through voltage sensitive Ca<sup>2+</sup> channels (11; 28).

Recent studies with selective EET agonists and antagonists indicate that EETs are diffusible factors interacting with G-protein coupled receptors. Downstream effects of EETs are inhibited by anti-Gs $\alpha$ , but not anti-Gs $\beta\gamma$  or anti-Gi $\alpha$ , antibodies (11). K<sub>Ca</sub> channel activity may be regulated by cAMP through activation of protein kinase A and 11,12-EET has also been implicated in the activation of protein kinase A (28). Evidence that receptors mediate the actions of EETs come from studies of silica bead bound EETs that prevent crossing cellular membrane, but that do not inhibit downstream activities. In monocytes and human leukemic monocyte lymphoma cell lines, <sup>3</sup>H-14,15-labeled EET exhibited high affinity saturable binding that was noncompetitive with other eicosanoids and fatty acids (11). These data suggest receptors mediate the actions of EETs although the receptors have yet to be identified.

The attenuation of EET mediated relaxation by endothelium removal could be indicative of either endothelial production or that the endothelium is the site of action (100). In endothelial cells, EETs have been shown to activate the non-selective cation channel, transient receptor potential (TRP) channels, in a autocrine manner to increase Ca<sup>2+</sup> influx (98). In endothelial cells, this increase in intracellular Ca<sup>2+</sup> not only increases NOS and COX activity, but also activates endothelial small (SK<sub>Ca</sub>) and intermediate (IK<sub>Ca</sub>) conductance K<sub>Ca</sub> to cause membrane hyperpolarization. (75). Endothelial hyperpolarization can be conducted to the vascular smooth muscle through gap junction to further cause relaxation (80). Inhibition of EET synthesis has been demonstrated to inhibit conducted hyperpolarization of the vascular smooth muscle (23; 27). Taken together, these studies suggest EETs regulate vascular tone through effects on both endothelium and vascular smooth muscle.

### *Endothelins*

ETs are synthesized as prepro-hormones that are enzymatically cleaved twice by the endothelin converting enzyme (ECE) to form active 21 amino acid peptides. The three isoforms, ET-1, -2, -3 are each encoded by different genes and possess autocrine, paracrine and endocrine activity. Vascular endothelial cells are the main site of ET-1 synthesis and can also produce ET-2 but not ET-3. Both ET-1 and ET-2 are additionally generated in the heart, kidney and central nervous system. Endocrine, gastrointestinal and central nervous system cells have been found to express ET-3 (16). ET-1 can be immediately released or stored in Weibel-Palade bodies, which upon stimulation fuse with the plasma membrane to release their contents into the myoendothelial cleft (39).

ET-1 is the most potent vasoconstrictor of the three isoforms, followed by ET-2 and ET-3 which also has vasodilator properties. ETs bind to ET receptors A and B (ET<sub>A</sub>R and ET<sub>B</sub>R, respectively), which are found on vascular endothelial cells and smooth muscle cells (figure 2). These two G protein coupled receptors activate phospholipase C, which increases intracellular calcium via generation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and accumulation of 1,2-diacylglycerol (16). ET<sub>B</sub>R located on the surface of endothelial cells function to clear ET from plasma and to stimulate vasodilator generation to help regulate vascular tone (36; 39). Endothelial vasodilators that can be increased by ET-1 activation of ET<sub>B</sub>-receptors include NO, COX products, and several poorly defined hyperpolarizing factors. Both receptor subtypes are also found on vascular smooth muscle cells where activation of either receptor leads to vasoconstriction, mediated in part by generation of O<sub>2</sub><sup>-</sup> (53; 99).

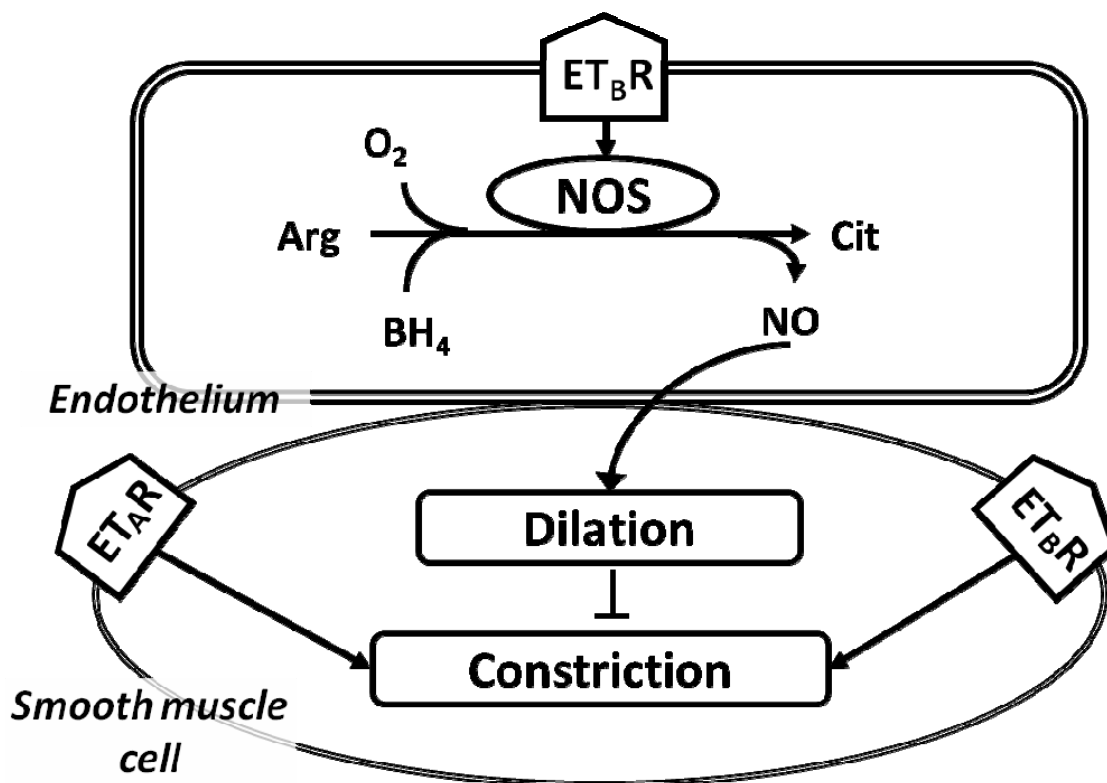


Figure 2: Endothelial and vascular smooth muscle effects of ET-1. L-citrulline (Cit), endothelin A and B receptor (ET<sub>A/B</sub>R).

### *Diesel Exhaust Generates Reactive Oxygen Species*

One potential factor linking DE to cardiovascular events is an apparent increase in oxidative stress observed as elevated levels of reactive oxygen species (ROS). ROS can participate in normal cellular signaling by modifying kinases and phosphatases and regulating transcription factors (76; 102). Xanthine oxidases, NADPH oxidases, uncoupled endothelial nitric oxide (NO) synthase (eNOS), and mitochondrial respiration are endogenous sources of ROS within the vasculature. Cellular defenses against excess ROS include antioxidants (i.e. glutathione, bilirubin) and scavenging enzymes (i.e. superoxide dismutase (SOD) and catalase) (104). However, when antioxidant systems are overwhelmed, excess ROS contributes to the development of atherosclerosis, heart failure and other cardiovascular diseases by reacting with proteins, sugars and lipids to alter the function of these targets (54; 104).

Chemicals found in DE and other air pollutants may spontaneously release ROS in tissues and cultured cells and to potentially stimulate endogenous ROS generating enzymes. *In situ* measurements in hearts and lungs from rats exposed to air pollutants showed cumulative increases in ROS accompanied by mild tissue damage, edema and activation of antioxidant pathways (33). DEP and organic extracts from DEP also dose-dependently increase hydrogen peroxide generation in human airway epithelial cell lines (7). DEP and whole DE may thus augment ROS generation beyond the antioxidant capacity, damaging susceptible target tissues.

### *Endothelin and Reactive Oxygen Species*

In addition to tissue damage, ROS in EC can increase production of ET-1. Angiotensin-II-mediated increases in ET-1 production and blood pressure were prevented



by an SOD mimetic, tempol, or the antioxidant, vitamin E (70). In addition, exogenous ROS-induced increases in ET-1 transcripts and protein were inhibited by ROS scavenging (42; 43). Thus oxidative stress may mediate ET-dependent cellular responses and induce ET-1 synthesis.

#### *Endothelial Dysfunction and Reactive Oxygen Species*

Endothelial cells aid in regulating vascular tone by releasing various vasodilators such as PGI<sub>2</sub>, NO and endothelium-derived hyperpolarizing factors as described above. ROS such as O<sub>2</sub><sup>-</sup> react with NO to form peroxynitrite and reduce NO bioactivity (60). The antioxidant superoxide dismutase scavenges O<sub>2</sub><sup>-</sup> to prevent the reaction of O<sub>2</sub><sup>-</sup> and NO (61; 69) and inadequate levels of endogenous superoxide dismutase lead to impaired endothelium-dependent dilation (104). Another antioxidant pathway induced by oxidative stress is hemoxygenase-1, which catalyses the degradation of heme to biliverdin (89; 103). Biliverdin is subsequently reduced to bilirubin, a potent antioxidant (85). Inadequate levels of these endogenous antioxidants can thus diminish NO bioavailability, further contributing to endothelial dysfunction. Endothelial dysfunction in the coronary vasculature increases the risk of myocardial and coronary artery diseases (10).

#### *Endothelin and Coronary Function*

Elevated levels of ET are associated with endothelial dysfunction and many cardiovascular disease states including left ventricular hypertrophy and heart failure (6; 38). ET<sub>A</sub>R is the predominate receptor in the human heart and coronary arteries (17; 37; 59). It binds ET-1 to maintain basal coronary vasomotor tone (51; 57). Compared to healthy individuals, hypertensive patients have increased ET<sub>A</sub>R-mediated vasoconstrictor tone (34) and treatment with the ET<sub>A</sub>R antagonist BQ-123 prevented endothelial

dysfunction to improve coronary circulation (51). Thus, elevated peptide and receptor expression and/or function could predispose individuals to the negative cardiovascular sequelae of air pollutants such as DE, which further activate the ET constrictor pathway.

### *Summary and Hypothesis*

Exposure to air pollutants such as DE increases ROS generation in cultured cells and in animals. Furthermore, DE can increase the synthesis of vasoconstrictor ET peptides (90-92) and the incidence of cardiac events (19; 82). However, it is unknown if ROS generation and increased ET levels contribute to the coronary events associated with DE exposure. Furthermore, it is not known if DE alters coronary artery contractility to potentially contribute to increased cardiac events. The following studies evaluated the effect of DE on coronary artery vasoactivity and the underlying mechanisms of the changes to determine if ROS generation with subsequent endothelial dysfunction contributes to negative cardiac consequences of DE inhalation. The following specific aims addressed our hypothesis that **inhaled DE generates ROS to augment coronary artery constriction** (figure 3).

**Specific Aim 1:** Determine the effect of inhaled DE on coronary artery contractility.

**Specific Aim 2:** Determine how DE-induced ROS production affects coronary artery contractility.

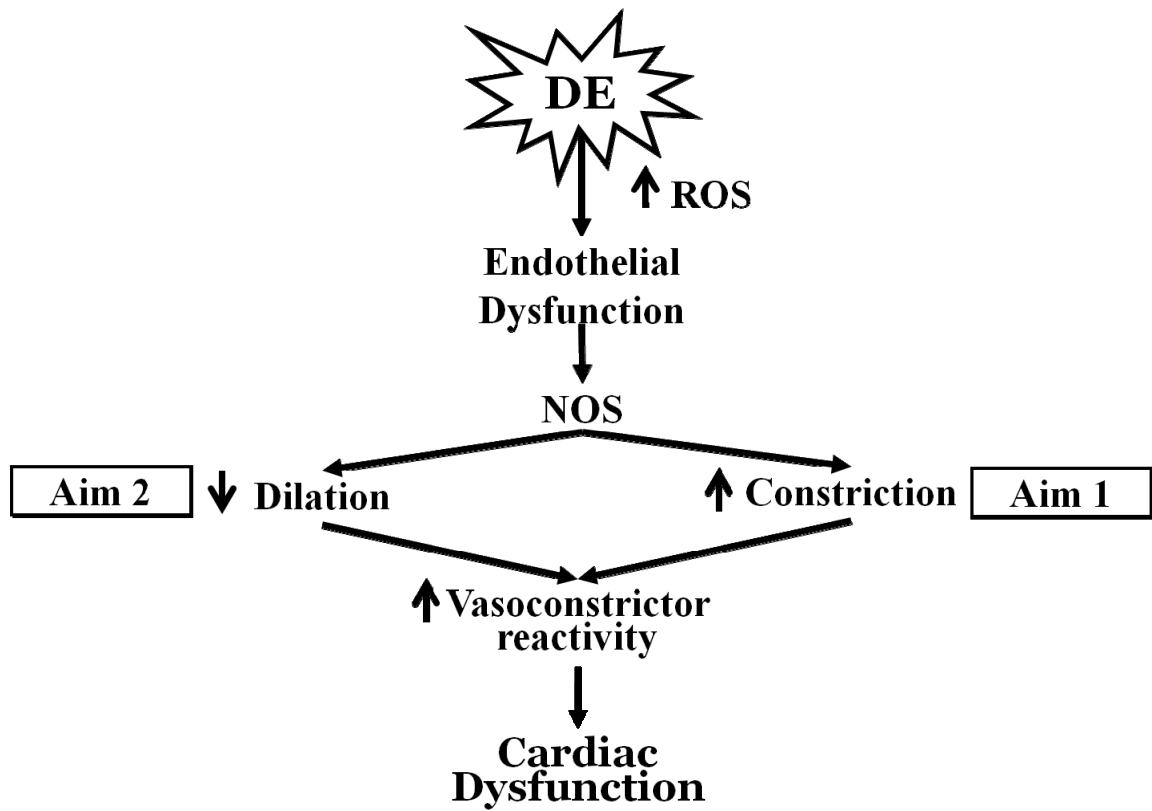


Figure 3: Overall hypothesis.

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## CHAPTER 2. EXPERIMENTAL METHODS

### *Diesel Exhaust Exposure*

Diesel exhaust was produced from a single-cylinder, 5500-watt, Yanmar diesel-engine generator using nationally certified diesel fuel at the Lovelace Respiratory Research Institute facility. Electrical current was drained from the engine to provide a constant load (90%) during operation. Desired PM concentrations were attained by diluting the direct exhaust with filtered air. Exposure chamber temperature and humidity were monitored throughout exposures, and temperatures were maintained in the range of 20–25° C. The particle concentration was monitored by sampling on 47-mm Pallflex (Pall-Gelman) filters. Pre-filter and post-filter weights were measured by a Mettler MT5 microbalance with static discharged before weighing to avoid any interference from electrical charge on the filters. Filter samples were collected two times a day (every 3 h) for each DE exposure chamber; one filter sample per day was collected from the control chamber.

Male Sprague-Dawley rats (250-300 g) were exposed to DE by placement in a sealed chamber to maintain a 300  $\mu\text{g}/\text{m}^3$  DE exposure concentration. This exposure has been previously shown not to cause pulmonary inflammation (5) and reflects 24 h limits of PM set by the Environmental Protection Agency. Control rats were housed in the same chamber but exposed only to filtered air. Rats were housed in identical cages under the same light-dark cycle.

### *Mean Arterial Pressure*

Rats were instrumented with telemetry devices as instructed by the manufacturer (Data Sciences Instruments, Minneapolis, MN) to record mean arterial pressure (MAP),

HR and electrocardiograms (ECG). After one week surgical recovery, rats were exposed to either filtered air or DE as described above.

### *Heart Rate Variability*

The clinical relevance of HRV as a prognostic tool for adverse cardiovascular outcome in various disease states was first realized in the 1960s (4). HRV, in its simplest definition, is the variance between successive heart beats. The intrinsic pacemaker property of the sinoatrial node maintains a relatively constant HR in the absence of extrinsic intervention. However, the heart is under continuous control of the autonomic nervous system to respond to ever-changing environmental stimuli by constantly adjusting HR and contractility of cardiac muscles. Parasympathetic modulation of heart rate is mediate by release of acetylcholine (ACh). The effects of parasympathetic influence are brief as the high concentration of cholinesterase present in the sinoatrial node degrades ACh rapidly, thus higher frequency HR changes are associated with parasympathetic activity (6). Contrary, lower frequency HR variations are linked to sympathetic responses mediated by the slower catabolism of epinephrine and norepinephrine (1). The inability to respond to the changing demand on the heart (diminished HRV) may signify cardiac dysfunction.

RR interval (RRI, msec/beat), the time interval between heart beats, was extracted from ECG recordings from radiotelemetry implanted rats. RRI series were broken into 15 min intervals and transformed using custom software as previously described (3). The low frequency (LF) variability range, representing a composite of sympathetic nervous system activity (SNSA) and parasympathetic nervous system activity (PSNSA), was set at 0.2–1.2 Hz, while the high frequency variability (HF) range, representative of PSNSA,

was set at 1.2–4.0 Hz. Sympathetic heart rate variability, expressed as the ratio of LF/HF of transformed RR intervals, was used as an index of SNSA. HR was calculated from the recorded RRI (1 min/RRI = HR, BPM) or from telemetry recordings.

### *Isolated Artery Preparation*

Intraseptal coronary arteries with an average inner diameter of  $200 \pm 2 \mu\text{m}$  were isolated within 30 minutes following the end of exposure and placed in chilled physiological saline solution (PSS); ([in mM] 129.8 NaCl, 5.4 KCl, 0.83 MgSO<sub>4</sub>, 19 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, and 5.5 glucose). Each of the two open ends of the arteries were cannulated onto glass micropipettes in a tissue chamber (Living Systems, CH-1) and secured with silk sutures. Tissue chambers were placed on the stage of an inverted Nikon Eclipse TS 100 microscope fitted with a video camera connected to a computer. Inner diameter changes were recorded using edge detection software (IonOptix). Decreases in measured diameter represent constriction of the artery while increases in diameter reflect dilation of the artery.

Vessels were stretched to approximate *in situ* length and pressurized to 60 mmHg with PSS in the lumen absent of flow and superfused at a rate of 5 mL/min with 37°C oxygenated (21%) PSS. Prior to the start of an experiment, vessels were equilibrated for 45 minutes at 37 °C with recirculated superfused PSS. Spontaneous or myogenic tone would develop in these arteries during the time of equilibration. At the end of the experiment, Ca<sup>2+</sup> free PSS was superfused for 60 minutes to fully relax the vessel. The endothelium was disrupted in some experiments to directly assess VSM function without the presence of endothelial influences. The endothelium was disrupted by inserting a strand of moose mane fiber into the free distal end of the vessel. Any loose EC were then



flushed from the vessel before cannulating the distal end and securing it with silk sutures. Endothelial inactivation was verified by the lack of a dilator response to ACh and a maintained constrictor response to the thromboxane mimetic agonist, U-46619 (2).

#### *Contractile Studies*

Arteries were treated with and without inhibitors of NOS (L-NNA, 100  $\mu$ M) in the superfusate and in the lumen for at least 30 minutes prior to the start of the experiment during equilibration to assess the role of endothelium-derived relaxing factors. The involvement of the endothelin pathway was evaluated with antagonists of the ET<sub>A</sub>R (BQ123, 10  $\mu$ M) and ET<sub>B</sub>R (BQ788, 10  $\mu$ M). Treated vessels were then constricted with increasing concentrations of ET-1 (0.1 to 10 nM) in the recirculating superfusate. VSM function was determined in endothelium disrupted arteries and also with the VSM depolarizing agent KCl (5-90 mM). Percent constriction was expressed as agonist induced tone.

#### *Dilator Studies*

ACh-induced dilation was recorded in cannulated and pressurized, endothelium intact arteries precontracted with U46619 (to ~50% of Ca<sup>2+</sup>-free diameter) to normalize starting tone between groups. Studies were conducted in the presence or absence of NOS, COX (indomethacin or aspirin, 10  $\mu$ M), EDHF and ROS (PEG-SOD, 150 U/mL) inhibitors as described above. The effects of BH<sub>4</sub> depletion was assessed with supplementation of sepiapterin (1  $\mu$ M) which through the salvage pathway of BH<sub>4</sub> biosynthesis generates BH<sub>4</sub>. Agonist induced dilation was evaluated using increasing concentrations of ACh added to the superfusate in 0.5  $\mu$ M steps. Dilation was recorded as

percent of  $\text{Ca}^{2+}$ -free diameter (i.e. 100% =  $\text{Ca}^{2+}$ -free diameter) determined at the end of each study. Each artery was used for only one curve.

#### *Quantitative PCR*

Freshly isolated coronary arteries were placed in RNAlater (Qiagen) RNA stabilization reagent overnight at 4°C before long term storage at -80°C. Total RNA was extracted from coronary arteries using the RNeasy Fibrous Tissue Mini kit (Qiagen) and concentration of the elution was determined with a NanoDrop technology instrument (ND-1000). Reverse transcription was used to generate cDNA, and subsequently real time PCR for, ET-1,  $\text{ET}_A\text{R}$ ,  $\text{ET}_B\text{R}$  and the endogenous control 18s was performed using an Applied Biosystems Fast 7500 PCR machine and TaqMan Gene Expression Assays.

#### *Plasma $\text{NO}_x$ , NE and ET-1*

Blood was collected via cardiac puncture from anesthetized rats, chilled on ice and centrifuged at 1000 rpm within 15 minutes of collection. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C. NO released from the endothelium is rapidly oxidized to nitrite ( $\text{NO}_2^-$ ) and nitrate ( $\text{NO}_3^-$ ). Plasma  $\text{NO}_x$  (sum of  $\text{NO}_2^-$  and  $\text{NO}_3^-$ ) is a crude measure of NO release. Total  $\text{NO}_x$  levels were assayed using a nitrate/nitrite colorimetric assay (Cayman Chemicals) based on the Griess assay. Plasma norepinephrine (NE) levels were assessed using a commercially available ELISA (BA 10-0200, Rocky Mountain Diagnostics, Inc). Circulating plasma ET-1 peptide was assayed using chemiluminescent immunoassay (R&D Systems).

#### *Inflammatory Markers*

Plasma levels of granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein 1 alpha (MIP-1 $\alpha$ ), interleukin 1 beta (IL-1 $\beta$ ), interleukin 4 (IL-4),

interleukin 2 (IL-2), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- $\alpha$ ), monocyte chemotactic protein 1 (MCP-1), interferon-gamma (IFN- $\gamma$ ) and growth-related oncogene (GRO-KC) were used as markers of inflammation and assayed using Milliplex<sub>MAP</sub> rat cytokine immunoassay (Millipore).

#### *ROS Detection*

Oxidation of the cell permeable fluorescent probe, dihydroethidium (DHE), by  $O_2^-$  generates ethidium<sup>+</sup> which then intercalates into DNA of cells (7; 8). Septum from both Air and DE exposed animals were embedded in OCT (TissueTek) without fixation and flash frozen with liquid nitrogen. Septal sections (10  $\mu$ m) were allowed to dry on glass slides for 30 minutes at room temperatures proceeded by treatment with PBS, LNNA (100  $\mu$ mol/L) or the superoxide dismutase mimetic, tiron (10  $\mu$ mol/L) for 30 minutes at 37°C followed by incubation with DHE (10  $\mu$ mol/L) with inhibitors for 45 minutes at 37°C. Coverslips were mounted on each slide with Vectashield. Images of coronary arteries were captured on a Nikon Optiphot fluorescence microscope using a Chroma TRITC filter set (excitation: 510-560 nm; emission: 570-650 nm). Data were expressed as average intensity (integrated intensity per area of region of interest) from one artery per rat for four rats in each group using Metamorph software (v7.0).

#### *Statistical Analysis*

Change in coronary contractility was compared between treatments and groups using two-way ANOVA for all protocols. If this test revealed differences, the Student-Newman-Keuls post hoc test was performed to make pairwise comparisons. p values  $\leq 0.05$  were accepted as statistically significant.

*Calculations*

Active tone (myogenic or drug-induced) and agonist induced dilation is calculated as the percent change in inner diameter as shown below.

$$\text{Myogenic tone: } \frac{\text{Diameter } (-\text{Ca}^{2+}) - \text{Diameter } (+\text{Ca}^{2+})}{\text{Diameter } (-\text{Ca}^{2+})} * 100$$

$$\text{Percent constriction: } \frac{\text{Diameter } (-\text{drug}) - \text{Diameter } (+\text{Drug})}{\text{Diameter } (-\text{drug})} * 100$$

$$\text{Percent dilation: } \frac{\text{Diameter } (+\text{ACh}) - \text{Diameter } (50\% \text{ Precontracted})}{\text{Diameter } (\text{Ca free}) - \text{Diameter } (50\% \text{ Precontracted})} * 100$$

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### CHAPTER 3

**Specific Aim 1:** Determine the effect of inhaled DE on coronary artery contractility

**Hypothesis:** DE acutely augments coronary artery vasoreactivity via ET receptor-mediated pathways.

**Rationale:** Contrary to our hypothesis, autonomic balance was not different between groups with DE inhalation (*see appendix*). However, the normal diurnal fall in MAP was absent in rats exposed to DE and DE appeared to augment expression of several components of the endothelin system. The lack of change in sympathetic activity indices and heart rate with a relative elevation in MAP suggested vascular resistance mediated by endothelin constriction may have been increased by DE exposure to maintain arterial pressure. However, systemic changes are minimal and the majority of the morbidity changes in epidemiological studies suggest the heart is the primary target. Therefore future studies focused on the effects of endothelin in coronary arteries.

Endothelins contributes significantly to coronary vascular tone through actions at both ET<sub>A</sub> and ET<sub>B</sub> receptors. Endothelin-mediated increases in coronary vascular resistance can result from enhanced coronary artery constriction through activation of the vascular smooth muscle ET<sub>A</sub> and ET<sub>B</sub> receptors and diminished release of endothelium dependent vasodilators mediated by endothelial ET<sub>B</sub> receptors. Therefore the following study evaluated coronary reactivity to endothelin. Because previous work had found that inhalation of DE causes NOS mediated endothelial dysfunction in both humans and animals, we investigated basal and ET-1-activated NOS modulation of coronary artery

function following DE exposure. We hypothesized that DE acutely augments coronary artery vasoconstrictor reactivity via ET receptor-mediated pathways.



**Impairment of coronary endothelial cell ET<sub>B</sub> receptor function following short-term inhalation exposure to whole diesel emissions**

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**Running Head:** Diesel exhaust and coronary vasoconstriction

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**Abstract**

Air pollutant levels positively correlate with increases in both acute and chronic cardiovascular disease. The pollutant diesel exhaust (DE) increases endothelin (ET) levels, suggesting this peptide may contribute to DE-induced cardiovascular disease. We hypothesized that acute exposure to DE also enhances ET-1-mediated coronary artery constrictor sensitivity. Constrictor responses to KCl, U46619, and ET-1 were recorded using video microscopy in pressurized intraseptal coronary arteries from rats exposed for 5 h to DE (300  $\mu\text{g}/\text{m}^3$ ) or filtered air (AIR). ET-1 constriction was augmented in arteries from DE-exposed rats. Nitric oxide synthase (NOS) inhibition (*N*<sup>o</sup>-nitro-L-arginine, L-NNA, 100 $\mu\text{M}$ ) and endothelium inactivation augmented ET-1 responses in arteries from AIR but not DE rats so that after either treatment, responses between groups were not different. DE exposure did not affect KCl and U46619 constrictor responses while NOS inhibition equally augmented KCl constriction in both groups. Thus basal NOS activity does not appear to be affected by DE exposure. The ET<sub>B</sub> receptor antagonist BQ-788 (10  $\mu\text{M}$ ) inhibited ET-1 constriction in DE but not AIR arteries and constriction in the presence of the antagonist was not different between groups. Cytokines levels were not different in plasma from DE and AIR rats suggesting acute exposure to DE does not cause an immediate inflammatory response. In summary, a 5-h DE exposure selectively increases constrictor sensitivity to ET-1. This augmentation is endothelium-, NOS- and ET<sub>B</sub> receptor-dependent. These data suggest DE exposure diminishes ET<sub>B</sub> receptor activation of endothelial NOS and augments ET<sub>B</sub>-dependent vasoconstriction. This augmented coronary vasoreactivity to ET-1 following DE, coupled with previous reports

that DE-induces production of ET-1, suggests that ET-1 may contribute to the increased incidence of cardiac events during acute increases in air pollution levels.

## **Introduction**

Epidemiological studies have linked air pollution to an increased incidence of cardiovascular morbidity and mortality (23; 38; 39; 44; 48). Short-term exposures exacerbate cardiovascular and respiratory diseases and may induce acute myocardial infarction in susceptible individuals (37). Recently, several reports have noted a strong association between traffic exposure and cardiac outcomes (19; 40), suggesting vehicular pollutants influence cardiovascular health.

The worldwide use of diesel engines in road and non-road machinery and in passenger vehicles contributes substantially to ambient air pollution and, potentially, to associated health problems. Indeed, previous studies have shown that inhaled diesel exhaust (DE) adversely affects cardiovascular parameters (31; 35; 45), but little is known about the mechanism underlying these effects. DE is a complex mixture of particulate and gaseous phases, both of which exert unique adverse health effects. Diesel engines emit 30 to 100 times more particulates than gasoline engines (46), and the United States Environmental Protection Agency (EPA) estimates that approximately 20% of particulates in urban air in the U.S. Mid-Atlantic region originates from DE (47). This particulate phase has been associated with direct vasoconstrictor effects (27), exacerbation of atherosclerosis (2), and inhibition of endothelial function (16; 42). In addition, DE contains gaseous compounds including nitrogen oxides (NO<sub>x</sub>), CO, and volatile organics that are also associated with arrhythmias and adverse cardiac events (9; 36; 40).

Metabolic changes associated with DE exposure include increased plasma endothelin (ET) (28; 35) that has been associated with many cardiovascular disease states

including heart failure and left ventricular hypertrophy (3; 21). In human heart and coronary arteries, endothelin A receptor (ET<sub>A</sub>R) is the predominant ET receptor (8; 20; 33) and its activation by ET potently stimulates both cardiac hypertrophy and vasoconstriction (26; 29). Compared with healthy individuals, hypertensive patients (15) and coronary bypass patients (26) have increased ET<sub>A</sub>R-mediated vasoconstrictor tone. Although smooth muscle cell endothelin B receptors (ET<sub>B</sub>Rs) can contribute to vasoconstriction in some vascular beds (17; 24), their primary contribution to vascular tone in the coronary circulation is not well characterized. In contrast, endothelial ET<sub>B</sub>Rs have beneficial effects on the vascular wall by clearing ET from plasma and by stimulating generation of the vasodilator nitric oxide (NO) (18; 22). Thus, elevated receptor expression and/or function could predispose individuals to the negative cardiovascular sequelae of DE, which appears to activate the ET constrictor pathway (28).

We have previously shown diesel exhaust exposed rats maintained a higher heart rate and displayed markers of ventricular arrhythmias (7). Furthermore, exposure of isolated murine coronary arteries to diesel emissions augments vasoconstriction (6); however, the effect of inhaled diesel emissions and the underlying biological mechanisms for such effects remain unknown. Thus, the present study exposed healthy rats to inhaled DE to test the hypothesis that DE acutely augments coronary artery vasoreactivity via ET receptor-mediated pathways.

## **Methods**

*Animals:* All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Lovelace Respiratory Research Institute (LRRI)

and conform to National Institutes of Health (NIH) guidelines for animal use. Male Sprague-Dawley rats (250–300 g, Charles River Laboratories) were exposed to 300  $\mu\text{g}$  particulate matter (PM)/ $\text{m}^3$  DE in a sealed chamber for 5 h. This exposure is approximately equal to the 24-h limit for PM set by the EPA for humans. The DE system has been previously characterized and produces levels of CO and  $\text{NO}_x$  at approximately 3 ppm and 4 ppm, respectively (30). Control rats (AIR) were housed identically but exposed to filtered air. Within chambers, rats were housed in standard cages and maintained on a 12:12 h light:dark cycle with food and water available ad libitum prior to exposures. Food was withdrawn during DE exposure.

*DE exposure:* DE was generated from a single-cylinder, 5500-watt, Yanmar diesel generator using nationally certified diesel fuel at the LRRRI facility. Electrical current was drained from the engine to provide a constant 90% load during operation to assure consistent emissions. The particle concentration was monitored by sampling on 47-mm Pallflex (Pall-Gelman) filters. Filters were collected two times a day (every 3 hours) for each DE exposure chamber and once per day from the control chamber. Pre-filter and post-filter weights were measured with a microbalance and desired concentrations of the emissions were attained by diluting the direct exhaust with filtered air. Exposure chamber temperature and humidity were monitored throughout exposures and temperatures were maintained at 20–25°C (30).

*Isolated artery preparation.* At the end of the 5-h exposure, fumes were off-gassed for 30 minutes and then the rats euthanized with sodium pentobarbital (200 mg/kg, i.p.). Hearts were immediately removed and intraseptal coronary arteries with an inner diameters of equivalent size between groups (AIR:  $184 \pm 8$ , DE:  $187 \pm 8$   $\mu\text{m}$ ) were

isolated and placed in chilled physiological saline solution (PSS, in mM, 129.8 NaCl, 5.4 KCl, 0.83 MgSO<sub>4</sub>, 19 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, and 5.5 glucose) aerated with 21% O<sub>2</sub>, 6% CO<sub>2</sub> and 73% N<sub>2</sub>. Each artery was used for only one experimental treatment. Both ends of the arteries were cannulated onto glass micropipettes in a tissue chamber (Living Systems, CH-1) and secured with silk sutures within 30 minutes of isolation from the heart. Vessels were stretched to approximate *in situ* length and pressurized to 60 mmHg with PSS in the lumen absent of flow and superfused at a rate of 5 mL/min with 37°C oxygenated PSS. At the end of the experiment, Ca<sup>2+</sup>-free PSS was superfused for 60 min to fully relax the vessel. The endothelium was disrupted in some experiments by rubbing the lumen of the artery with a strand of moose mane inserted into the free distal end of a vessel attached at the proximal end to a cannula. Loose endothelial cells were then flushed from the artery before cannulating the distal end and securing it with silk sutures. Endothelial inactivation was verified by lack of a dilator response to ACh and a maintained constrictor response to U46619 (5).

*Contractile studies.* Vessel chambers were placed on the stage of an inverted Nikon Eclipse TS 100 microscope fitted with a video camera connected to a data acquisition computer. Inner diameter changes were recorded using edge detection software (IonOptix) as described previously (10). Arteries were treated for at least 30 minutes with nitric oxide synthase (NOS) inhibitor (*N*<sup>o</sup>-nitro-L-arginine, L-NNA, 100 μM), ET<sub>B</sub>R antagonist BQ-788 (10 μM), ET<sub>A</sub>R antagonist BQ-123 (10 μM), or vehicle in the superfusate and in the lumen prior to constriction with increasing concentrations of endothelin-1 (ET-1, 0.1–10 nM), KCl, (10–90 mM), or thromboxane A<sub>2</sub> mimetic U46619 (0.1 nM–1 mM), added to the recirculating superfusate.

*Quantitative PCR:* Total RNA was extracted from coronary arteries using the RNeasy Fibrous Tissue Mini Kit (Qiagen). Reverse transcription-generated cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems), and real-time PCR for ET<sub>A</sub>R, ET<sub>B</sub>R, and the endogenous control 18s was performed using an Applied Biosystems' Fast 7500 PCR machine and TaqMan Gene Expression Assays. Data expressed as relative change from AIR group.

*Plasma NO<sub>x</sub>, ET-1 and inflammatory response:* Blood was collected via cardiac puncture from anesthetized rats, chilled on ice, and centrifuged at 1000 rpm within 15 min of collection. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C. NO released from the endothelium is rapidly oxidized to nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>) so that a change in plasma NO<sub>x</sub> (sum of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup>) is a crude measure of NO release. Total NO<sub>x</sub> levels were assayed using a nitrate/nitrite colorimetric assay (Cayman Chemical) based on the Griess assay. Circulating plasma ET-1 peptide was assayed using chemiluminescent immunoassay (R&D Systems). Plasma levels of granulocyte colony-stimulating factor (G-CSF), macrophage inflammatory protein 1 alpha (MIP-1α), interleukin 1 beta (IL-1β), interleukin 4 (IL-4), interleukin 2 (IL-2), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), monocyte chemotactic protein 1 (MCP-1), interferon-gamma (IFN-γ) and growth-related oncogene (GRO-KC) were used as markers of inflammation and assayed using Milliplex<sub>MAP</sub> rat cytokine immunoassay (Millipore).

*Statistical analysis and calculations:* Constrictor responses were analyzed using two-way repeated-measures ANOVA with Student-Newman-Keuls post hoc analysis for differences between groups, concentrations, and interactions. Differences in ET receptor



mRNA, plasma NO<sub>x</sub>, and ET-1 between groups were analyzed by Student's t-test. P < 0.05 was considered statistically significant for all analyses. Active tone (myogenic or drug-induced) is calculated as the percent change in inner diameter as shown below.

$$\text{Myogenic tone: } \frac{\text{Diameter } (-\text{Ca}^{2+}) - \text{Diameter } (+\text{Ca}^{2+})}{\text{Diameter } (-\text{Ca}^{2+})} * 100$$

$$\text{Percent constriction: } \frac{\text{Diameter } (-\text{drug}) - \text{Diameter } (+\text{Drug})}{\text{Diameter } (-\text{drug})} * 100$$

## Results

*DE augments endothelium-dependent ET-1 constriction.* Coronary arteries from rats exposed to DE had enhanced vasoconstrictor responses to ET-1 compared with arteries from air-exposed rats (Figure 1A, p < 0.001). Maximal constriction was greater in the DE group compared to AIR (80 ± 7% versus 49 ± 7, p < 0.001). The inner diameter of fully relaxed (Ca<sup>2+</sup>-free PSS) coronary arteries was not different between AIR and DE-exposed animals (167 ± 9 versus 191 ± 21 μm, respectively). ET-1 vasoconstriction was augmented only in the AIR group by inactivating the endothelium and the response was not different between groups in endothelial disrupted arteries (Figure 1B). Maximal constriction following endothelial inactivation was 74 ± 4 versus 72 ± 5% in AIR and DE arteries respectively (p = 0.687).

*KCl-mediated constriction unchanged following DE.* Receptor-independent vasoconstriction was assessed by increasing extracellular KCl (10–90 mM). Constrictor responses in endothelium-intact arteries from AIR and DE-exposed rats were similar (Figure 2A). Removal of the endothelium augmented KCl-mediated constriction in both groups and responses were still not different between groups (Figure 2B and 2C).

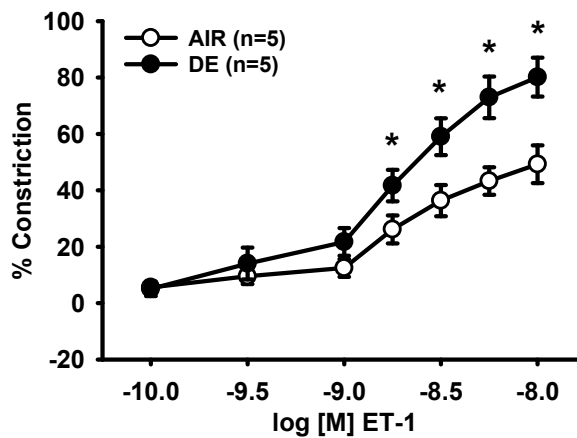
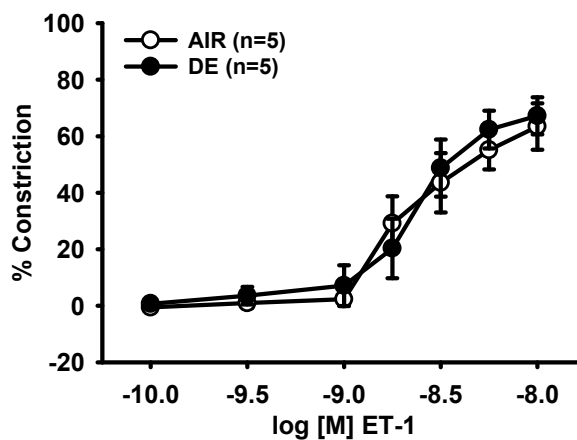
**A. Endothelium-intact****B. Endothelium-disrupted**

Figure 1: Percent vasoconstriction to increasing concentrations of ET-1 in the presence (A) and absence (B) of a functional endothelium in coronary arteries from AIR and DE-exposed animals. Endothelium-intact arteries displayed augmented vasoconstriction to ET-1 following DE exposure (\* different from AIR,  $p < 0.05$ ). This difference in ET-1 reactivity was abolished with endothelium removal.

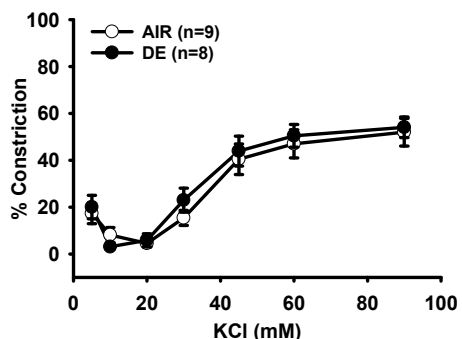
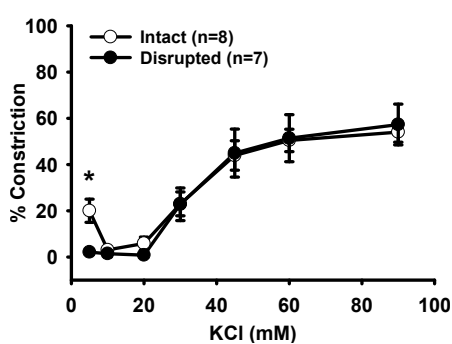
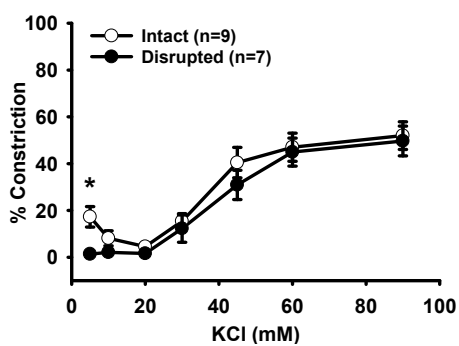
**A. Endothelium-intact****B. AIR****C. DE**

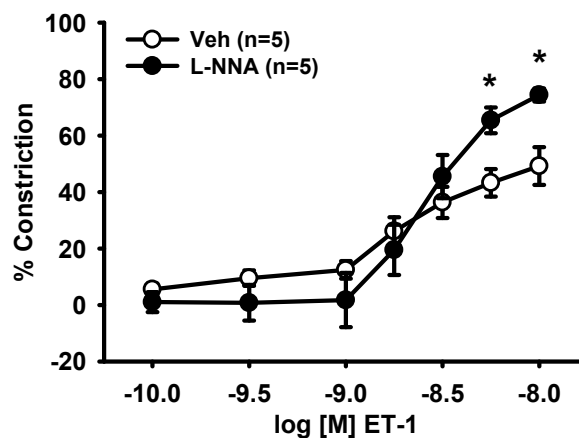
Figure 2: Comparison of KCl-induced constriction in endothelium-intact and -disrupted coronary arteries. There were no differences in vasoconstriction to increasing concentrations of KCl in intact arteries (A). Removal of the endothelium also did not affect KCl-induced constriction in arteries from either the AIR (B) or DE-exposed (C) groups

Coronary arteries from AIR and DE-exposed rats displayed myogenic tone that was not different between groups ( $17 \pm 4\%$  and  $20 \pm 5\%$ , respectively) that was abolished with endothelium removal (Figure 2). In endothelium-intact arteries, low concentrations of KCl ( $<30$  mM) maximally dilated arteries from both groups and arterial diameter in  $\text{Ca}^{2+}$ -free PSS was not different between AIR and DE-exposed groups ( $192 \pm 9$  versus  $188 \pm 9$   $\mu\text{m}$ , respectively).

*Enhanced ET-1 vasoconstriction is NOS-dependent.* NOS inhibition with L-NNA ( $100$   $\mu\text{M}$ ) augmented constrictor sensitivity to ET-1 in the AIR group (Figure 3A) compared with vehicle. In contrast, NOS inhibition diminished ET-1 constriction in the DE-exposed group at low concentrations without affecting the maximum constriction (Figure 3B). L-NNA treatment augmented the KCl response in both groups so that, as in the untreated arteries, KCl-induced constriction was not different between groups in the presence of the NOS inhibitor (Figure 4).

*ET<sub>B</sub>R mediates augmented ET-1 constriction following DE.* BQ-788 did not alter ET-1-mediated vasoconstriction in the AIR group (Figure 5A). However, ET<sub>B</sub>R antagonism with BQ-788 diminished ET-1-mediated vasoconstriction in coronary arteries from the DE-exposed rats (Figure 5B), normalizing constrictor responses to those in the AIR group. In the absence of a functional endothelium, BQ-788 slightly diminished ET-1-mediated constriction in both groups (Figure 6). The majority of ET-1-mediated vasoconstriction was prevented following ET<sub>A</sub>R blockade with BQ-123 in endothelium-intact coronary arteries from both groups. Arteries from DE-exposed animals still displayed a small constrictor response to  $10$  nM ET-1 in the presence of ET<sub>A</sub>R blockade

### A. AIR



### B. DE

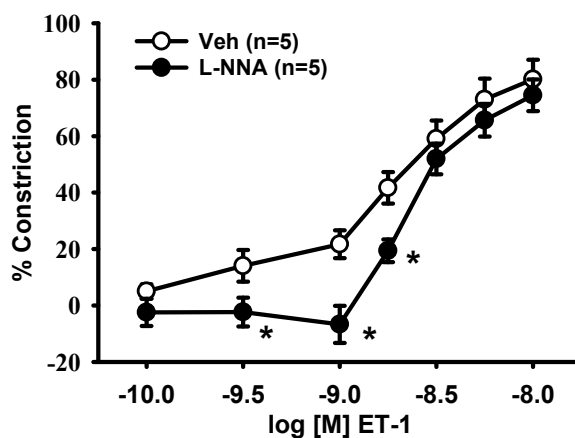


Figure 3: Constrictor response to ET-1 in the presence or absence of NOS inhibition. At higher concentrations of ET-1, L-NNA (100  $\mu$ M) augmented vasoconstriction compared with vehicle (\*  $p < 0.05$ ) in the AIR group (A). NOS inhibition did not affect maximal ET-1 constriction in the DE-exposed group (B) but blunted constriction at lower concentrations of ET-1 (\* different from vehicle,  $p < 0.05$ ).

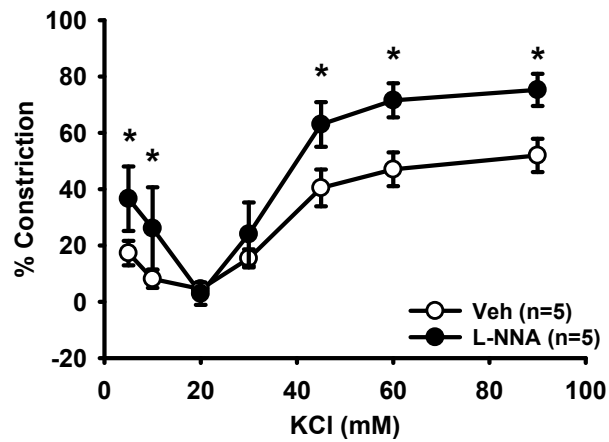
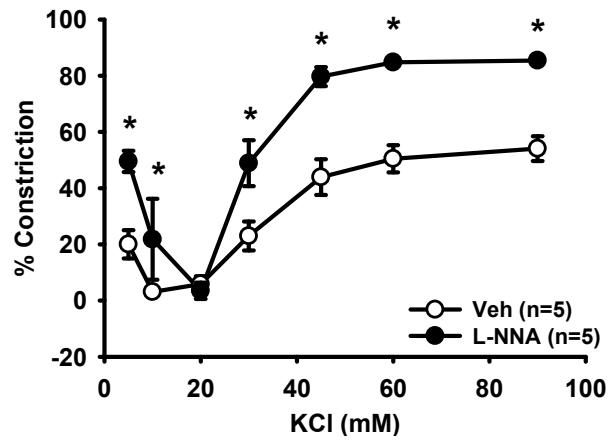
**A. AIR****B. DE**

Figure 4: Constrictor responses to KCl in the presence or absence of L-NNA (100  $\mu$ M). NOS inhibition augmented KCl-induced constriction similarly in the AIR (A) and DE-exposed (B) groups (\* different from vehicle,  $p < 0.05$ ).

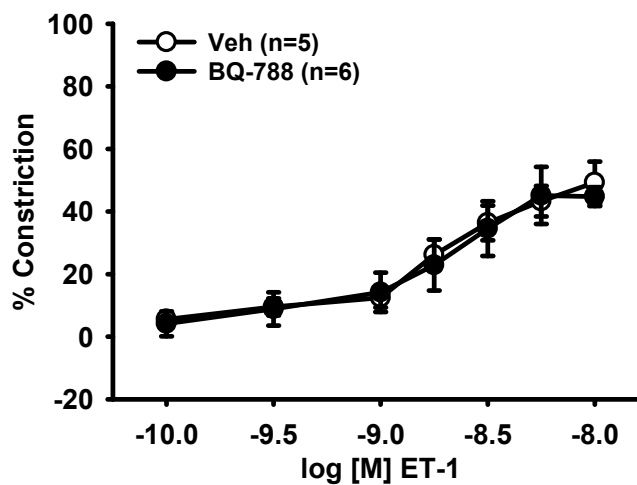
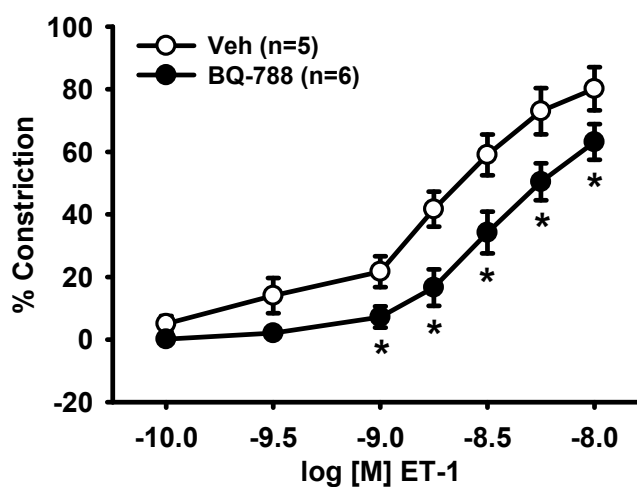
**A. AIR****B. DE**

Figure 5: Constrictor response to ET-1 following ET<sub>B</sub>R antagonism in endothelium-intact coronary arteries. BQ-788 (10  $\mu$ M) did not affect ET-1 constriction in arteries from the AIR group (A). Vasoconstriction was significantly diminished in arteries from DE-exposed animals (B) following BQ-788 treatment (\* different from vehicle,  $p < 0.05$ ).

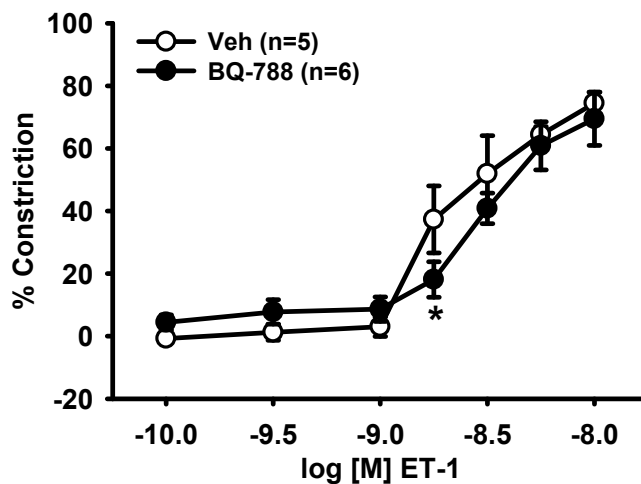
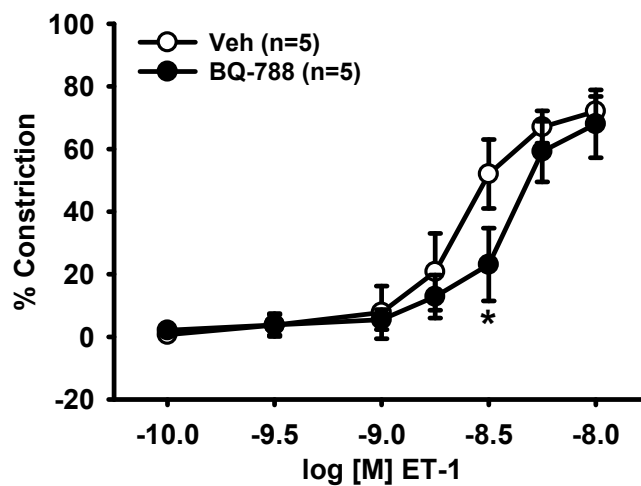
**A. AIR – Endothelium-disrupted****B. DE – Endothelium-disrupted**

Figure 6: Constrictor response to ET-1 following  $ET_B$ R antagonism in endothelium-disrupted coronary arteries. Compared with vehicle, BQ-788 (10  $\mu$ M) diminished ET-1 constriction in arteries from AIR (A) and DE-exposed (B) animals (\*  $p < 0.05$ ).



(Figure 7) that was abolished with endothelial disruption (supplemental figure S1). Constriction to the thromboxane A<sub>2</sub> mimetic U46619 (0.1 nM–1 mM) was not different between groups (supplemental figure S2, p=0.87).

*ET-1 and its receptors not altered by DE.* Changes in AIR and DE coronary artery expression of ET<sub>A</sub>R (1.01 ± 0.07 versus 1.08 ± 0.09, p=0.77) and ET<sub>B</sub>R (1.08 ± 0.20 versus 0.97 ± 0.20, p=0.68) were evaluated using quantitative real-time PCR and no differences in transcript levels between groups were found (supplemental figure S3). Circulating ET-1 was also not different between groups (Figure 8A).

*Plasma NO<sub>x</sub> elevated following exposure.* Following a 5-h exposure to DE (Figure 8B), plasma levels of NO<sub>x</sub> (9.5 ± 0.5 μM) were elevated almost two-fold compared with NO<sub>x</sub> levels in animals exposed to air (4.0 ± 0.5 μM, p < 0.05).

*Inflammatory cytokine levels.* Plasma levels of MCP-1, IL-4, IL-2, IL-6, IFN-γ and GRO-KC were not different between AIR and DE rats while G-CSF, MIP-1α, IL-1β and TNF-α were below detection limits (supplemental table S1, n = 4-5 per group).

## **Discussion**

The major finding in this study is that inhaled DE caused a dramatic increase in constrictor responses to ET-1 in endothelium-intact coronary arteries. The enhanced response was only seen in arteries when the endothelium was intact, NOS was not inhibited and ET<sub>B</sub>R were not blocked. Furthermore, DE exposure did not affect constrictor responses to KCl-induced depolarization or to the thromboxane A<sub>2</sub> mimetic, U46619. Therefore it appears that DE inhalation suppresses the generation of an endothelial vasodilator that opposes ET-1 constriction. Because inhibiting NOS and

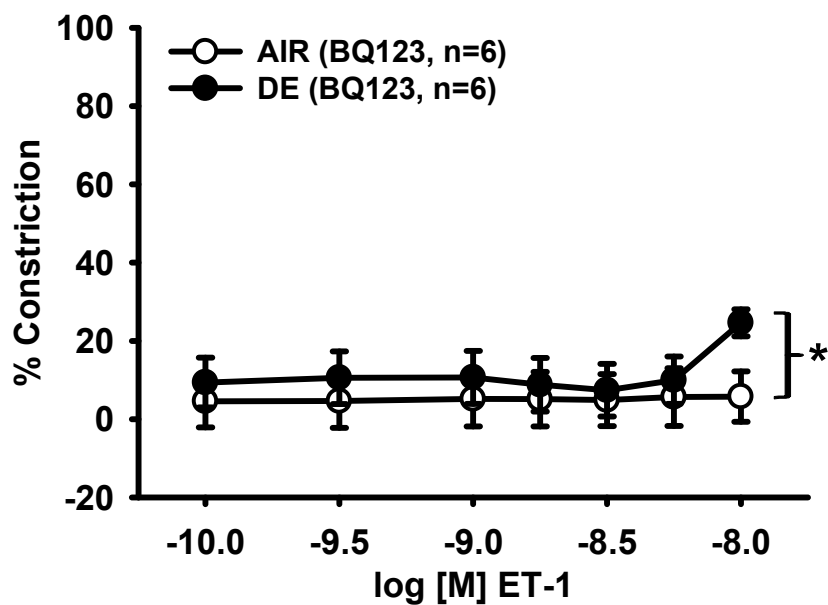


Figure 7: Constrictor response to ET-1 following  $ET_A$ R antagonism in endothelium-intact coronary arteries. BQ-123 abolished ET-1 constrictor response in arteries from the AIR animals. Vasoconstrictor response to ET-1 was inhibited in the arteries from the DE-exposed animals except at the highest concentration tested (10 nM). \* different from AIR,  $p < 0.05$ .

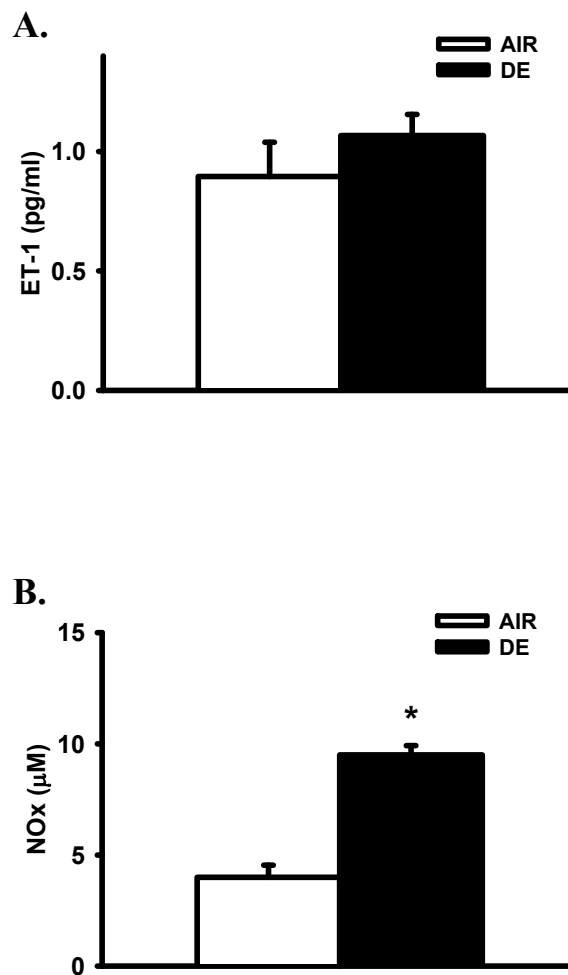


Figure 8: Plasma ET-1 peptide (A) and NO<sub>x</sub> (B) levels. Plasma ET-1 was not different between groups following a single 5-h DE exposure. However, the NO<sub>x</sub> concentration in plasma from DE-exposed animals was almost double that of plasma from the AIR rats (\*  $p < 0.05$ ). n=4-5 per group.

removing the endothelium similarly augmented ET-1-mediated constriction in arteries from AIR animals but did not affect ET-1-mediated constriction in arteries from DE-exposed animals, it appears that the lost vasodilator opposing ET-1 constriction is endothelial NO. Although NOS inhibition did not augment responses to ET-1 in arteries from DE-exposed animals, it did have the unexpected affect of diminishing ET-1-mediated constriction at low concentrations, implying ET-1 causes a NOS-dependent constriction (Figure 3B). Thus, acute DE exposure appears to augment ET-1 constriction by reducing bioavailable NO and also by generating an endothelial-derived NOS-dependent constrictor.

On the other hand, NOS inhibition might have reduced ET-1-dependent constriction in the DE arteries by enhancing the production of a cyclooxygenase (COX) derived vasodilator such as PGI<sub>2</sub>. NO has been shown to inhibit PGI<sub>2</sub> production by decreasing Ca<sup>2+</sup> entry (43). Therefore increased NO production following DE exposure may have inhibited PGI<sub>2</sub> to cause the elevated ET-1 constriction in the untreated arteries. Inhibition of endothelial NOS (eNOS) would restore PGI<sub>2</sub> synthesis leading to the observed diminished constriction in the presence of the NOS inhibitor. However, if NO-induced COX inhibition was the cause of the augmented constriction, then inhibiting NOS should also have reduced constriction to KCl. This was not observed. In fact, NOS inhibition increased KCl-induced constriction. Furthermore, blocking ET<sub>B</sub>R or removing the endothelium also should not have diminished the constriction unless these were a source of the constrictor. Therefore the loss of a COX-derived vasodilator following DE exposure does not appear likely but can only be truly defined by further investigation into this pathway.

An alternative possibility is that eNOS is actually the direct cause of the augmented constriction. NOS-derived vasoconstrictors can be generated when the enzyme is uncoupled. Usually, the heme-containing homodimer eNOS generates NO (1; 12; 34). However, components of DE can generate reactive oxygen species (ROS) (4; 14) that react with NO to form peroxynitrite and diminish NO bioavailability. ROS, including peroxynitrite, oxidize the requisite NOS co-factor BH<sub>4</sub>, leading to NOS uncoupling (12). The superoxide generated by uncoupled NOS can further augment tone both by exacerbating NOS uncoupling and directly mediating vasoconstriction (41; 49). Therefore, DE-generated ROS could lead to NOS uncoupling, depleting bioavailable NO and generating a NOS-dependent constriction.

Unlike ET-1-stimulated NOS activation, basal NO production does not appear to be different between groups. This is concluded from the observation that constrictor responses to KCl were the same in endothelium-intact coronary arteries from AIR and DE-exposed animals, and NOS inhibition similarly augmented KCl constriction in both groups (Figure 2A). The enhanced KCl-induced constriction following NOS inhibition indicates basal NO diminishes vascular tone. However, ET-1-stimulated NOS activation appears to be disrupted in the coronary arteries from the DE-exposed rats. That is, ET-1 activates ET<sub>B</sub>R on both the vascular endothelial and smooth muscle cells (22) to elicit smooth muscle-dependent constriction and endothelium-dependent dilation (18; 22). The final level of tone is the sum of these two responses. With KCl-induced depolarization and thromboxane receptor activation, the response is primarily mediated by activation of smooth muscle pathways that are modulated by basal rather than stimulated endothelial dilators. Thus, the selective augmentation of ET-1 constriction that is prevented by

removing the endothelium, blocking ET<sub>B</sub>R, or inhibiting NOS suggests that DE affects ET-1-stimulated endothelial NOS but not basal activity of the enzyme.

Vascular smooth muscle cells express both ET<sub>A</sub>R and ET<sub>B</sub>R which have been shown to form functional dimers modulating intracellular signaling. ET-1 stimulation of a homodimer of either receptor elicits a transient elevation of intracellular Ca<sup>2+</sup> while activation of a heterodimer causes a sustained intracellular Ca<sup>2+</sup> elevation (11). Therefore increased heterodimer expression would lead to increased vasoconstriction. However, selective inhibition of either ET<sub>A</sub>R or ET<sub>B</sub>R alone does not affect heterodimerization and does not diminish the ability of heterodimer pairs to mobilize Ca<sup>2+</sup> (11; 13). Therefore in the presence of heterodimerization, both receptors must be blocked to prevent ET-1-induced constriction. Because either ET<sub>A</sub>R or ET<sub>B</sub>R blockade alone diminished constriction in both AIR and DE endothelium-denuded arteries, this suggests the smooth muscle effect is not through a heterodimer effect. Furthermore, the effect of ET<sub>B</sub>R blockade was much greater in the endothelium-intact DE arteries further supporting an endothelial ET<sub>B</sub>R mediated dysfunction.

A lack of effect of DE on basal NOS activity is also suggested by the observation that there is an increase rather than a decrease in circulating NO<sub>x</sub>. Although the origin of the plasma NO<sub>x</sub> was not determined, the elevated plasma NO<sub>x</sub> is likely due to the high level of NO in DE (30). It is intriguing to speculate that this elevated exogenous NO might be the factor that uncouples endogenous NOS to contribute to the impaired NO generation. Previous studies have suggested that exposure to high levels of NO can indeed nitrosylate eNOS (32), but future studies with DE and NO will be needed to directly address this possibility.

The role of ET<sub>B</sub>R in the activation of the NOS pathway was evaluated using the selective receptor antagonist BQ-788. In endothelial disrupted arteries, BQ-788 had a modest effect in diminishing the ET-1 constriction in both groups (Figure 6), indicating a small contribution of smooth muscle ET<sub>B</sub>R to the ET-1 response. BQ-788 did not affect ET-1-mediated constriction in endothelium-intact coronary arteries from the AIR group (Figure 5A), suggesting the combined blockade of endothelial ET<sub>B</sub>R-mediated dilation and smooth muscle ET<sub>B</sub>R-mediated constriction may cancel each other out, leading to no observed effect in endothelium-intact arteries. Supporting this possibility is the observation that ET-1-mediated constriction in endothelium-intact arteries from the AIR group is abolished in the presence of the ET<sub>A</sub>R antagonist BQ-123 (Figure 7), such that opposing actions of activated endothelial and smooth muscle ET<sub>B</sub>R would be seen as no ET-1 constrictor response. In contrast, ET<sub>B</sub>R blockade diminished vasoconstriction (Figure 5B) and a modest ET<sub>B</sub>R-dependent constriction remained following ET<sub>A</sub>R antagonism (Figure 7) in endothelium-intact arteries from DE-exposed animals. Therefore DE exposure appears to impair ET<sub>B</sub>R-mediated dilator function so that only a constrictor effect remains. Furthermore, at low ET-1 concentrations, BQ-788 and L-NNA both diminished constriction in endothelium-intact arteries, suggesting endothelial ET<sub>B</sub>R stimulate NOS to produce vasoconstrictors in the DE arteries. In endothelial disrupted arteries, very high concentrations of ET-1 were required to activate smooth muscle ET<sub>B</sub>R and mediate constriction directly. Therefore, the overall effect of BQ-788 in endothelium-intact arteries from DE-exposed animals is to normalize constriction to that of the AIR arteries.

The observed alteration in coronary artery vascular function does not appear to be mediated by an inflammatory response to DE. Plasma levels of cytokines shown to be activated in acute inflammation were not different between groups following exposure. This is in agreement with previous observations that exposure to similar levels of whole DE alter vasoconstriction of mesenteric veins in the absence of inflammation (25). Additionally, Exposure to DE bubbled PSS enhanced ET-1 mediated constriction in coronary arteries (6) and mesenteric veins (25) similarly to the effects of whole body exposure. These observations support a direct effect of component(s) of DE to alter vascular function.

### **Perspectives and Significance**

Enhanced vasoconstriction following DE exposure has been previously reported in other animal models and in human studies. In ApoE knockout mice, DE inhalation enhanced coronary artery constriction to ET-1 and depressed T-waves, indicating myocardial ischemia (6). Similarly, DE exposure during stress testing in patients with prior myocardial ischemia elicited dramatic changes in ST-segment voltage compared with controls (31). Other systemic targets of DE include the mesenteric circulation where enhanced constriction was normalized with NOS inhibition, suggesting NOS is uncoupled in this bed as well (25). In human volunteers exposed to inhaled DE, brachial artery diameter decreased and plasma ET-1 increased (35). Thus, DE effects on the endothelium may augment coronary constriction to the endogenous vasoconstrictor ET-1 to decrease blood flow to the heart, contributing to cardiac events seen epidemiologically. These negative effects of DE can be persistent and long-standing (45), possibly contributing to the biphasic cardiovascular effects of elevated pollution levels.



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## Supplemental Data

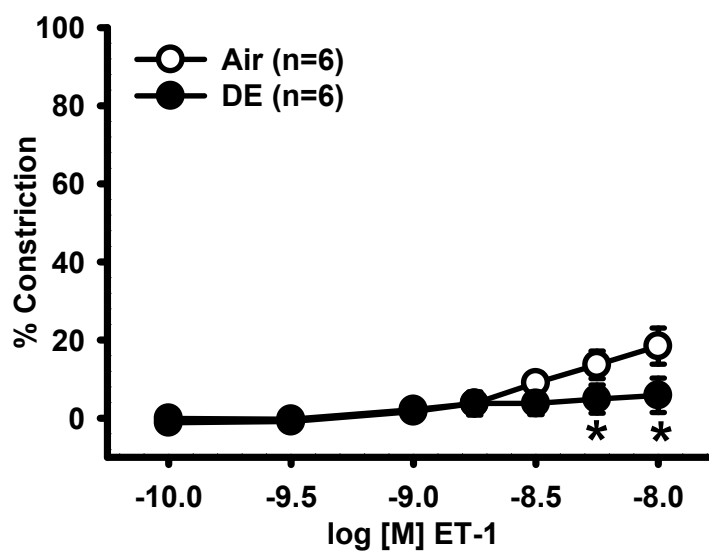


Figure S1: Constrictor response to ET-1 following  $ET_A$ R antagonism in endothelium-disrupted coronary arteries. BQ-123 abolished ET-1 constrictor response in arteries from the DE animals. Vasoconstrictor response to ET-1 was inhibited in the arteries from the AIR-exposed animals except at the higher concentrations. \* different from AIR,  $p < 0.05$ .

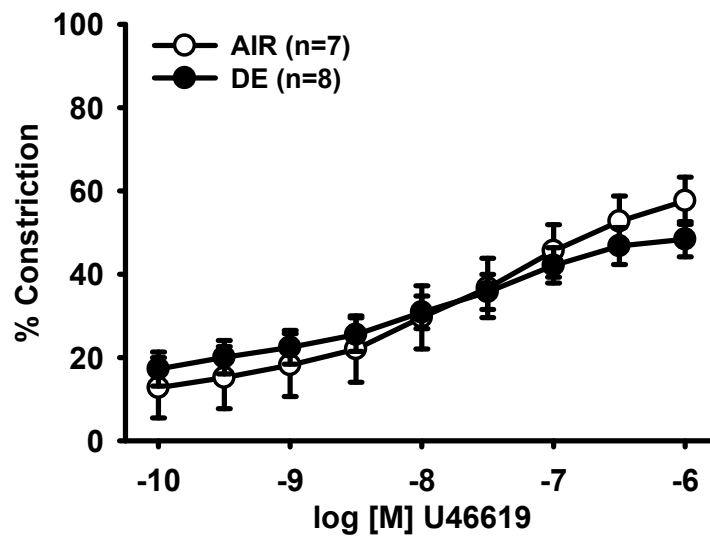


Figure S2: Constriction to the receptor mediated agonist U46619 in endothelium intact arteries. Vasoconstrictor response to the thromboxane mimetic agonist was not different between groups.

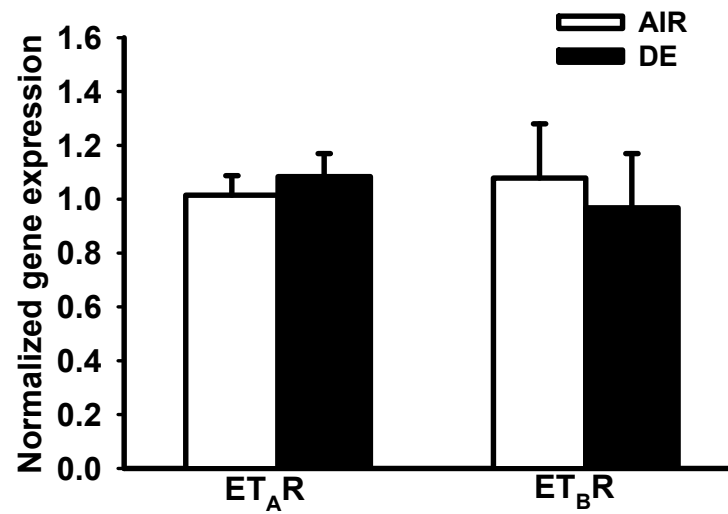


Figure S3: Endothelin receptor expression level. Transcript levels of ET<sub>A</sub>R and ET<sub>B</sub>R from coronary arteries were not different between AIR and DE groups.

	<b>G-CSF</b>	<b>MCP-1</b>	<b>MIP-1<math>\alpha</math></b>	<b>IL-4</b>	<b>IL-1<math>\beta</math></b>
<b>AIR</b>	<b>OOOR&lt;</b>	<b>353 <math>\pm</math> 195</b>	<b>OOOR&lt;</b>	<b>1837 <math>\pm</math> 1179</b>	<b>OOOR&lt;</b>
<b>DE</b>	<b>OOOR&lt;</b>	<b>363 <math>\pm</math> 217</b>	<b>OOOR&lt;</b>	<b>1268 <math>\pm</math> 893</b>	<b>OOOR&lt;</b>
	<b>IL-2</b>	<b>IL-6</b>	<b>IFN-<math>\gamma</math></b>	<b>GRO-KC</b>	<b>TNF-<math>\alpha</math></b>
<b>AIR</b>	<b>1499 <math>\pm</math> 1410</b>	<b>24822 <math>\pm</math> 22184</b>	<b>7542 <math>\pm</math> 5870</b>	<b>1085 <math>\pm</math> 307</b>	<b>OOOR&lt;</b>
<b>DE</b>	<b>777 <math>\pm</math> 795</b>	<b>10383 <math>\pm</math> 8726</b>	<b>4775 <math>\pm</math> 3092</b>	<b>1015 <math>\pm</math> 544</b>	<b>OOOR&lt;</b>

**OOOR< = Out of Range, Below**

**Expressed as pg/ml of plasma  $\pm$  S.E.M**

**n = 4-5 per group**

Table S1: Plasma cytokine levels from AIR and DE rats.

## CHAPTER 4

**Specific Aim 2:** Determine how DE-induced reactive oxygen species production affects coronary artery contractility.

**Hypothesis:** DE-induced ROS production uncouples NOS to diminish NO-dependent vasodilatation in coronary arteries.

**Rationale:** We have demonstrated that DE inhalation-augmented ET constrictor sensitivity is endothelium dependent and NOS mediated. DE constituents are able to induce ROS generation. This increased oxidative stress could deplete bioavailable BH<sub>4</sub>, a necessary cofactor of NOS activity, to uncouple NOS resulting in further generation of superoxide in place of NO. Augmented NOS mediated oxidative stress could therefore contribute to the scavenging of NO to form peroxynitrite, a reducing agent of BH<sub>4</sub>. The oxidation of BH<sub>4</sub> could uncouple NOS to enhance the response to vasoconstrictors such as ET-1. To address the role of DE-induced NOS dysfunction, we investigated coronary artery vascular function using the endothelium-dependent vasodilator, ACh. Agonist stimulation of NOS activity is usually accompanied by generation of other endothelial dependent vasodilators. Therefore DE exposure may also affect COX and EDHF vasodilator pathways, contributing further to the observed endothelial dysfunction and DE effects on these pathways were also evaluated. We hypothesize that DE-induced ROS uncouples NOS to diminish NO-dependent vasodilatation in coronary arteries.

## **Mechanisms of Diesel-Induced Endothelial Nitric Oxide Synthase Dysfunction in Coronary Arterioles**

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**Running title:** Diesel Exhaust and NOS Dysfunction

**Keywords:** nitric oxide synthase, exhaust, *N*<sup>o</sup>-nitro-L-arginine, arteries, rat, particulate matter, engine emissions, cardiovascular

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**Abbreviations:**

ACH - ACETYLCHOLINE

BH2 - 7,8-DIHYDROBIOPTERIN

BH4 - TETRAHYDROBIOPTERIN

COX - CYCLOOXYGENASE

DE - DIESEL ENGINE EXHAUST

DHE - DIHYDROETHIDIUM

ET-1 – ENDOTHELIN-1

NO – NITRIC OXIDE

NOS – NITRIC OXIDE SYNTHASE

O<sub>2</sub><sup>-</sup> - SUPEROXIDE

PM – PARTICULATE MATTER

ONOO<sup>-</sup> - PEROXYNITRITE

**Abstract**

Background and objective: Increased air pollutants correlate with increased incidence of cardiovascular disease potentially due to vascular dysfunction. We have reported that acute diesel engine exhaust (DE) exposure enhances vasoconstriction and diminishes acetylcholine (ACh)-induced dilation in coronary arteries in a nitric oxide synthase (NOS)-dependent manner. We hypothesize that acute DE inhalation leads to endothelial dysfunction by uncoupling NOS.

Methods: Rats inhaled fresh DE (300  $\mu\text{g PM}/\text{m}^3$ ) or filtered air (AIR) for 5 hrs. After off-gassing, intraseptal coronary arteries were isolated and dilation to ACh recorded using videomicroscopy.

Results: Arteries from DE exposed animals dilated less to ACh than arteries from AIR exposed animals. NOS inhibition did not affect ACh dilation in control arteries but increased dilation in the DE group, suggesting NOS does not normally contribute to ACh-induced dilation in coronary arteries but does contribute to endothelial dysfunction following DE inhalation. COX inhibition did not affect ACh dilation in the DE group, but combined inhibition of NOS and COX diminished dilation in both groups and eliminated inter-group differences suggesting the two pathways interact. Superoxide scavenging increased ACh dilation in DE arteries eliminating differences between groups. Tetrahydrobiopterin ( $\text{BH}_4$ ) supplementation with sepiapterin restored ACh-mediated dilation in the DE group in a NOS dependent manner. Superoxide generation (dihydroethidium staining) was greater in DE arteries and superoxide scavenging or NOS inhibition reduced the DHE signal in DE but not AIR arteries.

Conclusion: Acute DE exposure appears to uncouple NOS increasing ROS generation and causing endothelial dysfunction, potentially due to depletion of BH<sub>4</sub> limiting its bioavailability.

## Introduction

Exposure to vehicular pollutants is associated with exacerbation of both cardiovascular and respiratory diseases (13; 29; 30; 36; 39). Diesel engine exhaust (DE) is an important contributor to urban air pollution (4; 17; 31). Although the exact components of DE responsible for its effects have yet to be defined, both short term and chronic DE exposure is associated with arrhythmias, adverse cardiac events (9; 28; 33) and endothelial dysfunction that diminishes vasodilator response in systemic arteries (12). Multiple pathways regulate endothelium dependent vasodilatation including the release of nitric oxide (NO) and prostacyclin (PGI<sub>2</sub>) from the nitric oxide synthase (NOS) and cyclooxygenase (COX) pathways, respectively. Additionally, activation of small (SK) and intermediate (IK) Ca<sup>2+</sup> dependent potassium channels can result in the hyperpolarization and ultimately the relaxation of the vascular smooth muscle. However, short-term inhalation of dilute DE has been shown to inhibit forearm vasodilation to both acetylcholine (ACh) and the NO donor, or sodium nitroprusside, in healthy volunteers (21; 37) suggesting that DE exposure may specifically affect signaling downstream of NO.

Endothelial NOS (eNOS) is a homodimeric protein that generates NO from the conversion of L-arginine to L-citrulline. NO inhibits platelet aggregation, leukocyte adherence, and vascular smooth muscle proliferation to regulate vascular homeostasis (2; 16; 24). Synthesized NO diffuses into adjacent vascular smooth muscle where it activates soluble guanylate cyclase to reduce intracellular Ca<sup>2+</sup> concentrations and decrease vascular tone, leading to vasodilation. The eNOS cofactor 5,6,7,8-tetrahydrobiopterin (BH<sub>4</sub>) is required for the production of NO, possibly by stabilizing

the physical and/or electrochemical coupling of the NOS dimer, and is generated from guanosine-5'-triphosphate through a *de novo* pathway or is recycled from the oxidized form of BH<sub>4</sub>, 7,8-dihydrobiopterin (BH<sub>2</sub>) by dihydrofolate reductase (34).

DE exposure increases oxidative stress within the vasculature (20), which can potentially increase the oxidation of BH<sub>4</sub> to BH<sub>2</sub>, thereby limiting the bioavailability of this essential cofactor. Depletion of BH<sub>4</sub> or increases in BH<sub>2</sub> electrochemically uncouple eNOS, resulting in the generation of superoxide radical (O<sub>2</sub><sup>-</sup>) rather than NO (8; 34). In a cascade fashion, peroxynitrite (ONOO<sup>-</sup>) production from the reaction of O<sub>2</sub><sup>-</sup> and NO not only depletes bioavailable NO but can also oxidize BH<sub>4</sub> to catalyze further eNOS uncoupling. We have previously reported that vasoconstriction is augmented in systemic arteries from DE exposed rodents and that the augmented constriction is endothelium dependent and can be reversed with NOS inhibition (5; 6; 15). The aim of this study was to evaluate agonist-mediated vasodilation in coronary arteries from healthy rats exposed to DE. We hypothesized that DE-induced reactive oxygen species (ROS) uncouple NOS to diminish NO-dependent vasodilatation in coronary arteries.

## **Methods**

*Animals:* All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Lovelace Respiratory Research Institute (LRRRI) and the University of New Mexico and conform to National Institutes of Health (NIH) guidelines for animal use to ensure animals were treated humanely and with regard for the alleviation of suffering. Male Sprague-Dawley rats (250–300 g, Charles River Laboratories) were housed in quarantine for 14 days following receipt, then acclimated to exposure chambers for 7-14 days. Within the Hazelton 2000 chambers, rats were housed

in standard shoebox cages and maintained on a 12:12 h light:dark cycle with food and water available ad libitum prior to exposures. Food was withdrawn during DE exposure.

*DE exposure:* Rats were exposed to 300  $\mu\text{g}$  particulate matter (PM)/ $\text{m}^3$  DE in a sealed chamber for 5 h representing the daily PM exposure limit set by the US Environmental Protection Agency. Although daily exposure in the majority of the US population is far lower in terms of PM, occupations requiring the use of diesel engines have exposure conditions similar to this study (32). The DE system has been previously characterized and produces levels of CO and  $\text{NO}_x$  at approximately 3 ppm and 4 ppm, respectively (19). Control (AIR) rats were housed identically but exposed to filtered air. DE was generated from a single-cylinder, 5500-watt, Yanmar diesel generator using nationally certified diesel fuel at the LRRI facility. Electrical current was drained from the engine to provide a constant 90% load during operation to assure consistent emissions. The particle concentration was monitored by sampling on 47-mm Pallflex (Pall-Gelman) filters. Filters were collected two times a day (every 3 hours) for each DE exposure chamber and once per day from the control chamber. Pre-filter and post-filter weights were measured with a microbalance and desired concentrations of the emissions were attained by diluting the direct exhaust with filtered air. Exposure chamber temperature and humidity were monitored throughout exposures and temperatures were maintained at 20–25°C (19).

*Isolated artery preparation:* At the end of the 5-h exposure, chambers were off-gassed for 30 minutes and the rats removed and euthanized with sodium pentobarbital (200 mg/kg, i.p.). Hearts were immediately removed and intraseptal coronary arteries (resting inner diameter, AIR:  $176 \pm 7$ , DE:  $180 \pm 7$   $\mu\text{m}$ ) were isolated and placed in

chilled physiological saline solution (PSS, in mmol/L, 129.8 NaCl, 5.4 KCl, 0.83 MgSO<sub>4</sub>, 19 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, and 5.5 glucose) aerated with 21% O<sub>2</sub>, 6% CO<sub>2</sub> and 73% N<sub>2</sub>. Each artery was used for only one experimental protocol. Both ends of the arteries were cannulated onto glass micropipettes in a tissue chamber (Living Systems, CH-1) and secured with silk sutures within 30 minutes of isolation from the heart. Vessels were stretched to approximate *in situ* length and pressurized to 60 mmHg with PSS in the lumen absent of flow and superfused at a rate of 5 mL/min with 37°C oxygenated PSS. At the end of the experiment, Ca<sup>2+</sup>-free PSS (3.7 mmol/L EGTA) was superfused for 60 min to fully relax the vessel (Ca<sup>2+</sup> free inner diameter, AIR: 219 ± 4, DE: 224 ± 4 μm).

*Vasodilator studies:* Vessel chambers were placed on the stage of an inverted Nikon Eclipse TS 100 microscope fitted with a video camera connected to a data acquisition computer. Inner diameter changes were recorded using edge detection software (IonOptix) as described previously (10). Arteries were treated for at least 30 minutes with various drug treatments or vehicle (Veh) in the superfusate and in the lumen prior to dilation with increasing concentrations of acetylcholine (ACh, 0.001–100 μmol/L) in U46619 precontracted arteries (constricted to ~50% of fully relaxed diameter). The contribution of key endothelial dilator pathways were determined using nitric oxide synthase (NOS) inhibitor (*N*<sup>o</sup>-nitro-L-arginine, L-NNA, 100 μmol/L), cyclooxygenase inhibitor (aspirin, 10 μmol/L) and small (apamin, 100 nmol/L) and intermediate (Tram-34, 1 μmol/L) Ca<sup>2+</sup> activated K<sup>+</sup> channels. The role of NOS uncoupling and ROS mediated endothelial dysfunction following exposure was assessed with BH<sub>4</sub> donor (sepiapterin, 1 μmol/L) and the cell permeate superoxide dismutase (PEG-SOD, 150 U/mL), respectively.

*ROS Measurement:* Oxidation of the cell permeable fluorescent probe, dihydroethidium (DHE), by  $O_2^-$  generates ethidium<sup>+</sup> which then intercalates into DNA of cells (40; 41). Septum from both AIR and DE exposed animals were embedded in OCT (TissueTek) without fixation and flash frozen with liquid nitrogen. Septal sections (10  $\mu$ m) were allowed to dry on glass slides for 30 minutes at room temperatures proceeded by treatment with PBS, LNNA (100  $\mu$ mol/L) or the superoxide dismutase mimetic, tiron (10  $\mu$ mol/L) for 30 minutes at 37°C followed by incubation with DHE (10  $\mu$ mol/L) with inhibitors for 45 minutes at 37°C. Coverslips were mounted on each slide with Vectashield. Images of coronary arteries were captured on a Nikon Optiphot fluorescence microscope using a Chroma TRITC filter set (excitation: 510-560 nm; emission: 570-650 nm). Data are expressed as average intensity (integrated intensity per area of region of interest) from one artery per rat for four rats in each group and were gathered using Metamorph software (v7.0).

*Statistical analysis and calculations:* Dilator responses were analyzed using two-way repeated-measures ANOVA with Student-Newman-Keuls post hoc analysis (Sigma Stat, v3.5) for differences between groups, concentrations, and interactions. Probability levels less than 0.05 were considered significant. Data were tested for normality as part of the two way ANOVA analysis. All dilation data are expressed as a percent reversal of active tone (50% PE precontracted) and is calculated as the percent change in inner diameter as shown below.

$$\text{Percent dilation: } \frac{\text{Diameter (+ACh)} - \text{Diameter (50\% Precontracted)}}{\text{Diameter (Ca}^{2+} \text{ Free)} - \text{Diameter (50\% Precontracted)}} * 100 \quad [1]$$



## Results

*DE inhibits ACh-mediated vasodilatation.* First, we determined if diesel exposure affected endothelium-dependent responses to ACh. Coronary arteries from DE exposed rats (n=9) had diminished vasodilation to ACh compared to arteries from air-exposed rats (n=5,  $p = 0.006$ , figure 1a). Maximal agonist induced dilation in AIR ( $94 \pm 4\%$ ) was greater than DE arteries ( $44 \pm 12\%$ ,  $p = 0.007$ ). In Ca-free PSS, the inner diameter of fully relaxed coronary arteries was not different between AIR and DE groups ( $200 \pm 6$  versus  $210 \pm 13 \mu\text{m}$ , respectively). These findings note a substantial reduction in dilatory response after a single acute exposure to DE.

*NOS, but not COX, inhibition restores dilation following DE.* To elucidate which endothelium dependent pathways were altered by DE exposure, we investigated the roles of NOS, COX and SK and IK channels. NOS inhibition (n=6) augmented ACh-mediated dilation in the DE group compared to vehicle treatment ( $p = 0.011$ , figure 1b), restoring dilation to that in untreated AIR arteries ( $p = 0.945$ ) suggesting a NOS-dependent inhibition of dilation. Unexpectedly, L-NNA ( $100 \mu\text{mol/L}$ ) did not alter dilation in the AIR group compared to untreated controls (n=6,  $p = 0.456$ , figure 1c). In contrast, COX inhibition with aspirin ( $10 \mu\text{mol/L}$ ) modestly inhibited dilation in the AIR arteries (n=6,  $p = 0.040$ , figure 2a) but did not affect ACh mediated responses in the DE group (n=9, figure 2b).

The combined inhibition of NOS and COX blunted ACh-mediated dilation in both DE and AIR group (n=10 and n=11, respectively, figure 3a) compared to vehicle treatment within group. Blockade of SK and IK with Tram-34 ( $100 \text{ nmol/L}$ ) and apamin ( $1 \mu\text{mol/L}$ ), respectively, diminished ACh-mediated dilation significantly and similarly in

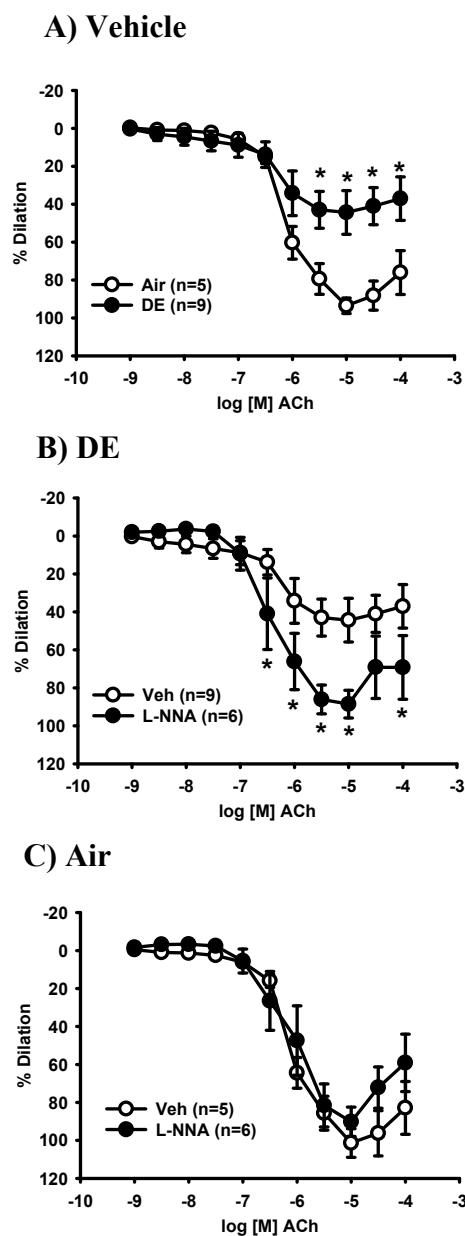


Figure 1: ACh vasodilation was diminished following exposure to DE compared to AIR control in vehicle treated coronary arteries (A). NOS inhibition with L-NNA (100  $\mu\text{mol/L}$ ) restored the blunted ACh mediated dilation (B) without affecting dilation in the AIR group (C). \* different from AIR or Veh,  $p < 0.05$ .

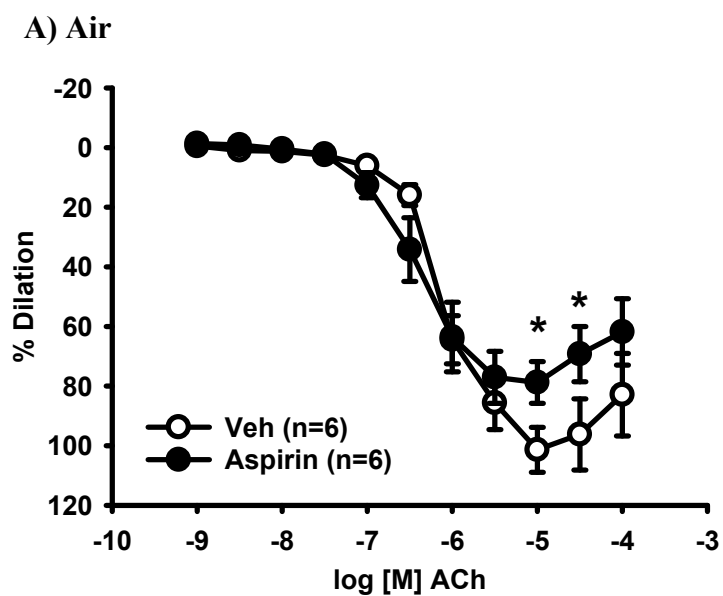


Figure 2: Pretreating coronary arteries with aspirin (10  $\mu\text{mol/L}$ ) to inhibit COX diminished ACh mediated dilation in the AIR group (A) but had no effect in the DE exposed group (B) compared to vehicle treatment. \* different from Veh,  $p < 0.05$

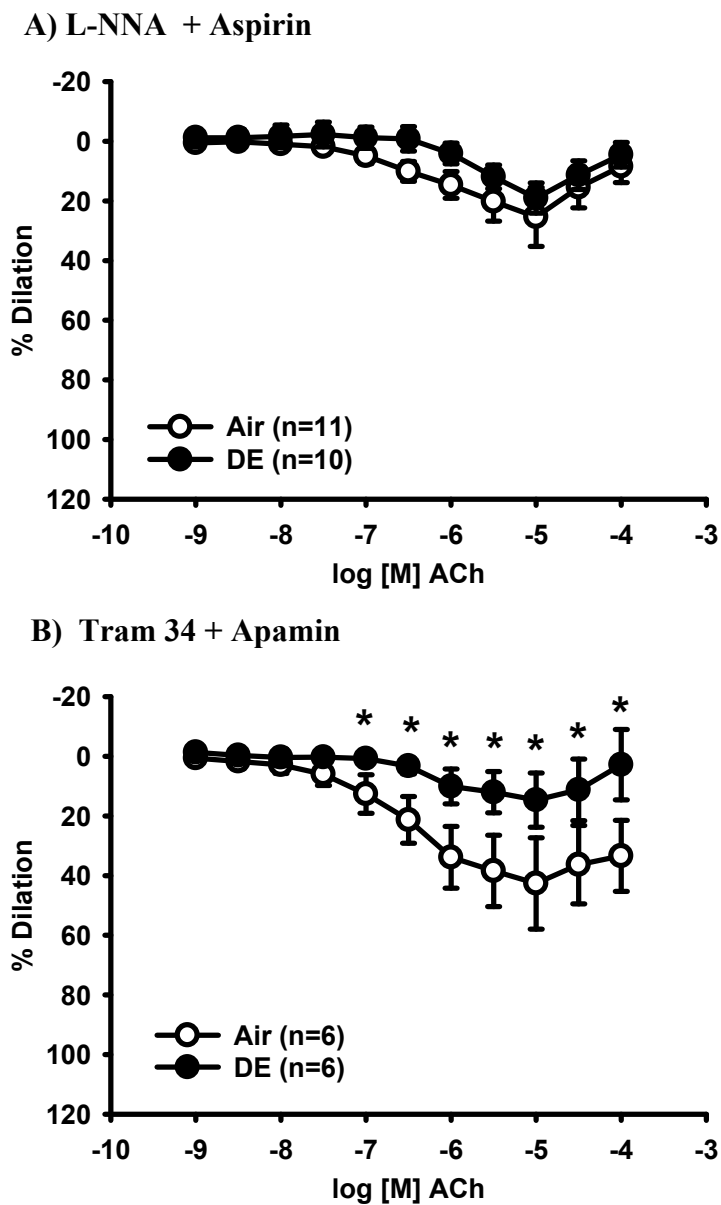


Figure 3: Inhibition of both NOS (L-NNA, 100  $\mu\text{mol/L}$ ) and COX (aspirin, 10  $\mu\text{mol/L}$ ) diminished dilation to ACh in both groups and eliminated between groups differences (A). Although blockade of small and intermediate  $\text{Ca}^{2+}$  dependent  $\text{K}^+$  channels blunted dilation in arteries from both groups, dilation was still less in the DE compared to the AIR group (B). \* different from AIR,  $p < 0.05$ .

both groups (n=6 per group, figure 3b) compared to vehicle treatment but the residual dilation was less in DE than AIR arteries. Finally, combined inhibition of NOS, COX, SK and IK completely abolished dilation in both groups (data not shown). Although inhalation of DE alters other endothelium-dependent dilator pathways, the impairment of NOS function, primarily, results in diminished ACh-mediated dilation.

*Diminished dilation following DE is restored with BH<sub>4</sub>.* To determine if supplementation with the cofactor BH<sub>4</sub> could rescue NOS function, arteries were treated with sepiapterin (1 μmol/L) which increases BH<sub>4</sub> levels via the salvage pathway of BH<sub>4</sub> biosynthesis. Sepiapterin treatment of isolated arteries augmented and restored ACh-mediated dilation in DE arteries (n=5, figure 4a) while blunting dilation in the AIR group (n=8, Figure 4b). The effects of sepiapterin on dilation were blocked by concurrent NOS inhibition (n=5 per group, figure 4b and 4d) such that there was no difference from vehicle treatment within each group. Following exposure, BH<sub>4</sub> levels appear to be insufficient to maintain NOS function.

*Superoxide scavenging prevents effects of DE exposure.* DE exposure can increase oxidative stress that can reduce both NO and BH<sub>4</sub> levels (14; 20). Coronary artery superoxide levels were evaluated with the cell permeable fluorescent probe DHE. Septal coronary arteries from rats exposed to DE induced greater DHE fluorescence than arteries from air exposed rats. Scavenging O<sub>2</sub><sup>-</sup> with tiron or inhibition of NOS had no effect on DHE fluorescence in AIR, but diminished and normalized fluorescence in DE compared to AIR (n=4 per group, Figure 5) indicating increased O<sub>2</sub><sup>-</sup> generation following DE exposure that is NOS dependent.

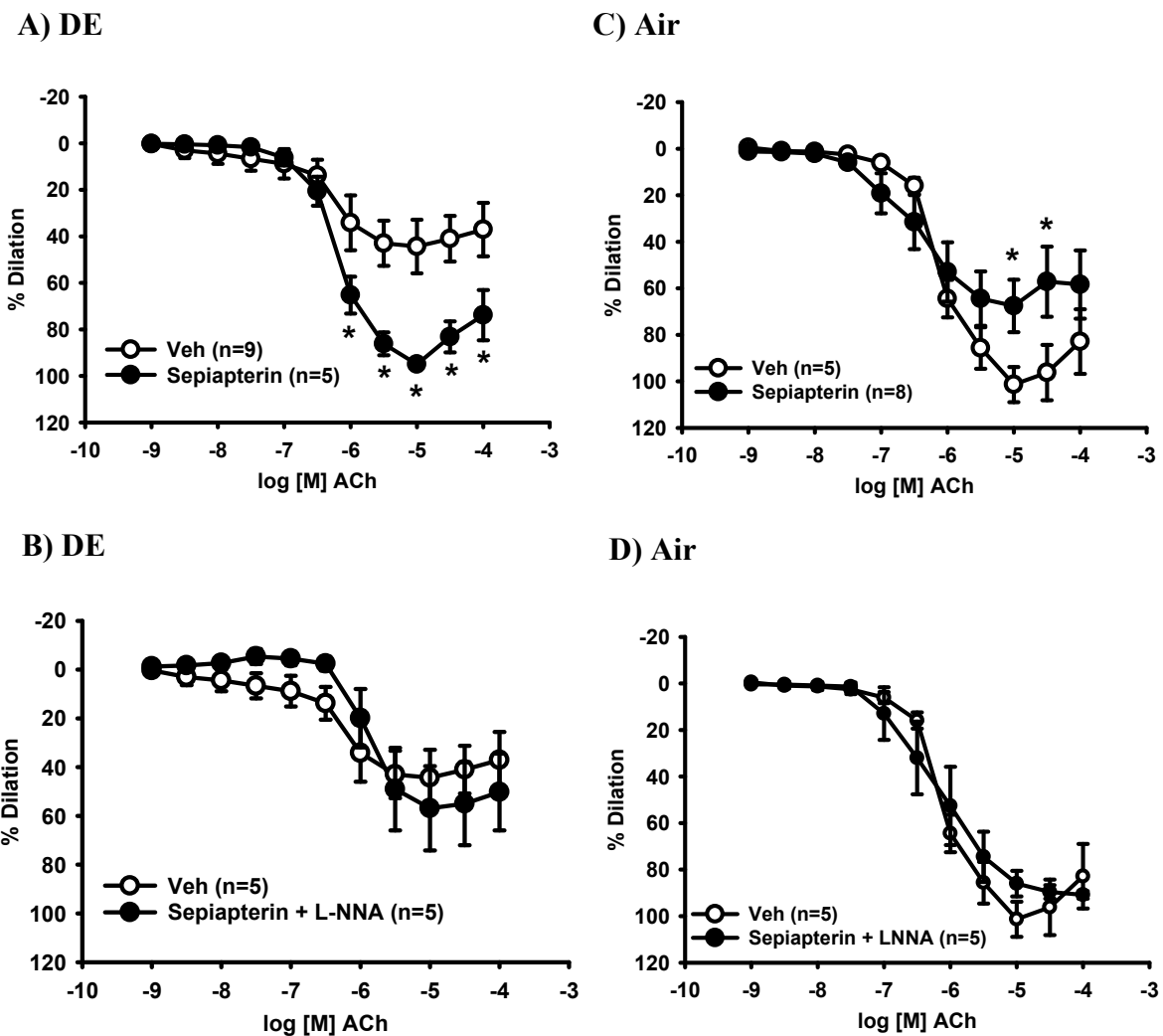


Figure 4: Dilation to ACh was augmented in arteries from the DE group following BH<sub>4</sub> supplementation (sepiapterin, 1 μmol/L) compared to Veh treatment (A). In the presence of NOS inhibition (L-NNA, 100 μmol/L), the effect of sepiapterin was blocked (B). Dilator response to ACh was blunted in arteries from the AIR group following BH<sub>4</sub> supplementation (sepiapterin, 1 μmol/L) compared to Veh treatment (C). In the presence of NOS inhibition (L-NNA, 100 μmol/L), the effect of sepiapterin was blocked (D). \* different from Veh, p < 0.05.

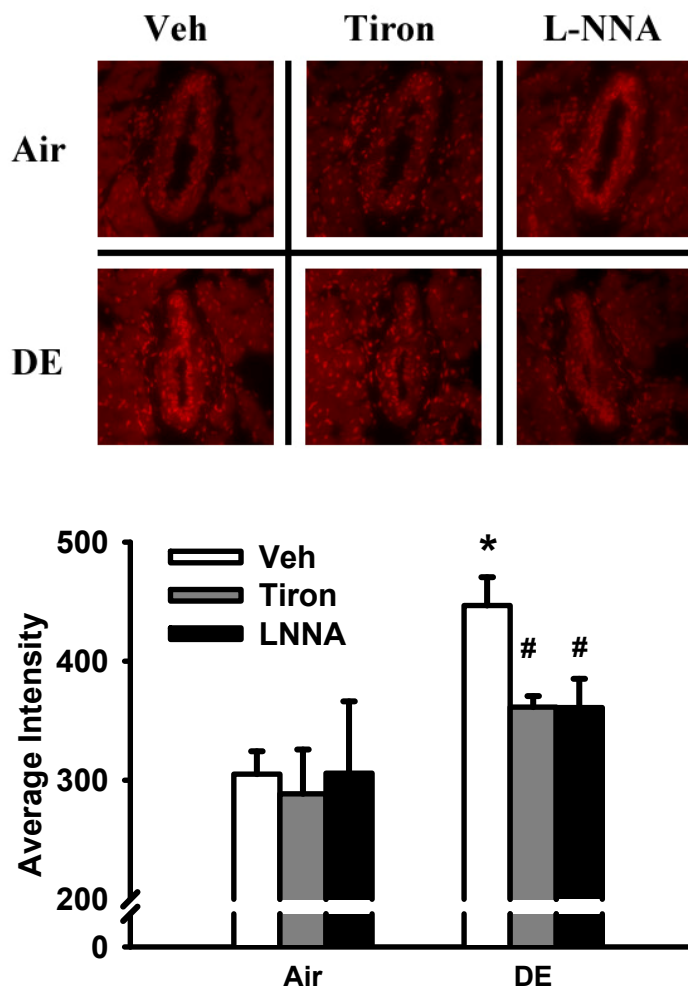


Figure 5: DHE fluorescence was greater in PBS treated (Veh) coronary arteries from DE exposed compared to AIR rats. Either tiron (10  $\mu\text{mol/L}$ ) or L-NNA (100  $\mu\text{mol/L}$ ) treatment *ex vivo* prevented the DE induced increase in fluorescence without effect in AIR arteries. \* different from AIR Veh,  $p < 0.05$ . # different from DE Veh,  $p < 0.05$ .  $n = 4$  per group.

We scavenged ROS with superoxide dismutase (SOD) to determine if oxidative stress mediates the vascular changes of DE exposure. Treatment of AIR arteries with PEG-SOD (150 U/ml) had no effect on ACh-mediated vasodilation compared to vehicle treatment. Similar to NOS inhibition or BH<sub>4</sub> supplementation, superoxide scavenging with PEG-SOD restored dilation in the DE group so that dilation was not different from arteries in the AIR vehicle treatment group (n=4 per group, Figure 6). Exposure to DE appears to augment superoxide generation that inhibits agonist induced dilation.

## **Discussion**

In the present study, acute exposure to DE impaired ACh-mediated dilation in coronary arteries from healthy rats, an effect that was reversed by NOS inhibition. Supplementation with the BH<sub>4</sub> precursor sepiapterin or scavenging ROS *in vitro* also completely restored ACh mediated dilation after DE exposure. Although COX inhibition did not alter dilation in either group, combined blockade of NOS and COX significantly blunted dilation in arteries from both AIR and DE exposed animals. Additional inhibition of SK and IK channels completely abolished dilation, with no difference between DE and filtered air groups. Combined, these observations suggest that DE exposure diminishes ACh-mediated dilation by selectively disrupting NOS-mediated responses.

The most likely mechanism for loss of ACh-induced dilation in the DE arteries is oxidative stress-induced uncoupling of NOS. As sepiapterin supplementation antagonized the effects of DE, the current results suggest NOS may be uncoupled due to a loss of



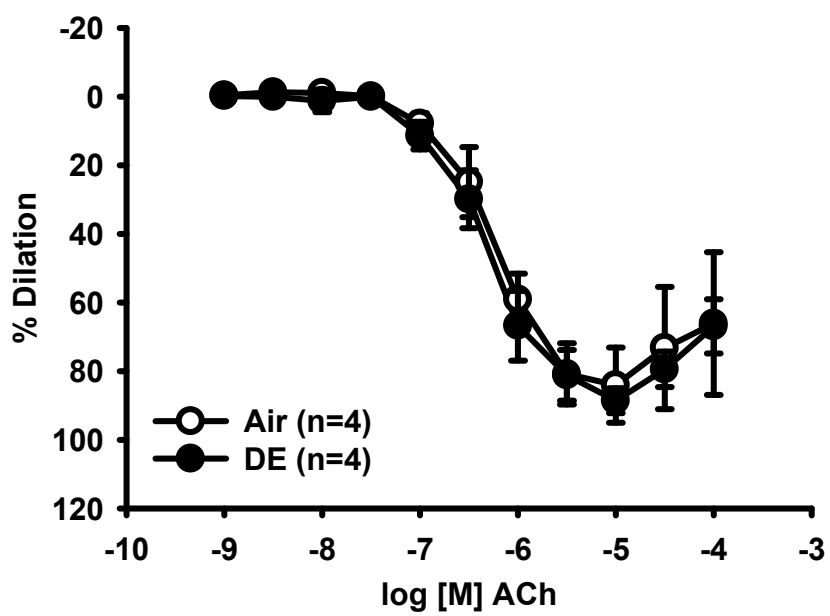


Figure 6: Scavenging of superoxide with PEG-SOD (150 U/mL) restored the dilator response to ACh in the DE group but did not affect responses in the AIR group.

BH<sub>4</sub>. Uncoupled NOS generate O<sub>2</sub><sup>-</sup> rather than NO, which can further scavenge BH<sub>4</sub> (14; 23; 26). BH<sub>4</sub> is necessary for physical and electrochemical coupling of NOS, and the depletion of this cofactor in an oxidative environment further exacerbates NOS and endothelial dysfunction. In the present study, supplementation with sepiapterin, a precursor to BH<sub>4</sub>, fully restored ACh-mediated dilation in coronary arteries from the DE exposed group suggesting loss of this co-factor led to the impaired dilation.

Inhibition of NOS in DE-exposed rats prevented the effects of sepiapterin, suggesting the effect of BH<sub>4</sub> was to recouple NOS. In contrast, BH<sub>4</sub> supplementation in coronary arteries from air exposed animals had the opposite effect, diminishing endothelium-mediated dilation. These effects also appear to be NOS dependent as treatment with L-NNA prevented sepiapterin blunting of dilation in the AIR arteries. Sepiapterin increases levels of BH<sub>2</sub>, the oxidized form of BH<sub>4</sub>, which is converted to BH<sub>4</sub> by dihydrofolate reductase through the salvage pathway (35). If the salvage pathway is saturated by excess BH<sub>2</sub> levels, sepiapterin release of BH<sub>2</sub> can inhibit NOS function by competing with BH<sub>4</sub> for NOS binding to mimic BH<sub>4</sub> depletion and increase ROS production by uncoupling NOS (8; 34). In this manner, sepiapterin may generate an oxidative environment in the AIR arteries that is partially NOS driven, similar to the effects of DE exposure. Inhibition of NOS in arteries from air exposed animals treated with sepiapterin blunts ROS generation and restores endothelial function.

Basal levels of superoxide detected by DHE staining in coronary arteries were elevated following DE exposure compared to the AIR group. This increase in superoxide generation was further demonstrated by increased sensitivity to tiron and was blocked with NOS inhibition supporting NOS mediated dysfunction resulting in generation of

ROS. It was recently described that the specificity of DHE fluorescence may not represent the intracellular levels of superoxide accurately using fluorescent microscopy (42). Nonetheless, the current study attempted to address the flaws of this methodology by comparing the tiron, a SOD mimetic, sensitive component rather than the raw values. Further supporting that DE effects were mediated by elevated levels of ROS, scavenging ROS with PEG-SOD in coronary arteries from DE exposed animals restored agonist mediated dilation but did not alter dilation in the AIR group. SOD catalyses the conversion of  $O_2^-$  to hydrogen peroxide, which is further broken down to  $H_2O$  and  $O_2$  by catalase. The restoration of ACh-induced dilation with PEG-SOD may be partially mediated by increased  $H_2O_2$  which has been shown to be a vasodilator (22). However, taken with the observations that NOS inhibition and  $BH_4$  supplementation also restore endothelial function, the present data are more consistent with the conclusion that following DE inhalation, uncoupled NOS generates  $O_2^-$ . The increase in  $O_2^-$  depletes bioavailability of both NO and  $BH_4$  to potentiate further uncoupling of NOS. In contrast to our findings, Courtois and colleagues found impaired NO-dependent relaxation following intratracheal PM instillation that was secondary to inflammation altered smooth muscle sensitivity to NO and ROS independent (7). As previously reported, acute inhalation of DE at moderate levels (i.e.,  $300 \mu g/m^3$ ) does not induce measureable changes in pulmonary inflammatory markers nor does it alter vascular smooth muscle sensitivity to basal NO (6). A major difference between our study design and that of Courtois as well as Nurkiewicz et al 2006 is that we investigated effects immediately following exposures rather than 6-72 hours later. Thus, inflammatory contributions to the systemic vasculature are minimal in our model as evidenced by lack of cytokine

induction, while at later timepoints evidence of rolling and adhering leukocytes and dexamethasone-sensitive vascular impairments can be seen. The differences in exposure method, instillation of urban PM versus inhalation of dilute whole DE, and of vascular bed of interest, pulmonary versus systemic, between Courtois and the present study may lead to the different mechanisms of NO impairment observed in these two studies. In fact, these differences provide insight into the multiple mechanisms leading to endothelial dysfunction following inhaled versus instilled exposure to air pollution.

Interactions between the NOS and COX pathways have been demonstrated previously in both cultured cells and isolated arteries (3) and are apparent in the current study. In AIR arteries, COX inhibitors only modestly diminished ACh-mediated dilation while NOS inhibition had no effect; however, the combined inhibition greatly blunted agonist induced dilation. These results suggest the two pathways exhibit functional redundancy, such that pharmacological blockade of one pathway can be offset by activation through the alternate pathway. Thus, inhibition of both NOS and COX blunts dilation more than the sum of inhibiting either pathway alone. The synergistic effect of combined inhibition of NOS and COX was seen in both AIR and DE arteries suggesting the NOS/COX interaction is not lost after DE exposure and plays an essential role in the coronary artery.

Blockade of the SK and IK channels inhibited dilation in both groups but dilation was still blunted in the DE compared to AIR arteries in the presence of the inhibitors. Therefore, activation of SK and IK channels contribute to ACh-induced dilation but do not appear to be altered by DE exposure. Interpretation of these results is complicated because these potassium channels regulate endothelial  $\text{Ca}^{2+}$  entry, which affects both

NOS and COX activity. Inhibition of these channels induces membrane depolarization that reduces the electrochemical driving force for  $\text{Ca}^{2+}$  entry upon agonist stimulation, (11) potentially reducing both NOS and COX activation. In sum, the data show that ACh-induced dilation in coronary arteries is mediated by NOS, COX, SK, and IK, as combined inhibition of these pathways was necessary to completely abolish dilator responses.

Both human and animal studies suggest the NOS pathway is impaired by DE exposure but mechanistic details have not been fully elucidated. Recently, Nurkiewicz and colleagues (25) found that NO availability is reduced in vessels isolated from rats exposed acutely to ultrafine particulate matter. However, it was not clear whether NOS was uncoupled or whether generated NO was scavenged; both mechanisms appear likely. Previously, we demonstrated that inhaled DE enhances ET-1 vasoconstriction in rat coronary arteries by stimulating uncoupled NOS-dependent constriction via endothelial endothelin B receptor (6). Langrish and colleagues demonstrated similar findings in healthy human volunteers exposed to DE, where circulating ET-1 was not different but ET-1 constrictor sensitivity was enhanced, apparently via endothelin B receptor pathways (18). In the present study, DE exposure also diminishes ACh-mediated dilation, which was restored by NOS inhibition or  $\text{BH}_4$  supplementation, further supporting the conclusion that DE exposure uncouples eNOS. ROS scavenging also reversed the effects of DE inhalation supporting a role for increased oxidative stress which can decrease NOS mediated dilation through multiple pathways. Elevated ROS levels presumably oxidize  $\text{BH}_4$  thereby decreasing availability of this necessary cofactor for NO generation resulting in NOS uncoupling.  $\text{BH}_4$  supplementation provides enough excess cofactor that NOS stays in a coupled state preventing the effects of DE inhalation. Furthermore, NO

can be scavenged by ROS to form peroxynitrite, which not only depletes NO bioavailability but can nitrosylate proteins to alter function (1). Further studies are needed to determine if the initial source of ROS is DE or uncoupled NOS. It may be that DE provides the initial oxidative burst that leads to the uncoupling of NOS resulting in a feed forward cycle leading to endothelial dysfunction. Alternatively, DE may provide a substrate for NOS uncoupling that is not related to ROS.

It is clear that pharmacologically-induced vasodilatation is blunted in healthy volunteers (21; 37) and in animal models (6; 15) following acute inhalation of DE. It is unclear if the alterations in vascular function in the current study persist at later time points, which future studies will address. We speculate similar changes will be present but waning over the following 24-72 hours as the vascular alterations in humans, which have rapid onset (21) and parallel our observations in the coronary arteries, persist at least 24 hours after cessation of exposure (37). Potentially, activation of uncoupled NOS following DE inhalation may result in prolonged endothelial dysfunction that is only slowly reversed after exposure. Likely vascular endothelium must recover by the *de novo* production of NOS protein along with cofactors such as BH<sub>4</sub> which, depending on the potency of exposure, abundance of precursors and nutrients, and genetic factors, could take several days to recuperate. Furthermore, DE inhalation can increase circulating levels of the endogenous vasoconstrictor, ET-1, in both animal (38) and human studies (27) and enhance vascular responsiveness to ET-1 (6). Such changes would be expected to further diminish dilation and enhance constriction in coronary vessels, thereby increasing risk and/or severity of coronary occlusive sequelae. Thus, susceptible individuals with underlying cardiovascular conditions may have augmented coronary

vasoconstriction following DE exposure, contributing to the increased cardiovascular events seen epidemiologically following elevated pollution levels.

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## CHAPTER 5. DISCUSSION

Numerous epidemiological studies have demonstrated that deteriorating air quality levels are associated with increased incidence of cardiovascular morbidity and mortality. Exposure to air pollution elevates the risk of arrhythmias and myocardial infarctions (70; 71). A major contributor to air pollution is DE from the use of on- and off-road diesel engines worldwide (91). Short term inhalation of dilute DE has been shown to exacerbate myocardial ischemia (56) and impair brachial artery vasodilator function in humans (57), possibly contributing to the increase in hospital admissions for cardiovascular events following exposure to traffic related air pollution (78). However, the mechanism by which DE elicits cardiovascular alterations is poorly understood and the elucidation of the cellular signaling that potentially leads to decreased oxygen delivery to the heart has been the focus of this dissertation.

Only a handful of studies are available to date examining the *in vivo* human vascular changes following DE exposure. Furthermore, the technical difficulty of studying human coronary arteries *in vivo* limits investigation of this vascular bed. However, the brachial artery has been established as a noninvasive surrogate for the study of the coronary artery function because of the similarities in endothelium-mediated vasomotor function between these two artery beds (6; 83). Peretz *et al*, recently demonstrated that acute inhalation of 200  $\mu\text{g PM}/\text{m}^3$  DE in healthy volunteers decreased resting diameter of the brachial artery compared to arteries in controls breathing filtered air inhalation (69). In similar studies, agonist-induced brachial artery dilation of healthy volunteers was blunted by a brief exposure to 300  $\mu\text{g PM}/\text{m}^3$  DE compared to control, and the impairment persisted for up to 24 hours post exposure. In contrast, endothelium



independent dilation was not altered between groups (57; 90). Furthermore, DE exposure elicited brachial artery vasoconstriction to infused ET-1 that was absent in the control group (51). These studies suggest endothelial function is impaired following acute inhalation of DE.

Various animal studies with DE have observed effects that reflect the vascular changes observed in human studies. ACh mediated relaxation was attenuated in rat aortic rings bathed in diesel exhaust particles (DEP) suspended solution (55). Likewise, Ikeda and colleagues found that DEP exposed rat aortic rings demonstrated diminished endothelium-dependent relaxation to ACh (43). ET-1 mediated vasoconstriction was augmented in mesenteric veins from mice exposed to DE ( $350 \mu\text{g PM}/\text{m}^3$ ) by inhalation compared to veins from filtered air-exposed animals (48). Correlating with both human and animal studies, we observed acute inhalation of  $300 \mu\text{g PM}/\text{m}^3$  DE augmented ET-1-mediated constriction and blunted ACh-mediated dilation of intraseptal coronary arteries from healthy rats compared to responses in arteries from filtered air-exposed rats.

Enhanced ET-1-mediated vasoconstriction after inhalation of DE was only observed in endothelium-intact coronary arteries. Furthermore, exposure did not alter endothelium-independent constrictor responses to KCl-induced depolarization or to the thromboxane  $A_2$  mimetic agonist, U46619. Thus, DE inhalation appears to suppress the generation of an endothelium derived vasodilator that opposes ET-1 constriction, rather than alter vasoconstrictor signaling in the vascular smooth muscle cell. NO is an important endothelium-derived regulator of coronary artery vascular tone. Pharmacological inhibition of NOS to reduce NO synthesis decreases coronary basal diameter and flow in addition to diminishing agonist-induced dilation (11; 61). Likewise,

DEP exposure has been demonstrated to directly reduce NO generation (55; 62) to enhance vascular tone (49). Thus, impairment of the coronary artery NO pathway was a likely target of DE exposure.

Consistent with endothelium disruption, NOS inhibition augmented coronary artery ET-1-mediated constriction only in the AIR group to normalize constrictor responses between groups. Therefore, there was a loss of endothelial NO to oppose ET-1 constriction following DE inhalation. Unexpectedly, NOS inhibition diminished vasoconstriction at low ET-1 concentration without altering maximal constriction which suggested ET-1 elicited the generation of a NOS dependent vasoconstrictor following DE exposure. Endothelial disruption similarly diminished ET-1 mediated vasoconstriction without altering maximal response in DE arteries. Therefore, acute DE exposure augments ET-1 constriction by generating an endothelial-derived NOS-dependent constrictor in addition to reducing bioavailable NO.

Contrary to the vascular reactivity data suggesting diminished bioavailable NO, we measured a 2-fold increase in plasma NO<sub>x</sub> levels, which has been used as an index of vascular NO levels. Although the origin of the plasma NO<sub>x</sub> was not determined in the present studies, the high level of NO in DE could contribute to the observed elevation in plasma NO<sub>x</sub> (54). Supporting this conclusion, rats exposed to pure NO by inhalation at a comparable concentration found in whole DE had similarly increased plasma NO<sub>x</sub> levels and enhanced vasoconstrictor sensitivity to ET-1 (47). Indeed, previous studies have suggested that exposure to high levels of exogenous NO can uncouple endogenous NOS (59), impairing NO generation. Although data is sparse linking ambient levels of NO to related mortalities, Filleul and colleagues found increased risk for non-accidental death

with elevated ambient  $\text{NO}_x$  levels (28). Future studies will address the possibility of excess NO in the whole DE as a contributing factor to the impairment of NO generation.

While systemic levels of  $\text{NO}_x$  were elevated with acute DE inhalation, basal NO production did not appear to be different in intraseptal coronary arteries. Constrictor responses to the depolarizing agent, KCl, were not different in endothelium-intact coronary arteries from AIR and DE-exposed animals. This is in contrast to a predicted greater response in DE arteries if there was a generalized loss of basal NO production. In addition, NOS inhibition augmented basal and KCl induced tone equally in both groups further supporting the contention that basal NO levels are not different between groups. In the KCl studies, low concentrations of KCl completely reversed basal tone (~20%) and maximally dilated arteries in both groups, presumably through the activation of inward rectifying potassium channels ( $\text{K}_{ir}$ ) and  $\text{Na}^+/\text{K}^+$  ATPase on the vascular smooth muscle (34). KCl induced constriction was also not different between groups after endothelial disruption supporting the observation that DE exposure specifically impairs agonist-induced endothelium-dependent dilation.

It appears that ET-1-mediated NOS activation was disrupted in the coronary arteries from the DE-exposed rats. Although ET-1 can bind to multiple receptors on the vasculature to elicit differential effects, ET-1 mediated constriction is primarily through the stimulation of  $\text{ET}_A\text{R}$  in the coronary vasculature (35). However, activation of vascular smooth muscle  $\text{ET}_B\text{R}$  also elicits contraction of the muscle, while endothelial  $\text{ET}_B\text{R}$  stimulate release of endothelium dependent dilators, including NO (38; 42). Although it is not clear if intraseptal coronary arteries express both receptors in the medial, muscle layer, the final level of tone after ET-1 stimulation should be the sum of these responses.

We used the selective receptor antagonist BQ-788 to assess the role of ET<sub>B</sub>R-mediated activation of the NOS pathway. ET-1 constriction was diminished in both groups with BQ-788 treatment in endothelial disrupted arteries indicating a minor contribution of smooth muscle ET<sub>B</sub>R to the ET-1 constrictor response. ET<sub>B</sub>R antagonism in endothelium-intact arteries from DE-exposed animals diminished the ET-1 constriction, reducing the response to that in untreated, intact AIR arteries. A modest BQ-788-sensitive constriction persisted following ET<sub>A</sub>R antagonism suggesting DE exposure impairs ET<sub>B</sub>R-mediated dilator function leaving only a constrictor effect. This constriction was nearly eliminated with endothelial disruption in the presence of BQ-123 indicative that ET<sub>B</sub>R-mediated constriction was endothelial in origin. Inhibition of ET<sub>B</sub>R and NOS similarly diminished constriction in DE arteries at low ET-1 concentrations suggesting NOS dependent vasoconstrictor production is stimulated by endothelial ET<sub>B</sub>R. Therefore, the overall effect of ET<sub>B</sub>R antagonism in endothelium-intact arteries from DE-exposed animals was to normalize constriction to that of the AIR arteries by apparent blockade of endothelial activation of a NOS-mediated constriction.

Alterations in the ET-1 system in various disease states have previously been shown to augment ET-1 constrictor sensitivity (25). In our studies, plasma ET-1 level was not different between groups after a brief 5 hour exposure. This is in contrast to previous reports of elevated plasma levels of ET-1 associated with air pollutant exposure. This may be due to differences in the air pollutant sources (i.e., urban ambient particles) affecting different molecular signaling pathways compared to DE. The discrepancy may also result from the excessive exposure dose, approximately 10-fold greater than the present study, used to elicit these observed changes (86; 94). Indeed, DE exposures

comparable to the current studies in humans also found enhanced constrictor reactivity to ET-1 infusion without differences in plasma ET-1 (51). However, Peretz observed increased plasma ET-1 an hour after cessation of DE exposure in humans (69) suggesting local elevations in ET-1 may require additional time to accumulate in the circulation, so that any potential increase was missed by the current study. Another possibility is species differences in responses and this may be more likely in the current study as preproET-1 transcript levels were not different between groups in a variety of tissues including cardiac tissue.

Augmented ET-1 constriction could also be mediated by increased ET receptor expression. In certain forms of hypertension, ET<sub>A</sub>R are unregulated and contribute to enhanced ET-1 mediated vasoconstriction. Blockade of these receptors with BQ-123 completely abolished vasoconstrictor responses (4; 5). Generally, we did not find alterations in ET<sub>A</sub>R or ET<sub>B</sub>R transcripts in various target tissues suggesting that acute DE exposure likely does not alter receptor expression. This was supported by isolated artery studies where endothelium disruption or NOS inhibition normalized and ET<sub>A</sub>R antagonism eliminated, ET-1 constrictor responses. Because transcript levels do not necessarily correlate with protein translation, additional studies to measure protein levels will be needed to address this discrepancy and to confirm the mRNA findings.

DE potentially can alter formation of ET receptor dimers which can regulate Ca<sup>2+</sup> mobilization. Formation of vascular smooth muscle ET<sub>A</sub>R and ET<sub>B</sub>R dimers has been shown to differentially modulate intracellular Ca<sup>2+</sup> signaling in the absence of receptor expression changes. Stimulation of endothelin receptor homodimers elicits a transient increase of intracellular Ca<sup>2+</sup> while heterodimer activation causes a sustained elevation in

intracellular  $\text{Ca}^{2+}$  (22). Increased heterodimer expression could therefore increase vasoconstriction without changes in receptor expression. Heterodimer formation and subsequent mobilization of  $\text{Ca}^{2+}$  is not affected by inhibition of either  $\text{ET}_A\text{R}$  or  $\text{ET}_B\text{R}$  alone and blockade of both receptors is necessary to prevent ET-1 induced dilation (22; 33). Because ET-1 constriction was diminished in endothelium-disrupted arteries from both groups with either  $\text{ET}_A\text{R}$  or  $\text{ET}_B\text{R}$  antagonism, our results imply smooth muscle ET receptor heterodimerization does not mediate DE induced changes in vasoreactivity. Further support of an endothelial  $\text{ET}_B\text{R}$  mediated dysfunction was the observation that the effect of  $\text{ET}_B\text{R}$  blockade was greater in endothelium-intact DE arteries. Therefore, the selective augmentation of ET-1 constriction that was prevented by endothelium disruption,  $\text{ET}_B\text{R}$  antagonism, or NOS inhibition suggests that DE affects ET-1-stimulated endothelial NOS, but not basal, enzyme activity.

The production of NOS-derived vasoconstrictors generated from uncoupling of the enzyme potentially contributed to augmented ET-1 constrictor response. Production of NO requires the dimerization of eNOS monomers and binding of various cofactors, including the redox sensitive  $\text{BH}_4$  (3; 29; 60). Endothelium dependent dilation was improved with ROS scavenging (55) implying DE induced ROS generation inhibits NOS function. DE has been shown to generate various ROS (8; 66) that can scavenge NO forming peroxynitrite to diminish NO bioavailability (49). Peroxynitrite can uncouple NOS by oxidizing  $\text{BH}_4$  to stimulate superoxide generation in place of NO (29). NOS derived superoxide can further augment tone both by exacerbating NOS uncoupling and directly mediating vasoconstriction (75; 98).

Glutathione (GSH) is one of the many endogenous antioxidant systems found in most cells that scavenge excess ROS. The tripeptide promotes proper enzymatic function of NOS by scavenging ROS and ONOO<sup>-</sup> to prevent the oxidation of BH<sub>4</sub>. Oxidation of GSH to GSSG can be reversed by GSSH reductase forming a regenerative redox cycle. Glutamate-cysteine ligase (GCL) catalyzes the rate limiting step of GSH synthesis and GCL polymorphisms are associated with lower plasma GSH and endothelial dysfunction (63). Populations with this mutation have a greater risk for myocardial infarction and potentially may be more susceptible to DE mediated effects (79). It is currently unknown if DE affects GCL expression or activity but inhibition of this, or other antioxidant, pathways would decrease the cellular capacity to modulate ROS levels and exacerbate the effects of DE.

Basal levels of superoxide were higher in coronary arteries from DE exposed rats compared to the AIR group supporting the hypothesis that ROS is a mediator of coronary artery dysfunction following DE exposure. The specificity of DHE fluorescence for O<sub>2</sub><sup>-</sup> was validated by the addition of tiron, a superoxide scavenger, which lowered the fluorescent signal to the levels in AIR arteries. NOS inhibition similarly lowered the DHE measured O<sub>2</sub><sup>-</sup> suggesting NOS dependent ROS generation was increased following DE exposure. Collectively, the data indicate that DE exposure alters NOS coupling and function with agonist stimulation and is not specific to ET. Therefore agonists that activate the NOS pathway should show similar impairment in vascular function.

To address endothelium dysfunction following DE exposure more directly, we assessed NOS function using the endothelium-dependent vasodilator ACh. ACh-mediated dilation was blunted in coronary arteries from DE exposed rats compared to

AIR rats. This impaired dilator response was reversed with NOS inhibition, in support of the generation and release of a NOS dependent constrictor following DE inhalation. NO has been shown to be an important regulator of vascular tone in the coronary vasculature, but NOS inhibition did not reduce ACh-induced dilation in the AIR group (11; 61). These data suggest either NOS is not a major pathway for ACh-mediated dilation in these small arteries or that compensatory dilator pathways were activated when NOS was blocked.

ACh mediates endothelium-dependent dilation by binding endothelial muscarinic receptors to initiate a  $Ca^{2+}$  signaling cascade that not only stimulates NOS function, but also activates other vasodilator pathways such as COX, SK and IK. Inhibition of COX activity with aspirin did not affect ACh dilator response in the DE group, but modestly diminished dilation in the AIR arteries compared to untreated AIR arteries. These data suggest a loss of COX derived vasodilator(s) following DE exposure and imply 1) neither NOS nor COX products mediate ACh dilation or 2) cooperativity between the two pathways in AIR coronary arteries that is lost after DE inhalation. The combined inhibition greatly blunted agonist induced dilation in both groups, suggesting there is indeed cross-talk between these two pathways.

Interactions between NOS and COX products apparent in the current study have been demonstrated previously in both cultured cells and isolated arteries (12; 93). Products from each of the two pathways have been shown to negatively inhibit the activity of the other, such that pharmacological blockade of one pathway alleviates the inhibition of the other. The net result of NOS inhibition is thus masked by the increased activity of the COX dilator pathway and vice versa. Therefore the concurrent inhibition of NOS and COX blunts dilation more than the sum of inhibiting either pathway alone.



The synergistic effect of combined inhibition of NOS and COX was seen in both AIR and DE arteries which suggests the NOS/COX interaction is not lost after DE exposure and plays an essential role in the regulation of coronary artery tone.

ACh-induced dilation was partially mediated by the activation of SK and IK channels but does not appear to be altered by DE exposure. SK and IK channel inhibition reduced dilation in both groups but dilation was still less in the DE compared to AIR arteries. Potassium channel blockers induce membrane depolarization that decreases the electrochemical driving force for  $\text{Ca}^{2+}$  entry into endothelial cells (23) potentially reducing NOS and COX activation. Therefore, these data alone do not implicate a role of SK and IK in ACh mediated dilation directly but because the combined inhibition of NOS, COX, SK and IK abolished dilation, it appears each pathway is an important contributor to ACh-mediated dilation.

Thus, parallel to the findings with ET-1, the diminished ACh-induced dilation in DE arteries appears to be mediated by NOS dysfunction. Supporting the previous speculation that DE inhalation leads to NOS uncoupling, sepiapterin supplementation antagonized the effects of DE insinuating the dysfunction results from a loss of endogenous  $\text{BH}_4$ . Both physical and electrochemical coupling of NOS requires sufficient cofactor bioavailability. DEP can generate an oxidative environment in a cellular system (65) and it is plausible that the increase in oxidative stress depletes  $\text{BH}_4$  levels. NOS derived  $\text{O}_2^-$  further contributes to additional  $\text{BH}_4$  scavenging and further exacerbates NOS and endothelial dysfunction (45; 60; 64).

Surprisingly, sepiapterin supplementation blunted endothelium dependent dilation in AIR arteries compared to untreated arteries. Sepiapterin indirectly elevates  $\text{BH}_4$  levels

by first releasing BH<sub>2</sub>, which is subsequently converted to BH<sub>4</sub> by dihydrofolate reductase through the salvage pathway (88). Saturation of dihydrofolate reductase activity shunts excess BH<sub>2</sub> to competitively bind to NOS, mimicking BH<sub>4</sub> depletion and NOS uncoupling (18; 81). The effects of sepiapterin supplementation were NOS dependent as L-NNA treatment prevented any changes in vascular reactivity of both groups. Therefore, sepiapterin treatment appears to have increased BH<sub>4</sub> levels to restore NOS function in DE-arteries while uncoupling NOS by elevating BH<sub>2</sub> levels in AIR-arteries.

Furthermore, BH<sub>4</sub> levels are regulated by a balance between the *de novo* and salvage pathways. Guanosine triphosphate cyclohydrolase I (GTPCH) is the rate limiting enzyme in the synthesis of BH<sub>4</sub> and is inhibited by lipid peroxidation products. One such product, 4-hydroxy-2-nonenal (4-HNE), promotes the proteosomal degradation of GTPCH (97). If DE exposure increases 4-HNE levels to decrease GTPCH and therefore reduce BH<sub>4</sub> availability, we would detect a decrease in GTPCH protein levels in conjunction with lower BH<sub>4</sub> levels. This may offer an alternative pathway by which DE mediates a decrease in this essential cofactor and NOS uncoupling (95).

Scavenging O<sub>2</sub><sup>-</sup> with PEG-SOD restored agonist-mediated dilation in DE coronary arteries without affecting dilation in AIR arteries. SOD catalyses the conversion of O<sub>2</sub><sup>-</sup> to hydrogen peroxide, which has vasodilator properties (58) and may partially

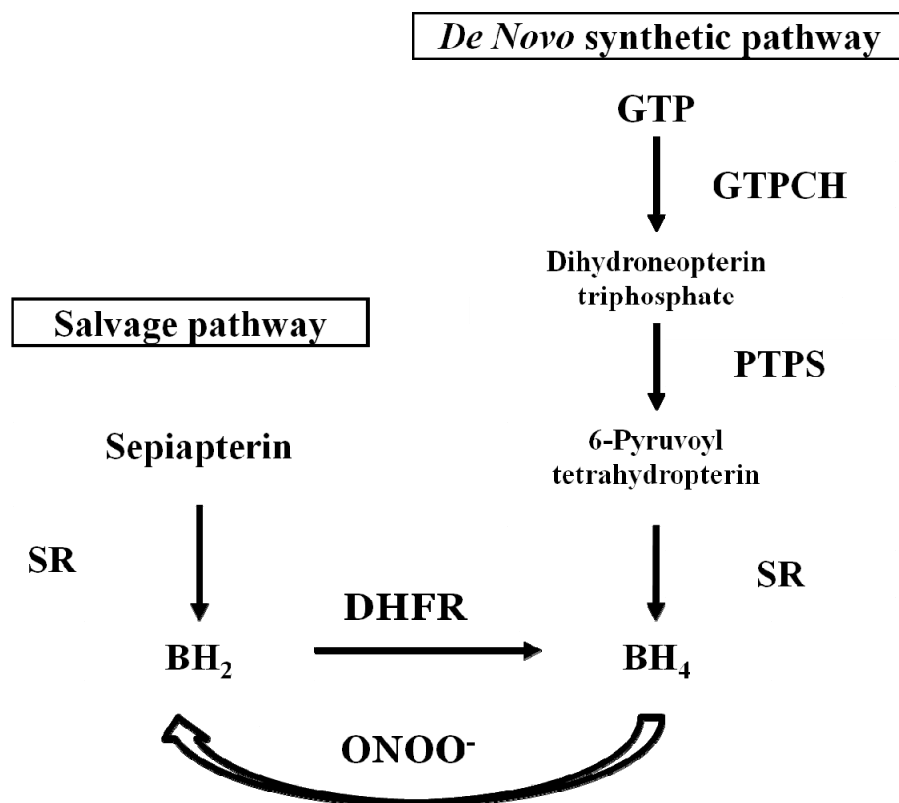


Figure 1. Biosynthesis of tetrahydrobiopterin (BH<sub>4</sub>). Dihydrobiopterin (BH<sub>2</sub>) Guanosine-5'-triphosphate (GTP), GTP cyclohydrolase I (GTPCH), sepiapterin reductase (SR), ONOO<sup>-</sup> (peroxynitrite), dihydrofolate reductase (DHFR).

mediate the restoration of ACh-induced dilation. However, given that NOS inhibition, BH<sub>4</sub> supplementation and ROS scavenging restored endothelial function, we draw the conclusion that DE inhalation uncouples NOS through increased ROS generation and not loss of H<sub>2</sub>O<sub>2</sub> production. It would be interesting to evaluate the combined effects of O<sub>2</sub><sup>-</sup> scavenging and BH<sub>4</sub> supplementation. If increased oxidative stress mediates NOS uncoupling, scavenging ROS would prevent uncoupling and BH<sub>4</sub> supplementation should blunt ACh mediated dilation similar to sepiapterin treatment alone in AIR arteries. To further evaluate our hypothesis that DE leads to NOS dysfunction, measurements of NO levels in the presence of PEG-SOD or sepiapterin would show increased NO levels compared to vehicle-treated DE arteries.

Although a single exposure to inhaled DE dramatically altered coronary artery vasoreactivity, changes in cardiac parameters were not as apparent. Calculated SNSA was not different between groups as there was withdrawal of both SNSA and PSNSA over the 5 hour exposure period in both groups. Similarly, plasma NE was not different between groups. Epidemiology studies of healthy participants further confirm the current findings by demonstrating that cardiac parameters, either immediately or hours post exposure, are not altered by exposure to air pollutants (20; 30). Furthermore, DE exposure-induced decreases in brachial artery diameter occurred in the absence of plasma catecholamine changes (69) indicating alterations in vascular reactivity can occur independent of changes in overall autonomic and cardiac parameters.

Individuals with preexisting cardiovascular disease and other susceptible conditions, however, do display changes in HRV after exposure to air pollutants. DE inhalation decreased HRV with persistent ventricular arrhythmia following exposure in a

chronic ischemic heart failure rat model (7). In the elderly population, decreased HRV was exhibited following elevated levels of air pollution (2). Interestingly, DEP instilled into rat lungs decreased HRV with an associated increase in inflammatory factors. However, inflammatory factors can augment SNSA and exacerbate changes in various heart rate variability and other cardiac parameters (24; 41; 53; 92). Therefore preexisting disease and local inflammation, absent in the current studies, appear to be requisite for DE-induced activation of SNSA.

Normal circadian patterns presumably contributed to the fall in heart rate observed in both AIR- and DE-exposed instrumented rats as the start of exposure coincided with the onset of the normal sleep period (9). However, the concurrent fall in MAP during the 5 hour period in the AIR rats was absent in DE-exposed rats. It is not evident if the maintained MAP is SNSA or PSNSA driven as changes in LF and HF were not different between groups. It is plausible that the differential MAP response results from increased vascular resistance as mediated by enhanced vasoconstriction and diminished vasodilation as observed in the cardiac (13; 15) and systemic vasculature (48) of isolated arteries following DE exposure.

In summary, DE inhalation augmented coronary artery ET-1 constrictor sensitivity and blunted ACh-mediated dilation through a NOS dependent pathway. Basal levels of NOS generated  $O_2^-$  are elevated following DE exposure and  $O_2^-$  scavenging, NOS inhibition or  $BH_4$  supplementation restored coronary function. Therefore our data suggest DE mediates endothelial dysfunction by depletion of  $BH_4$  to uncouple NOS (figure 2) without greatly altering hemodynamic parameters. Therefore, alterations in

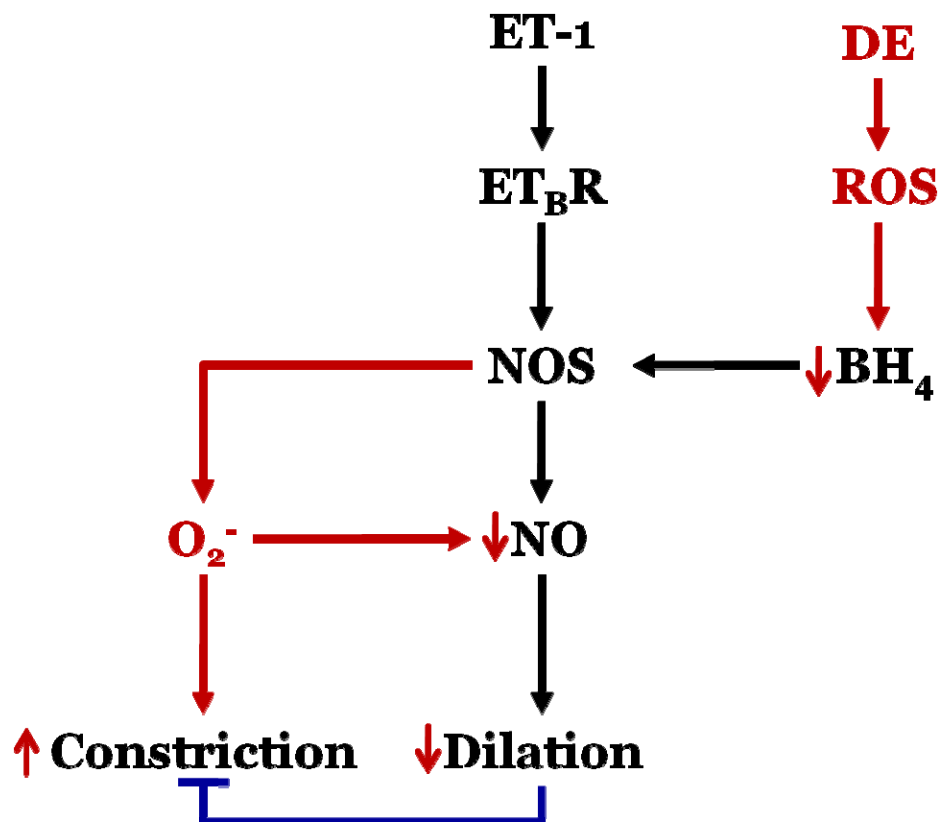


Figure 2. Mechanism of DE induced endothelial dysfunction of coronary arteries.

coronary and systemic artery function appear to drive the greatest initial insult on the vasculature which may not be apparent in healthy individuals..

### **Perspectives and Significance**

Metabolic syndrome is characterized by a cluster of risk factors that include elevated blood pressure, blood sugar, and plasma cholesterol and excess abdominal fat. Individuals diagnosed with metabolic syndrome are at greater risk for cardiovascular disease, stroke and diabetes (31; 32). Although air pollution exposure has been demonstrated to negatively impact cardiac parameters and vascular function independent of metabolic syndrome risk factors (68; 69), individuals with metabolic syndrome had a potentiated risk for the negative effects of particulate air pollution exposure (67). Populations with preexisting cardiovascular diseases, therefore, have greater risk of mortality following exposure and individuals with ischemic heart disease represent the most vulnerable subgroup for air pollution-related cardiovascular mortality (72). The increase in the onset of myocardial infarction an hour after exposure (71) suggests an immediate impairment of coronary artery function, possibly from a decrease of BH<sub>4</sub> bioavailability as observed in the current studies. Therefore, due to the preexisting impairment of coronary function, the subpopulation with CAD is at the greatest risk of mortality as air pollutants may exacerbate narrowing of the arteries to cause myocardial ischemia and infarction.

The progression of atherosclerosis is exacerbated by elevated levels of low density lipoprotein (LDL) and oxidative stress associated with many cardiovascular diseases (36). Formation of atherosclerotic plaques from the oxidation of LDL occludes coronary arteries to advance the development of CAD. Occlusions of the coronaries

decrease blood flow, reducing oxygen delivery to cardiac tissues. The increase in vascular resistance found in various forms of hypertension (5) can exacerbate the effects of CAD by increasing the workload of the heart and the demand for oxygen delivery. Without the compensatory increase in coronary blood flow to match the oxygen demands, chronic mild-to-moderate myocardial ischemia can result in progressive heart failure. Patients with CAD are often treated with statins to lower cholesterol synthesis and improve endothelial dysfunction by decreasing oxidative stress (19; 82). In addition to its effects on cholesterol regulation, statins can attenuate ambient air pollution induced inflammatory responses (77) which can slow the progression of atherosclerosis.

Furthermore, angiotensin-converting enzymes inhibitors are commonly used for the treatment of hypertension and have been suggested to improve CAD patient outcome by decreasing vascular resistance, blood pressure and improving endothelial function (26; 27). Lowering the level of angiotensin II may also reduce the impact of air pollution exposure by diminishing activation of NADPH oxidase to generate ROS, a source of NOS uncoupling (19). Coronary artery function can also be improved by exogenous NO treatment with nitroglycerin (85), a common treatment for angina pectoris. However, air pollution induced oxidative stress can potentially counteract the beneficial effects of nitroglycerin by scavenging NO and generating ONOO<sup>-</sup> to uncouple endogenous NOS. Our studies suggest that supplementation with antioxidants to scavenge ROS would improve the effectiveness of nitroglycerin following exposure to air pollution. Although these pharmacological interventions help manage and lower the risk of CAD, population studies still demonstrate an additional risk contributed from air pollution on cardiovascular mortality suggesting that further interventions are needed.



Based on the present studies, increasing biological levels of BH<sub>4</sub> in conjunction with current metabolic syndrome treatments may diminish the effects of air pollution on the cardiovascular system. Acute supplementation of BH<sub>4</sub> has been demonstrated to improve vascular function in various cardiovascular diseases. Coronary blood flow was improved in patients with CAD when BH<sub>4</sub> was co-infused with ACh (80). Patients with essential hypertension treated with BH<sub>4</sub> showed restoration of forearm blood flow to levels seen in normotensive controls (37). The beneficial effects of long term oral administration of BH<sub>4</sub> seen in patients with hypercholesterolemia may also protect from exhaust-induced vascular dysfunction. After 4 weeks of treatment, plasma BH<sub>4</sub> levels were elevated and NO-mediated forearm blood flow was augmented and normalized compared to healthy control subjects (16). Patients with uncontrolled hypertension on traditional stable antihypertensive therapy saw significant reduction in MAP with 4 weeks of oral BH<sub>4</sub> treatment. Brachial artery function was also improved with BH<sub>4</sub> supplementation although the effects on MAP and vascular function was reversible upon the cessation of the treatment (74). Currently, there are over half a dozen clinical trials in the U.S. evaluating the effectiveness of a synthetic BH<sub>4</sub>, sapropterin dihydrochloride (BioMarin Pharmaceutical), in patients with peripheral arterial disease, pulmonary arterial hypertension and coronary artery disease (46).

In addition to the potential benefits of oral BH<sub>4</sub> supplementation in subjects with preexisting cardiovascular diseases, the current findings suggest oral supplementation of BH<sub>4</sub> might blunt the detrimental coronary artery vascular effects of air pollution. Clinical studies with controlled exposures to diesel emissions in a manner similar to previous reports (56; 57) could easily confirm the relevance of supplemental BH<sub>4</sub> therapy.

However, further investigations to address the effects of supplemental BH<sub>4</sub> in healthy individuals are needed, as the data from the current studies caution against BH<sub>4</sub> “overload” as it may impair coronary artery function. Takeda and colleagues observed in the absence of plasma BH<sub>4</sub> level changes, elevated BH<sub>2</sub> levels correlated with a decrease in flow mediated dilation of the brachial artery. Consequently, an increase in BH<sub>4</sub>/BH<sub>2</sub> ratio positively correlated with flow mediated dilation and endothelial function (84). Therefore, additional clinical research is needed to determine the mechanisms of both the beneficial and detrimental effects of BH<sub>4</sub> supplementation.

DE constituents may be the initial source of the oxidative burst that leads to the oxidation of BH<sub>4</sub> to uncouple NOS, thereby initiating the feed forward cycle of endothelial dysfunction. The increase in DE-induced ROS may oxidize BH<sub>4</sub>, potentially hindering the beneficial effects of BH<sub>4</sub> supplementation. In conjunction with BH<sub>4</sub> supplementation, additional therapy with antioxidants should reduce air pollution mediated ROS generation to help stabilize and prolong BH<sub>4</sub> delivery to target organs. Romieu *et al.* showed decreased antioxidant function with exposure to air pollution in the elderly population of Mexico City (76). Omega-3 polyunsaturated fatty acid supplementation improved endogenous SOD activity and increased levels of plasma GSH thereby reducing air pollution induced oxidative stress. Increased dietary intake of omega-3 polyunsaturated fatty acids has also been associated with improved HRV parameters in the elderly (39). In hypercholesterolemia patients, acute infusion of vitamin C augmented agonist induced endothelium dependent vasodilatation of the brachial artery (89). Hornig *et al.* found oral administration of vitamin C for four weeks in patients with chronic heart failure restored flow dependent dilation that was L-NMMA sensitive (40).

These studies suggest endothelial dysfunction observed with metabolic syndromes are associated with elevated oxidative stress which can be attenuated with antioxidant treatment. However, it must be noted that long term follow up studies of vitamin supplementation found that increased plasma vitamin levels exhibited no significant reduction in mortality from cardiovascular disease (1).

More recently, we found that inhalation of comparable levels of exogenous NO found in DE elevated plasma NO<sub>x</sub> and enhanced ET-1 constrictor responses (47) paralleling the current findings with whole DE. In addition, depletion of BH<sub>4</sub> levels by oxidizing components in DE could contribute to exogenous NO induced uncoupling of endogenous NOS (59). In an oxidative environment induced by DE inhalation, exogenous NO may be transformed to ONOO<sup>-</sup> which then oxidizes and depletes the available BH<sub>4</sub>. This would lead to uncoupled NOS and propagation of ROS generation with resultant endothelial dysfunction. Therefore, increased antioxidant capacity may also be useful in the reduction of exogenous NO oxidation. However, all of this is conjectural and elucidation of the cell signaling changes initiated by air pollutants will greatly increase our understanding of possible interactions of pollutants and their products with both therapeutic medications and endogenous pathways that are altered by other cardiovascular diseases.

Alternatively, the cardiovascular effects of DE inhalation may not be a direct action of a constituent of DE (48; 52), but instead secondary to an inflammatory response (90). ROS-independent impairment of NOS-mediated relaxation following PM exposure has been shown to be reversed with anti-inflammatory drugs (17) implying effects are secondary to inflammation. However, we found that plasma levels of cytokines typically

associated with acute inflammation were not different between groups following DE exposure. This is in agreement with previous observations that C-reactive protein does not increase with DE exposure in healthy human subjects (14) corroborating our observation that vascular parameters can be altered in the absence of pulmonary inflammation.

Not surprisingly, the greatest impact in reducing air pollution related cardiovascular mortality to date has been by reducing non-natural sources of PM. In the mid 1960s, an 8.5 month copper smelter strike in 4 US Southwest states resulted in a 60% decline of sulfate PM that was associated with a significant drop in mortality. Likewise, the Harvard Six Cities study showed a strong association of air pollution with fine PM and increased cardiopulmonary mortality for a 15 year period from the mid 1970 in six (mid)eastern US cities (21). In an 8 year follow-up, the reduction in PM concentrations in the six cities was also associated with a decrease in mortality risk (50). Across the US, an examination of 51 metropolitan areas over 2 decade periods showed an increase, as much as 15%, in life expectancy associated with a  $10 \mu\text{g}/\text{m}^3$  reduction in PM (73). It should be acknowledged that while PM was the indexed metric, there were correlated declines in gaseous pollutants as well.

The US Environmental Protection Agency has implemented more stringent standards for both new highway diesel engines and fuels and nonroad diesel fuels. The reduction in diesel fuel sulfur (and therefore sulfur-containing emissions) allows the use of aftertreatment technologies to further reduce DE related pollutants, especially PM. However, other constituents of DE may remain relatively unchanged by these interventions, and warrant further study into potential health effects. It is estimated motor

vehicles account for half of nationwide NO<sub>x</sub> emissions and that diesel engines are responsible for 50% of those emissions. NO is oxidized readily in the atmosphere and promotes the synthesis of ozone. The cardiovascular effects of DE may thus be mediated in part by NO directly (47; 59) or indirectly through ozone, which potentially has adverse vascular effects (87; 96) and can potentiate the effects of DE (10; 44). Although these proposed and existing aftertreatment processes will reduce harmful emissions, our data suggest additional regulation of NO<sub>x</sub> products may be needed to truly mitigate the public and environmental health impact of DE exposure.

Epidemiology studies show the subpopulation with metabolic syndrome is most susceptible to the detrimental effects of air pollution exposure. Increases in oxidative stress and inflammation are underlying pathways that mediate the effects of air pollution on the cardiovascular system. Although standard-of-care pharmacological strategies to control the progression of cardiovascular disease may overlap with these underlying pathways affected by air pollution, our research suggests that exogenous BH<sub>4</sub> supplementation could be a complementary therapeutic in this subpopulation. More broadly, the general reduction of air pollution is still greatly beneficial to the immediate improvement of public health. Identification of key chemical pollutants of vehicular exhaust that are most harmful would guide advancements in aftertreatment technology and improve environmental policies.

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## APPENDIX

**Specific Aim:** Evaluate cardiac hemodynamic alterations during DE inhalation

**Hypothesis:** Acute inhalation of diesel engine exhaust augments heart rate and mean arterial pressure and decreases heart rate variability.

**Rationale:** Numerous epidemiological studies have demonstrated higher risk for cardiovascular mortality and morbidity with decreased air quality. It is estimated that on and off road diesel engines contribute approximately 20% of U.S. ambient air pollution. Cardiovascular changes in both human and animal models of DE exposure demonstrate alterations in cardiovascular function and homeostasis. Air pollutant exposure of susceptible animal models increases incidences of cardiac arrhythmias and ischemia along with alterations in blood pressure and cardiac autonomic balance as measured by heart rate variability. We therefore hypothesized that acute inhalation of DE augments heart rate and mean arterial pressure and decreases heart rate variability in healthy rodents. Additionally, augmented synthesis of endothelin following exposure has also been documented. Since the release of endothelin can be regulated by autonomic outflow we further investigated alterations in the endothelin system following exposure.

The manuscript is in preparation for submission to Cardiovascular Toxicology.



**Influence of inhaled diesel exhaust on heart rate variability, hemodynamics and endothelin Homeostasis.**

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Running head: Diesel exhaust and HRV

**Abstract**

Multiple studies show associations between diesel exhaust (DE) exposure and adverse cardiovascular sequelae. It has been proposed that inhaled pollutants can alter autonomic nervous system function, as well as the endothelin (ET) signaling pathway. We hypothesized DE inhalation elevates resting SNSA and ET vascular reactivity to mediate vascular and hemodynamic responses to this air pollutant. Rats inhaled DE (300  $\mu\text{g}$  particulate matter/ $\text{m}^3$ ) or AIR for 5 hours. Mean arterial pressure (MAP), electrocardiograms (ECG) and heart rate (HR) were recorded prior to and throughout the DE exposure using indwelling telemetry devices. Immediately following DE exposure, rats were euthanized and tissues obtained to evaluate ET expression. ECG data were analyzed to determine HR variability (HRV) as an index of sympathetic nervous system activity (SNSA). Rats inhaling DE exhibited a slight rise in MAP compared to baseline, while filtered air-exposed rats showed the typical circadian reduction of MAP. HRV analysis did not show differences between exposure groups. ET-3 and ET<sub>A</sub> receptor, but not ET-1 transcripts were increased in lungs from DE rats. In summary, elevated MAP following DE exposure may increase cardiac work load whereas increased expression of ET pathway components could diminish oxygen delivery. These data suggest that whole DE inhalation could be a contributing factor in the cardiovascular mortality associated with air pollution.

## Introduction

Multiple studies show associations between air pollution and the incidence of cardiovascular mortality and morbidity in urban populations (2; 14; 35). Cardiac arrhythmias, changes in blood pressure, and decreases in heart rate variability (HRV) are observed with decrements in air quality or controlled exposures to particulate matter air pollution in both humans and animal models (16; 27). However, the effects are often subtle and not always consistent, suggesting that several factors may play a role, from individual susceptibility to the physicochemical nature of the pollutant mixture. Recent evidence for near roadway exposures driving cardiovascular morbidity suggests that vehicular engine emissions, in total, may have a more potent effect than PM, alone (2; 28). In an elderly population, concentrated ambient air pollution particles (CAPS) caused a decrease in HRV (15) that was also observed with DE exposure(1). Vehicular emissions appear to impact the urban population significantly (18; 22; 29; 36) and are an obvious target to potentially improve health outcomes.

Of the sources of vehicular exhaust, diesel exhaust (DE) contributes significantly to the particulate load in urban settings (7). Several emerging studies indicate that specific components of DE acutely increase endothelin synthesis (25), endothelial dysfunction (20; 24), oxidative stress (4; 5), and blood pressure (33). Previously, we demonstrated coronary arteries from DE exposed rats displayed enhanced constriction to ET-1 and diminished dilation to ACh that was NOS dependent (12). Additionally, alterations in systemic arterial and venous function were also apparent following DE exposure (24). We have also observed that whole DE exposure in spontaneously hypertensive rats led to a persistent elevation in heart rate and increased AV nodal

conduction duration that lasted several days after exposures ended (9). Similar findings were observed in a model of chronic heart failure in rats where ventricular arrhythmias were persistent even after cessation of exposure (3). However, the effects of short term DE inhalation on cardiac parameters in healthy subjects are not clearly defined. We hypothesized that whole DE inhalation would decrease HRV and elevate MAP in healthy rodents.

## **Methods**

*Animals:* Animal protocols for this study were approved by the Institutional Animal Care and Use Committee of the Lovelace Respiratory Research Institute and followed the National Institutes of Health guidelines for animal use in research. Male Sprague Dawley and Fisher 344 (Charles River Laboratories, Reno, NV) rats weighing 250 to 300 g were used for all studies. Animals (n = 8 / group) were housed in chambers with free access to food and water and exposed to either 5 hours of filtered AIR or DE (300  $\mu\text{g}/\text{m}^3$  PM). MAP, HR and ECG were recorded before and throughout the DE/AIR exposure periods. Following the exposure, rats were deeply anesthetized with sodium pentobarbital (150 mg/kg) and tissues collected. Tissues were immediately placed in RNAlater (Ambion) overnight and stored at  $-80^\circ\text{C}$ .

*Mean arterial pressure:* Rats were instrumented with telemetry devices as instructed by the manufacturer (Data Sciences Instruments, Minneapolis, MN) to record mean arterial pressure (MAP), heart rate (HR) and electrocardiograms (ECG). After one week recovery, rats were exposed to either filtered AIR or DE as described above.

*Heart rate variability analysis:* RR interval (RRI, msec/beat), the time interval between heart beats, was extracted from ECG recordings from radiotelemetry implanted rats. RRI series was broken into 15 min intervals and transformed using custom software as previously described (10). The low frequency (LF) variability range, representing a composite of sympathetic nervous system activity (SNSA) and parasympathetic nervous system activity (PSNSA), was set at 0.2–1.2 Hz, while the high frequency variability (HF) range, representative of PSNSA, was set at 1.2–4.0 Hz. Sympathetic heart rate variability, expressed as the ratio of LF/HF of transformed RR intervals, was used as an index of SNSA. Heart rate (HR) was calculated from the recorded RRI (1 min/RRI = HR, BPM) or from telemetry recordings.

*Norepinephrine levels:* Immediately after the 5 hour exposure, blood was collected via cardiac puncture from anesthetized rats, chilled on ice, and centrifuged at 1000 rpm within 15 min of collection. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C. Plasma norepinephrine (NE) levels were assessed using a commercially available ELISA (BA 10-0200, Rocky Mountain Diagnostics, Inc).

*mRNA analysis:* Total RNA was extracted from coronary arteries using the RNeasy Fibrous Tissue Mini Kit (Qiagen). Reverse transcription-generated cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems), and quantitative real-time polymerase chain reaction (qRT-PCR) for ET<sub>A</sub>R, ET<sub>B</sub>R, ET-1, ET-3 and the endogenous control 18s was performed using an Applied Biosystems' Fast 7500 PCR machine and TaqMan Gene Expression Assays. Data are expressed as relative change from AIR group.

*Statistical analysis:* mRNA transcripts level was expressed as a percent of AIR. HR and HRV were expressed as actual values while MAP was expressed as a percent

change from the hour prior to exposure. All data are expressed as means  $\pm$  SEM. We compared changes between groups using two-way analysis of variance and  $p < 0.05$  was considered statistically different.

## Results

*Heart rate variability:* The effect of DE exposure on SNSA was estimated by calculating the LF/HF ratio as described above. AIR exposure did not alter SNSA compared to the hour prior to exposure. DE exposure also did not alter SNSA during the 5 hour exposure period nor was activity different from AIR (Figure 1). There was a drop in both the LF and HF in both groups during the exposure period that was more pronounced at specific time points in the DE-exposed rats than in the AIR-exposed (Figures 2 and 3). There were no differences in plasma NE levels at the end of the 5 hour exposure (Figure 6). The lack of change in LF/HF appears due to concomitant withdrawal of both HF and LF power in both groups.

*Heart rate:* In both groups, HR decreased from the pre-exposure to exposure period due to the normal morning circadian pattern. HR was not different between groups prior to exposure. HR dropped the greatest in the first two hours in both groups. Both groups displayed a reduction in HR that was sustained for the duration of exposure. The reduction in HR was significantly greater in the DE exposed animals compared to the AIR during the 2nd and 3rd hour of exposure (Figure 4).

*Mean arterial pressure:* MAP was greater in the DE group prior to the start of exposure. AIR animals displayed a slight fall in blood pressure during the five hour exposure compared to the hour prior to exposure. However, animals exposed to DE did not have a similar fall in MAP throughout the five hour period (Figure 5) but maintained

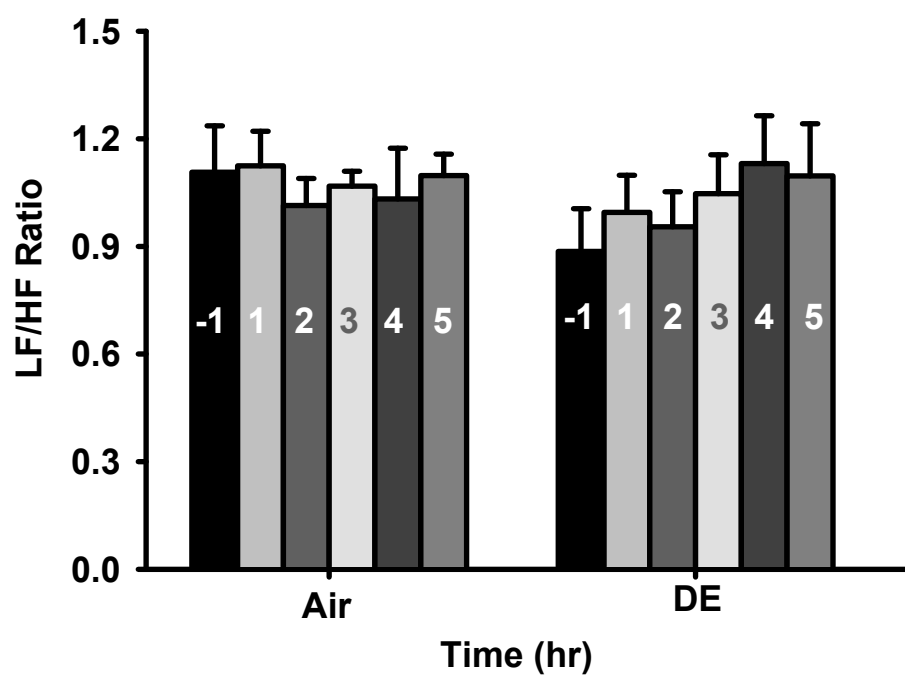


Figure 1: LF/HF from rats exposed to AIR or DE. Compared to the hour prior, animals exposed to either AIR or DE did not have HRV alterations.

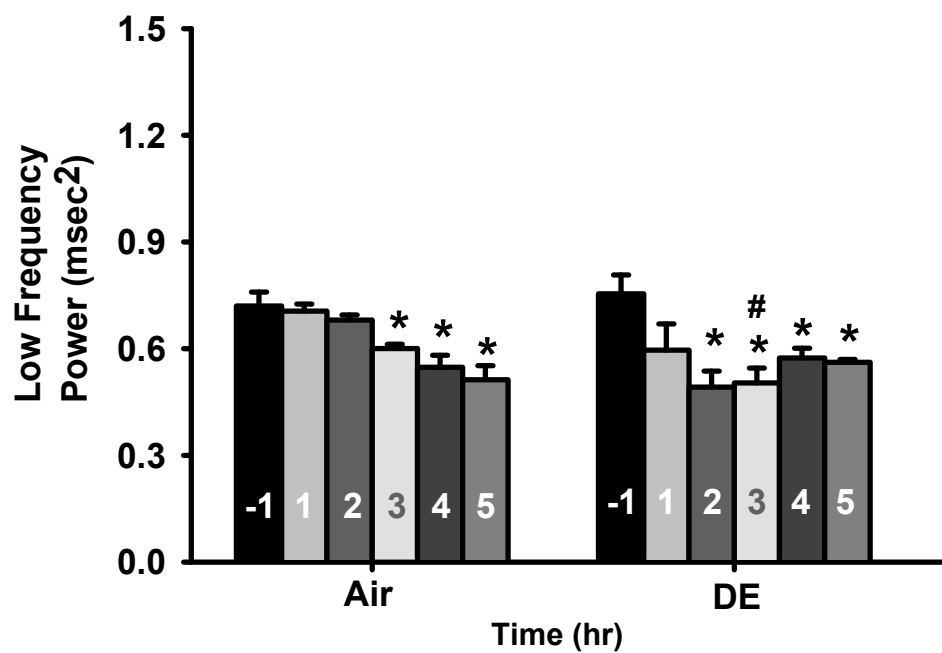


Figure 2: Low frequency component of HRV from rats exposed to AIR or DE. Both groups showed an equal decrease in LF power during the five hour exposure compared to the hour prior. \* < 0.05 compared to hour prior within group. # <0.05 compared between groups.



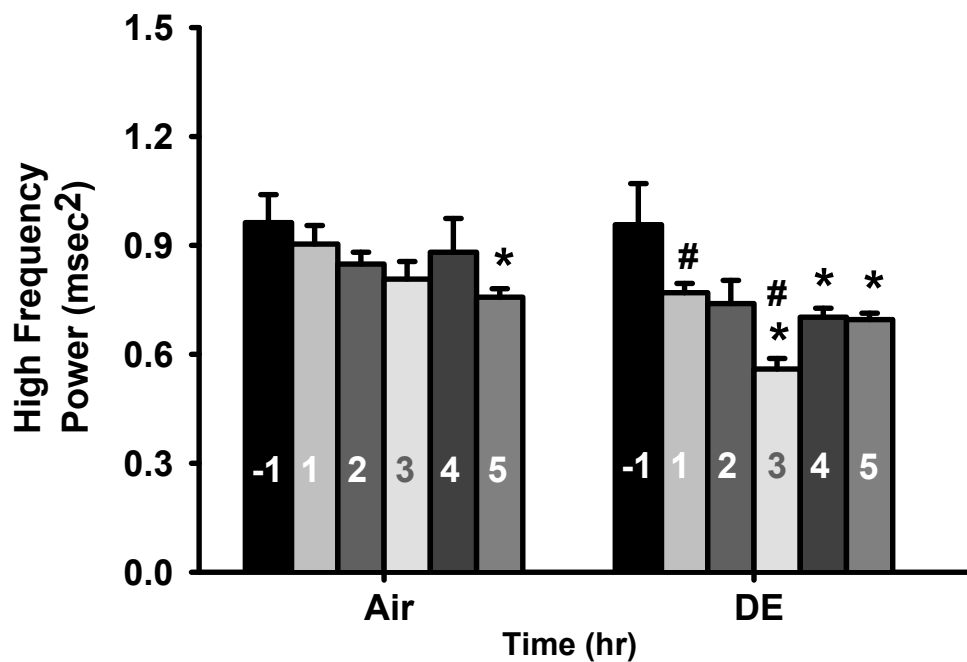


Figure 3: High frequency component of HRV from rats exposed to AIR or DE. Both groups showed an equal decrease in HF power by the end of exposure compared to the hour prior. DE animals tend to show greater parasympathetic withdraw compare to the AIR. \* < 0.05 compared to hour prior within group. # < 0.05 compared between groups.

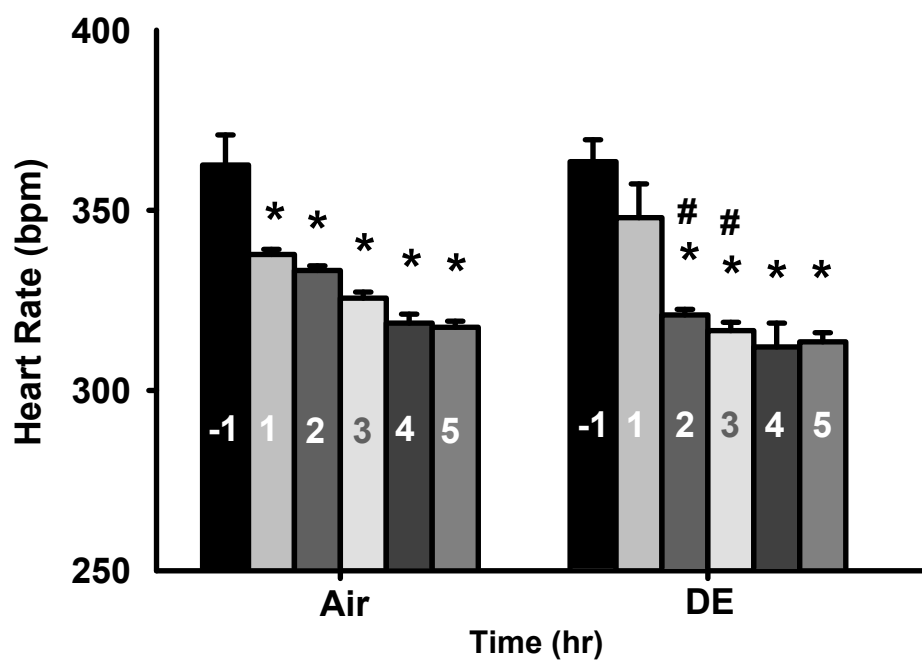


Figure 4: Heart rate showed an equal fall in both groups during the exposure period. \* < 0.05 compared to hour prior within group. # < 0.05 compared between groups.

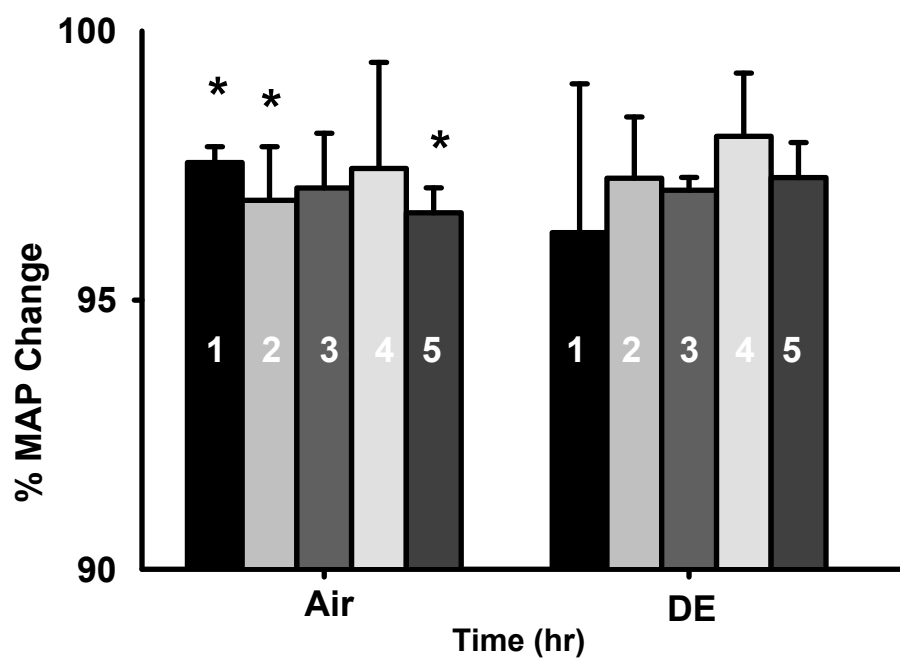


Figure 5: DE exposure prevented fall in mean arterial pressure. DE animals had an elevated MAP prior to the start of exposure and maintained the elevated pressure. \* < 0.05 compared to hour prior within group. # < 0.05 compared between groups.

higher MAP compared to AIR. Thus there was a significant difference between AIR and DE rats throughout the five hours of exposure.

*Endothelin system mRNA transcripts:* ET-1 transcripts were not different between groups from lung, aorta or renal tissue (Figure 6). DE exposure elevated ET-3 message in lung tissue but not in renal tissue compared to AIR. ET<sub>A</sub>R transcripts were elevated in lung, but not aorta. ET<sub>A</sub>R tended to be elevated in the renal medulla but not in the cortex.

## **Discussion**

In the present study we investigated HRV and hemodynamic alterations following inhalation to DE. We observed that, in healthy rats, acute DE exposure had little acute effect on HRV and MAP, consistent with these systems being under tight homeostatic control. However, the subtle effects of DE were consistent with a disruption in circadian control of the autonomic influence of HRV and MAP, which may be augmented by certain conditions such as hypertension, sleep-disordered breathing, or metabolic syndrome. We also found that DE augments the various components of the endothelin system, which may contribute to longer term effects on coronary reactivity and cardiac morbidity.

Numerous epidemiology studies report increased cardiopulmonary mortality rates proportional to pollution levels. We have previously shown that coronary arteries from rats exposed to DE for 5 hours have increased ET-1 sensitivity (12) and diminished ACh-mediated dilation (13) that was endothelial NOS-dependent. Endothelial dysfunction can be rescued by NOS inhibition or supplementation of necessary cofactors. Diminished NOS production of NO in coronary arteries would narrow the coronary arteries to

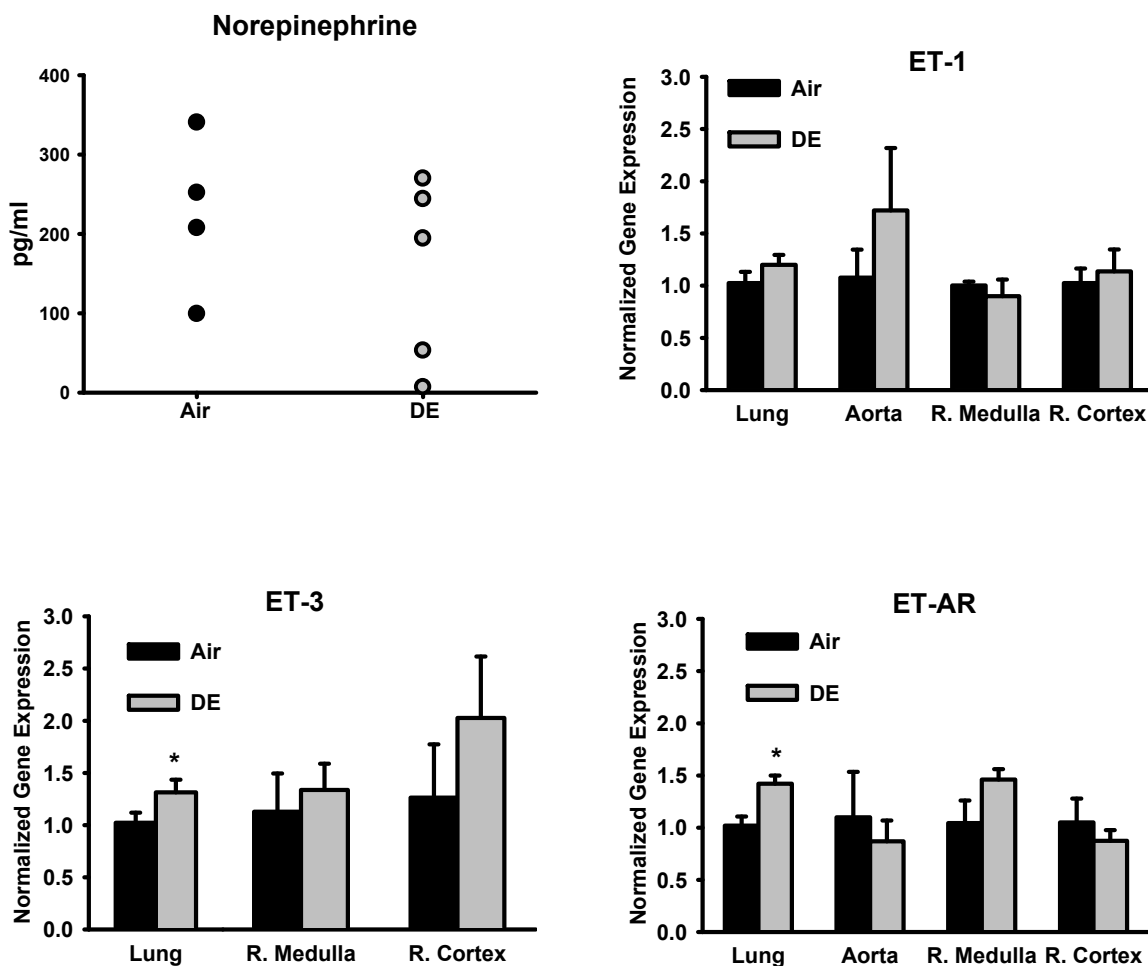


Figure 6: Plasma NE levels were not different between groups. ET-1, ET-3 and ET<sub>A</sub>R transcripts were altered by DE exposure in aorta, renal medulla and renal cortex tissue. ET-3 and ET<sub>A</sub>R were elevated but not ET-1 mRNA in lung tissue following acute exposure to DE. \*  $p < 0.05$  compared between groups.

decrease blood flow to the heart. Cardiac ischemia in turn could contribute to the increase in cardiovascular related mortality with decreases in air quality level.

Animals from both groups displayed a fall in LF and HF indicating both SNSA and PSNSA decrease at the onset of the sleep period even in the presence of DE inhalation. Thus the calculated SNSA was not different between groups prior to or during the 5 hours of DE exposure. DE-exposed animals tended to have a greater parasympathetic withdrawal compared to AIR animals as calculated by the HRV data, which correlated with HR changes during the first hour of exposures, where DE-exposed rats exhibited a delayed onset of circadian HR reductions. Beyond that timepoint, however, HR tended to decrease more in the DE rats than the AIR rats. In agreement with this observation, plasma NE, drawn at the end of the exposures, was not different between groups. These data are in agreement with studies of healthy individuals who do not have altered cardiac parameters either immediately or 24 hour after exposure to concentrated ambient air pollution particles (15). Similarly healthy volunteers exposed to ultrafine carbon particles at rest did not have measurable differences in cardiac parameters (17). Therefore it appears that exposure to multiple air pollutants does not cause acute cardiac dysfunction in the absence of pre-existing heart disease.

In contrast to several previous reports (1; 3; 11; 26), we did not find dramatic changes in HRV after exposure to air pollutants. DE inhalation caused a more profound withdrawal of both HF and LF power, but this was significant compared to the filtered air-exposed rats at only a few time points. In a rat model of chronic ischemic heart failure, DE inhalation decreased HRV during the exposure period with persistent ventricular arrhythmia following exposure (3). We have also previously shown that

spontaneously hypertensive rats exposed to DE exhibit mild but consistent changes in ventricular conduction, in terms of PR intervals (9). Decreased HRV is also apparent in the elderly population following increases in air pollution levels, primarily from parasympathetic withdrawal (1). Additionally, Huang et. al recently reported that rats instilled with DE particles (DEP) in the lungs had decreased HRV primarily from increased HF and not LF decreases (i.e. more SNSA without PSMSA withdrawal) (23). Along with decreased HRV, instillation of DEP also increased levels of inflammatory factors which have been shown to augment SNSA (34) through increased NADPH oxidase generation of reactive oxygen species (37). We previously reported that inhaled DE in healthy rats does not detectably induce circulating inflammatory factors (12). Therefore preexisting disease and local inflammation in response to direct installation of DEP appear to be requisite for DE-induced activation of SNSA.

In agreement with subtle changes in HRV, heart rate fell similarly in both groups during the exposure paradigm, with the exception of the first hour of exposures. This fall in heart rate is due to the normal circadian pattern, as the start of the DE exposure period coincided with the onset of the normal sleep period (6). Similarly, a matching decline in MAP was observed during the 5 hour period in the air-exposed rats. However, the expected fall in MAP was not observed in the rats exposed to DE. The lack of change in LF and HF suggests the prevention of MAP dipping is not overtly SNSA or PSNA driven. Alternatively, our previous report that acute DE inhalation uncouples NOS to enhance vasoconstriction and diminish vasodilation in the cardiac (12) and systemic vasculature (24) is consistent with elevated vascular resistance leading to the differential

MAP response. Augmented constriction in systemic resistance vessels may therefore contribute to the relatively elevated MAP.

In order to investigate a potential activation of the endothelin system, we examined transcripts from this system in lungs, aorta and kidneys from AIR and DE exposed rats. There were no detectable changes in ET-1 transcripts in the four tissue targets immediately after exposure, in agreement with the previous findings that plasma ET-1 levels are not acutely altered by DE (12). It has been shown that higher exposure levels of particles (32) or collection of tissue at later time points (31) does reveal DE-induced generation of endothelin.

In contrast to the ET findings, ET<sub>A</sub>R and ET-3 mRNA were both elevated in lung tissue by DE exposure. Greater endothelin receptor expression in the lung potentially increases vascular resistance to elevate pulmonary arterial pressure resulting in right ventricular hypertrophy. Unlike activation of the ET<sub>A</sub>R, activation of the ET<sub>B</sub>R primarily mediates vasodilation through activation of eNOS (21) ET-3 is a somewhat selective endogenous ligand for the ET<sub>B</sub>R and the elevation in ET-3 transcript could indicate greater activation of the uncoupled endothelial NOS pathway. We have previously shown that activation of this system in coronary arteries enhances vasoconstriction (12) and this effect may also contribute to the loss of the diurnal fall in MAP. Recent findings by Brook *et al* showed that ambient particulates could induce a modest increase in diastolic BP that was non-significantly attenuated by treatment with a general ET-1 receptor antagonist, Bosentan (8). The mild effects observed in that study are consistent with our findings, and suggest that ET-1 has either a partial role in driving cardiovascular



effects of air pollution or there are vulnerable subpopulations in which this pathway may be more active than in others.

Overall, acute inhalation of diesel emissions did not result in large HRV or hemodynamic changes, likely due to the overall health of the animal model. As reported by many epidemiologic studies, the populations at the highest risk for coronary events are the elderly and those with preexisting cardiovascular diseases. Therefore, rapid change in vascular function with short term exposure to pollutants may provide an explanation for the spike in hospital admission for various cardiac conditions while chronic elevations in air pollution and prolonged exposure maybe required to alter cardiac parameters enough in healthy individuals to result in heart failure (19; 30).

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