

The Role of Hypoxia in Air Pollutant-Induced Cardiovascular Dysfunction

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Abstract

CHRISTINA M. PEREZ: The Role of Hypoxia in Air Pollutant-Induced Cardiovascular Dysfunction

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Diesel exhaust (DE) is a major contributor to traffic-related urban air pollution and has been associated with cardiovascular dysfunction in humans, especially susceptible individuals. DE is a complex pollutant consisting of particles and toxic gases such as acrolein. Although the mechanisms that mediate adverse cardiovascular health effects are unclear, epidemiological evidence has linked exposure to air pollution to drops in blood oxygen saturation, suggesting that hypoxia may play a role. Acute and repeated hypoxia is associated with carotid body-mediated cardiovascular effects which overtime lead to hypertension and predisposition to cardiac arrhythmia. Thus, my overarching hypothesis for this dissertation project is that air pollutant-induced hypoxia mediates the adverse cardiovascular effects of air pollution exposure. To test this hypothesis, we 1) characterized the impacts of short-term exposure to DE on cardiovascular physiology in rats, 2) characterized the cardiovascular response to exposure to the DE component acrolein, and determined if acrolein exposure increases the risk of adverse cardiovascular responses to a cardiac stressor (i.e, hypoxic atmosphere) in hypertensive rats, and 3) determined if inhibition of the sensory response to hypoxia attenuated air-pollutant-induced cardiovascular dysfunction. Exposure to DE caused PR prolongation and ST depression, a marker of myocardial ischemia, only in hypertensive rats exposed to the gaseous components of DE. Because the gaseous components appeared to be driving the responses, studies were

conducted to assess the effects of acrolein. Exposure to acrolein caused increases in heart rate and blood pressure. These responses were confined to the hypertensive rat, and subsequent stress testing with hypoxic atmosphere (10% FiO₂) confirmed enhanced sensitivity with increased diastolic blood pressure in the hypertensive rat. We also found that acrolein exposure significantly decreased arterial pO₂, and carotid body inhibition prevented acrolein-induced blood pressure increases and impaired contractility responses in hypertensive rats. This suggests that air pollutants may cause hypoxia and that the cardiac responses following pollutant exposure may be mediated by the carotid body. This research describes a novel mechanism that mediates the adverse cardiovascular effects of air pollutant exposure and fills important data gaps in our understanding of air pollution-induced cardiovascular dysfunction.

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List of Abbreviations

AHA, American Heart Association

ANS, autonomic nervous system

APB, atrial premature beat

ApoE^{-/-}, apolipoprotein E-deficient

AVB, atrioventricular block

BP, blood pressure

BPM, beats per minute

Ca²⁺, calcium ion

CAPs, concentrated ambient particles

cDNA, complementary DNA

CEMs, continuous emission monitors

CIH, chronic intermittent hypoxia

CO, carbon monoxide

COPD, chronic obstructive pulmonary disease

CV, cardiovascular

DE, diesel exhaust

dP/dt_{max}, maximum up-slope in pressure

dP/dt_{min}, minimum down-slope in pressure

ECG, electrocardiogram

EDV, end diastolic volume

ESV, end systolic volume

fDE, filtered diesel exhaust

FIO₂, fraction of inspired oxygen

HAPs, hazardous air pollutants

Hct, hematocrit

HF, high frequency

HIF-1, hypoxia inducible factor 1

HO-1, heme oxygenase-1

HR, heart rate

HRV, heart rate variability

HVR, hypoxic ventilatory response

IL-6, interleukin 6

K⁺, potassium ion

LF, low frequency

MAP, mean arterial pressure

MI, myocardial infarction

Na⁺, sodium ion

NAAQS, National Ambient Air Quality Standards

NATA, National-scale air toxics assessment

NF-κB, nuclear factor- kappa B

NO, nitric oxide

NO₂, nitrogen dioxide

NOS, nitric oxide Synthase

NTS, nucleus of the solitary tract

O₃, ozone

OSA, obstructive sleep apnea

PAH, polyaromatic hydrocarbon

Pb, lead

pCO₂, partial pressure of carbon dioxide

PCR, polymerase chain reaction

PM, particulate matter

PM_{2.5}, “fine particulate matter”, less than 2.5 µm in diameter

PM₁₀, “coarse particulate matter”, between 2.5 and 10 µm in diameter

pO₂, partial pressure of oxygen

ppb, parts per billion

ppm, parts per million

QTc, heart rate-corrected QT interval

RARs, rapidly adapting pulmonary receptors

RAS, renin-angiotensin system

RNA, ribonucleic acid

RMSSD, square root of the mean of squared differences of adjacent RR intervals

ROFA, residual oil fly ash

ROS, reactive oxygen species

SAB, sinoatrial block

SaO₂, oxygen saturation

SARs, slowly adapting pulmonary receptors

SDNN, standard deviation of the RR interval

SEM, standard error of the mean

SH, Spontaneously Hypertensive

SHHF, Spontaneously Hypertensive Heart Failure

SO₂, sulfur dioxide

T_e, expiratory time

TEOM, tapered element oscillating microbalance

tHB, total hemoglobin

TLR, toll-like receptor

TRP, transient receptor potential channel

UFP, ultrafine particulate matter

US EPA, United States Environmental Protection Agency

VBP, ventricular premature beat

VOCs, volatile organic compounds

VT, ventricular tachycardia

wDE, whole diesel exhaust

WHO, World Health Organization

WKY, Wistar Kyoto

Chapter 1

Introduction

History of Air Pollution Epidemiology and Policy

Air is all around us. Air sustains life and brings growth, but it is also vulnerable to pollution from human industry and global progress. In the twenty-first century, we recognize that the decisions we make affect the environment around us, and numerous companies are investing in “green” energy and more efficient practices. Despite this invigorated desire to protect the air we breathe, the history of air pollution epidemiology is rich with stories of human progress mixed with environmental destruction and an ever evolving understanding of the relationship between human health and our air. It was not long ago that major industrialized cities of the world boasted some of the worst breathing conditions, a fact that still remains true for many developing countries. Revisiting these situations is important in realizing both how far we have come in our understanding of the health effects of air pollution exposure but also as a warning that our environment is an ever changing entity that must be consistently protected to ensure human health.

The British industrial revolution that began in the late 1770’s presents some of the most widely read accounts of the horrific air quality in London caused by excessive use of coal. Charles Dickens’s Bleak House, published in 1852, opens with this detailed description of the air in industrial London:

London. Michaelmas term lately over, and the Lord Chancellor sitting in Lincoln's Inn Hall. Implacable November weather ... smoke lowering down from chimney-pots, making a soft black drizzle, with flakes of soot in it as big as full-grown snowflakes—gone into mourning, one might imagine, for the death of the sun... Fog everywhere. Fog up the river, where it flows among green aits and meadows; fog down the river, where it rolls defiled among the tiers of shipping and the waterside pollutions of a great (and dirty) city. (Dickens, *Bleak House*).

Despite well documented accounts of severe air pollution during the 1800s and even for centuries before that time, the link between air pollution exposure and adverse effects on human health did not become apparent until the early twentieth century with three key air pollution events. In 1930, an acute air pollution event occurred in the Meuse Valley of Belgium leading to the death of sixty residents and numerous cattle (Firket, 1936). The narrow, 15 mile strip of land held 14 different industrial centers including coke ovens, steel mills, and zinc smelters. A temperature inversion in December caused abnormally high levels of air pollution that led to a 10.5 times increase in mortality and left almost 6000 residents ill with respiratory disease and complaints of eye and throat irritation (Jun, 2009). Despite the severity of this incident, additional events would have to occur to solidify the link between air pollution exposure and adverse effects on human health. In the fall of 1948, a heavy smog settled over Donora, Pennsylvania, a town located on a river with multiple industries including a steel mill and zinc production facility. The steady accumulation of air pollutants from the factories caused increased mortality and over 40% of the town's residents to be affected with acute illness during the high air pollution episode (Jun, 2009). Several years

later, the infamous “London smog” of 1952 forever linked the association between dangerous levels of air pollution and increased morbidity and mortality. This acute air pollution event led to over 4000 excess deaths and a dramatic rise in hospital admissions for cardiovascular and respiratory related injuries (Logan, 1953). Similar to the other classic acute air pollution events, many of the deaths occurred in patients with pre-existing cardiovascular or respiratory diseases. These three examples as well as many other events that occurred during the 1960’s and 1970’s led to the understanding that exposure to air pollution can increase morbidity and mortality in human populations and set the stage for both policy changes to monitor air pollution and more in-depth studies to decipher how air pollution effects human health.

By the late 1940’s in the United States, there were a few state-initiated laws involving air pollution control, but it was not until 1955 that the federal government passed the Air Pollution Control Act, the first federal legislation that allocated funds to study the effects of air pollution (Roman and Saundry, 2007). The Clean Air Act of 1963 was the first federal legislation involving the control of air pollution, and the subsequent Air Quality Act in 1967 expanded the federal government’s ability to monitor and control air pollution levels. These laws and others created the groundwork for the landmark Clean Air Act amendment in 1970 which resulted in a radical shift in the role of the federal government regarding air pollution control and research. The Clean Air Act of 1970 established emission standards for both stationary and mobile sources of air pollution and allowed the development of the National Ambient Air Quality Standards (NAAQS). On December 2, 1970, the United States Environmental Protection Agency (US EPA) was created to implement the new laws now governing the country. The US EPA established six criteria pollutants including ozone (O₃),

sulfur dioxide (SO₂), particulate matter (PM), nitrogen dioxide (NO₂), carbon monoxide (CO), and lead (Pb) that would be included in the NAAQS with appropriate standards being reviewed every five years in order to monitor the evolving understanding of the different pollutants and ensure human health (US EPA http://epa.gov/air/caa/caa_history.html). In addition to the criteria pollutants, the US EPA set standards for Hazardous Air Pollutants (HAPs) and stationary sources of air pollution. Since the implementation of this revolutionary act, our regulation of air pollution from multiple sources has greatly evolved and our understanding of the link between air pollution exposure and adverse effects on human health has increased exponentially.

Despite huge advancements in policy and scientific understanding, the regulation of air pollution levels in the United States and abroad remains a daunting necessity with a variety of challenges. In the United States, our landscape has changed from several large industrial cities surrounded by unspoiled expanses of rural lands to a suburban sprawl with air pollution spread diffusely over the landscape (Costa and Amdur, 1996). This change in air pollution dynamics tests the government's ability to regulate air pollution levels on such a vast scale and highlights the necessity of increased awareness and increased regulation outside of the traditional industrialized city centers. On a global scale, high levels of air pollution are still a common problem in developing countries. The World Health Organization (WHO) recently released more stringent standards for global levels of the six criteria pollutants, but most cities in developing countries such as Asia, Africa, and Latin America have baseline air pollution levels that greatly exceed WHO recommendations (Chen and Khan, 2008). These recommendations focus mainly on outdoor air pollution, but for many nations, indoor air pollution from biomass burning is a major source of particle and gas

exposure and greatly increases an individual's overall exposure to air pollution (Fullerton *et al.*, 2008). Although the following information will focus primarily on the health effects of air pollution in United States cities, it is critical to understand that regulating air pollution levels to protect human health is as relevant today as it was when the Clean Air Act was passed in 1967. The history of air pollution epidemiology is a continuing mosaic with pieces from a variety of nations and governments. Through past examples of acute air pollution events and the modern challenges we face domestically and abroad, it is critically important to understand that the air we breathe not only sustains our lives but presents a rich history of human evolution, environmental awareness, and the importance of public health policy in protecting human health.

Modern Epidemiology and Health Effects of Criteria Air Pollutants

Studies decades after the acute air pollution events in the 1930's to 1950's established strong links between exposure to air pollution and increased human morbidity and mortality. The exact health effects of air pollution exposure and what components were causing those responses still need to be deciphered. Numerous epidemiological studies have demonstrated clear adverse health effects associated with each of the criteria pollutants. The current NAAQS standard for O₃ is 75 ppb over an 8 hour period (NAAQS US EPA). Ozone exposure has been associated with increased mortality (Levy *et al.*, 2005), asthma exacerbation (Dockery and Pope, 1994), and increased hospital and emergency room visits (Choi *et al.*, 2011). In addition, individuals with underlying pulmonary and cardiovascular disease have increased susceptibility to ozone exposure (Burnett *et al.*, 2001). The current NAAQS standard for CO is 9 ppm over an 8 hour period (NAAQS US EPA), and prolonged

CO exposure has been associated with altered redox status and ion homeostasis as well as adverse effects on cardiovascular health (Reboul *et al.*, 2012). The national standard for NO₂ is 100 ppb during a 1 hour period (NAAQS US EPA), but epidemiological studies have calculated that each increase of 5 ppb NO₂ is associated with a 12% increase in mortality from cardiovascular disease (Chen *et al.*, 2013). Another criteria air pollutant strongly associated with adverse exposure effects is SO₂ with a national standard of 75 ppb for every 1 hour period (NAAQS US EPA). Exposure to SO₂ has been linked to increased risk of myocardial infarction and increased hospitalizations for cardiovascular events (Amancio and Nascimiento, 2012). In addition to these major components of air pollution, PM has been strongly associated with adverse health effects and a significant amount of research has characterized the response to this dynamic pollutant.

Health Effects of Particulate Matter (PM)

Since the establishment of the NAAQS, an enormous amount of research has been conducted to characterize the effects of each criteria pollutant. Although all criteria pollutants are dangerous to human health and important to consider in any air pollution exposure, PM has probably been the most intensely studied over the last two decades. There are three major categories of PM based on size. The first is PM₁₀, or particulate matter less than or equal to 10µm in aerodynamic diameter. The current NAAQS standard for coarse particulate matter is 150 ug/m³ over a 24 hour period (NAAQS US EPA). The second is PM_{2.5}, or particulate matter less than or equal to 2.5 µm in aerodynamic diameter. The current NAAQS standard for fine (PM_{2.5}) particulate matter is 35 µg/m³ over a 24-hour period (NAAQS US EPA). Finally, ultrafine particles (UFPs) are defined as particles less than or equal to 0.1 µm in

diameter (Brook *et al.*, 2010). There is no current standard for UFPs. While each of these particle size ranges has been associated with adverse health outcomes in humans, most of the evidence implicates fine and coarse PM.

Several key epidemiological studies defined the adverse health effects of PM exposure. The Harvard Six Cities Studies which began in 1974 estimated the effects of air pollution on mortality in 8111 adults in six different US cities including Portage, Wisconsin; Topeka, Kansas; Watertown, Massachusetts; St. Louis, Missouri; Harriman, Tennessee; and Steubenville, Ohio (Dockery *et al.*, 1993). The study found that residents in the most polluted city had a 1.26-fold higher likelihood of air pollution associated mortality than the least polluted cities, and this mortality was associated with PM_{2.5} and sulfate exposure (Dockery *et al.*, 1993). In addition, the increased mortality after air pollution exposure was most strongly associated with lung cancer and cardiopulmonary diseases after an individual's smoking habits were taken into account (Dockery *et al.*, 1993). Several years later, the link between PM exposure and cardiopulmonary disease was further demonstrated with the analysis of a cohort from an American Cancer Society study that found that 45% of the deaths associated with PM were due to cardiovascular disease and 8% of deaths were due to respiratory disease (Pope *et al.*, 2004). Specifically, the deaths due to cardiovascular disease were predominately due to ischemic heart disease, dysrhythmias, heart failure, and cardiac arrest (Pope *et al.*, 2004). The association between air pollution exposure and increased mortality due to cardiopulmonary disease has been further strengthened by subsequent epidemiological studies.

Additional studies demonstrated that inhalation of particulate matter (PM) associated with ambient air pollution causes adverse effects on cardiovascular function. Peters *et al.*

(2004) found that increases as small as 10-20 $\mu\text{g}/\text{m}^3$ PM_{2.5} caused significant increases in the risk of myocardial infarction up to 1 day after exposure. In addition, individuals who had been hospitalized for coronary artery disease showed increases in T wave area (suggesting impairment in myocardial repolarization) after exposure to ambient levels of black carbon (Zanobetti *et al.*, 2010). PM inhalation has also been associated with myocardial ischemia (Gold *et al.*, 2005; Wellenius *et al.*, 2003) and arrhythmias (Brook *et al.*, 2004), especially in susceptible populations such as the elderly and those with underlying cardiovascular disease. Epidemiological studies indicate that these effects are not immediate, usually manifesting some time after exposure. Patients with implanted cardioverter defibrillators had increased incidence of life-threatening arrhythmias up to 2 days after air pollution exposure (Peters *et al.*, 2000). These findings suggest that exposure to air pollution alters cardiovascular physiology and increases conditional susceptibility to triggers of thrombosis and arrhythmia. Moreover, the fact that these effects happen at lower exposure concentrations than those shown to elicit effects in animal studies indicates that the responses are more complicated than the standard monotonic dose-response relationship of traditional toxicology. Several mechanisms have been proposed to account for these responses to air pollution exposure including autonomic imbalance, systemic inflammation-mediated vascular dysfunction, and direct actions of particles on the heart and vasculature (Donaldson *et al.*, 2001; Schulz *et al.*, 2005; Utell *et al.*, 2002). Much work remains to further delineate these mechanisms in order to provide biological plausibility for the health effects observed after short-term fluctuations in ambient levels of PM.

Diesel Exhaust

There are many sources of PM in the environment, but diesel exhaust is one of the major contributors to fine and ultrafine PM air pollution (EPA/600/8-90/057F 2002). Vehicular traffic is a dominant source of ambient PM, particularly in urban environments, and studies have shown that proximity to traffic sources (e.g., highway or tunnel) is a major determinant of cardiovascular health outcomes (Hoek *et al.*, 2002; Hoffman *et al.*, 2006; Van Hee *et al.*, 2009). Peters *et al.* (2004) found that exposure to traffic with high levels of DE was associated with onset of myocardial infarction. Similarly, a study in eight European countries attributed hospitalizations for acute coronary syndrome in older patients to exposure to DE (Le Tertre *et al.*, 2002). In a controlled human exposure study, Mills *et al.* (2007) found that DE exposure accentuated exercise-induced electrocardiographic ST depression (potential indication of myocardial ischemia) in subjects with known coronary artery disease and exercise-induced ischemic ECG changes. While DE is a significant contributor to PM, it is also a source of other pollutants constituting a complex mixture of particulate and gaseous components. DE also consists of a mixture of gases including nitrogen oxides (NO_x), sulfur oxides (SO_x), carbon monoxide (CO), and volatile organics including aldehydes, benzene, and polycyclic aromatic hydrocarbons. The complex composition of DE makes attributing specific health effects to one or more components within DE challenging.

Acrolein

One notable toxic component of DE is acrolein, a pulmonary irritant that has been associated with reflex pulmonary irritation and changes in autonomic tone. Acrolein is an

α,β -unsaturated aldehyde that is formed during the combustion of organic materials and is a toxic components of cigarette smoke and internal combustion engine exhaust (Baeuchamp *et al.*, 1985). The most recent national scale assessment (NATA) released by the US EPA has listed acrolein as the number one national noncancer hazard driver (NATA 2005). Acrolein is a widespread environmental pollutant and ambient levels of 0.04 - 0.08 ppm have been measured (Costa and Amdur, 1996). Cigarette smoke, however, contains up to 90 ppm acrolein (Esterbauer *et al.*, 1991), and acrolein levels in sidestream tobacco smoke are as high as 10 ppm (Esterbauer *et al.*, 1991). Acrolein is highly reactive and will immediately bind and deplete glutathione levels in cells as well as form adducts by reacting with protein residues (Keher and Biswal, 2000). Acrolein can cause oxidative stress and inhibit cell proliferation *in vivo* and can also illicit a host of reflex responses due to its high reactivity. Acrolein exposure in primary human hepatocytes resulted in increased apoptosis, decreased intracellular glutathione, and activation of stress-signaling MAP-kinases (Mohammad *et al.*, 2012). In human bronchial smooth muscle cells, acrolein exposure causes induction of IL-8 and leads to phosphorylation of MAP kinase substrates (Moretto *et al.*, 2012). In addition, intravenous injection of acrolein in rats causes significant increases in blood pressure that are reversed with guanethidine, a sympatholytic drug (Green and Egle, 1983). DE and its components present highly relevant pollutants to utilize in controlled experiments to characterize the cardiovascular effects of air pollution exposure.

Susceptibility to Air Pollution

Many of the adverse responses to air pollution occur in susceptible subpopulations, groups with a heightened risk for an adverse endpoint compared with the general population

(Brook *et al.*, 2010). Multiple epidemiological studies have demonstrated that adverse events following PM exposure are heightened in the elderly (Pope *et al.*, 2002) and in individuals with diabetes (Zanobetti and Schawart, 2001), preexisting heart disease (Katsouyanni *et al.*, 2001) or heart failure (Goldberg *et al.*, 2001). Individuals with pre-existing coronary artery disease had a significant increase in the risk of a cardiac event after PM exposure even if they did not experience any symptoms of the underlying disease (Pope *et al.*, 2006). In addition, increases in PM_{2.5} have been associated with more hospitalizations for heart failure than any other cardiopulmonary disease (Dominici *et al.*, 2006), and daily increases in PM₁₀ have been shown to cause increases in heart failure hospitalizations in older adults (Schwartz and Morris, 1995). Obesity and current smoking status have also been suggested as susceptibility markers for air pollution exposure (Miller *et al.*, 2007). Because many of the adverse responses observed during and after air pollution exposure occur in susceptible groups, it is critical to model susceptibility in experimental studies to more accurately reflect physiological responses.

Modeling Susceptibility

To understand the mechanisms behind adverse effects of air pollution exposure in susceptible populations, researchers model exposures in the laboratory using rodents with underlying disease. One of the primary rodent models used to study the cardiovascular effects of air pollution is the Spontaneously Hypertensive (SH) rat. The mechanisms accounting for the elevated sensitivity of the SH rat are uncertain, but may relate to the structural, biochemical, and physiological characteristics of the cardiovascular system attendant to prolonged hypertension. Previous studies have shown that SH rats have, on

average, 40 mmHg higher mean arterial pressure than background control rats with normal blood pressure (El-Mas and Abdel-Rahman, 2005) as well as greater arterial wall thickness (Mulvany and Halpern, 1977). The hypertensive rat was developed in 1963 when two Wistar Kyoto rats with spontaneous hypertension were mated (Hulstrom, 2012). Although the exact genetic determinates of spontaneous hypertension in this model are not fully characterized, the hypertensive phenotype of the SH rat is believed to be related to vascular damage leading to arterial hypertension in the juxtamedullary cortex of the kidney resulting in dysfunction (Hulstrom, 2012). Over time, hypertension leads to structural and biological remodeling of the left ventricle characterized by hypertrophy, fibrosis, and changes in membrane channels, cellular energetics and ion regulation that combine to heighten myocardial sensitivity (Bernardo *et al.*, 2010). Such remodeling has been demonstrated in SH rats (Goltz *et al.*, 2007) and may account for the differences between the SH rat and rats with normal blood pressure. It has previously been demonstrated that exposure to both particulate or gaseous air pollutants causes exaggerated cardiovascular effects in rat models of hypertension and heart failure (Carll *et al.*, 2012; Farraj *et al.*, 2011; Hazari *et al.*, 2009; Lamb *et al.*, 2012).

Mechanisms of Adverse Cardiovascular Effects After PM Exposure

Our understanding of the mechanisms underlying air-pollution induced cardiovascular function has increased substantially since the first epidemiological studies suggested a link between PM exposure and increased cardiovascular morbidity/mortality. While multiple mechanisms have been postulated, there are three widely-acknowledged hypothesized mechanisms of action. One postulates that health effects result from the translocation of PM and/or its components into the systemic circulation with injury resulting

from the direct interaction of PM/components with blood vessel walls and/or the myocardium. Fine and ultrafine PM can deposit deep within the lung and penetrate through the lung tissue to the capillaries (Peters *et al.*, 2006). Researchers have found that some UFPs were able to translocate to the liver within 4 to 24 hours post exposure, and entered the olfactory bulb and possibly crossed the blood-brain barrier (Oberdorster *et al.*, 2004). Despite evidence for translocation of particles, this mechanism is probably the least understood with limited evidence to support such effects.

The second mechanism of PM-induced cardiovascular dysfunction postulates that PM inhalation triggers a local pulmonary oxidative injury and inflammation. This local response leads to systemic oxidative stress and inflammation characterized by an increase in activated white blood cells, platelets, and cytokine expression. This activation of immune components causes inflammation of the liver and increased vasoconstriction leading to higher thrombogenicity and coagulation (Brooks *et al.*, 2010). The exact pathway by which PM inhalation leads to oxidative stress and inflammation is multivariable and complex with particle size and site of deposition being large determinants of the response. UFPs may have the ability to enter lung cells directly and interact with mitochondria (Muhlfeld *et al.*, 2008) while larger particles are taken up by macrophages through the innate immune response (Møller *et al.*, 2010). Some particles, such as metals, may generate reactive oxygen species (ROS) directly or through biotransformation, while others may interfere with iron homeostasis and generate ROS's through Fenton reactions (Ghio and Cohen, 2005). This increased oxidative stress can lead to inflammation with increased signaling of toll-like receptors (TLR) and mitogen activated protein kinases that lead to the production of pro-inflammatory transcription factors such as NF- κ B (nuclear factor- κ B) and the increased

production of inflammatory cytokines (Nel *et al.*, 2006). Perhaps the major consequence of PM-induced systemic inflammation is the targeting of the vascular endothelium, the inner lining of the blood vessel wall. Under normal physiological conditions, the endothelium mediates vascular dilation to maintain normal blood pressure and ensure adequate organ perfusion, prevents platelet activation and clot formation, and inhibits adhesion of leukocytes (Madden, 2012). Evidence in both humans and animal models has shown that PM causes endothelial injury thus promoting vasoconstriction, thrombosis, and inflammation (Krishna *et al.*, 1998). These effects can lead to atherosclerotic plaque progression, coronary vasospasm, and myocardial ischemia (Madden, 2012).

The third mechanism for PM-induced cardiovascular dysfunction postulates that PM inhalation triggers lung reflexes that modify autonomic nervous system control of cardiovascular function (ANS). The ANS controls many of the visceral functions and is composed of two branches, the sympathetic branch that is responsible for the “fight or flight” response and the parasympathetic branch that is responsible for returning the body to a homeostatic baseline. One of the ways that researchers can measure changes in the ANS in both humans and experimental animal models is by measuring heart rate variability (HRV). HRV is the degree of difference in the interbeat intervals of successive heartbeats, and is an indicator of the balance between the sympathetic and parasympathetic arms of the autonomic nervous system (Rowan *et al.*, 2007). The heart is a dynamic organ and must be able to respond to changes in the body’s activity level. High HRV is traditionally considered positive because the heart has the ability to respond to rapidly changing environments. Low HRV, reflecting increased sympathetic tone (Rowan *et al.*, 2007), is associated with an increased risk of cardiac arrhythmia (Corey *et al.*, 2006) and an increased risk of mortality in people

with heart disease (Bigger *et al.*, 1993). An association between high ambient PM and low HRV has been observed in several different studies in both healthy individuals and those with cardiovascular disease (Chuang *et al.*, 2005; Schulz *et al.*, 2005; Vallejo *et al.*, 2006). Exposure to PM causes significant reduction in HRV and is associated with increased risk of myocardial infarction, cardiac arrhythmias, and sudden cardiac death, especially in susceptible populations (Sinnreich *et al.*, 1998; Singh *et al.*, 2003). The elderly and individuals with preexisting cardiovascular diseases have been shown to have significant decreases in HRV after exposure to PM (Liao *et al.*, 1999). The response of the autonomic nervous system is an orchestrated series of events that is influenced by a variety of sensory responses in the lungs and vasculature. The established link between air pollution exposure and changes to autonomic function presents an important relationship between reflex responses and environmental exposures.

Autonomic Reflex Arcs and Their Perturbation by Air Pollution Exposure

The body has a complex and multivariate system that responds to environmental stressors by triggering autonomic reflexes. One of the first responses that occurs after inhalation of certain air pollution components or different irritants is activation of pulmonary neural reflexes that send information to the brain through various airway sensory receptors. The activation of these receptors depends on location of particle deposition as well as chemistry and reactivity of the irritant. In addition to activation of pulmonary sensory mechanisms, the baroreceptor response may also be elicited. Baroreceptors are localized mechanoreceptors in the body that respond to changes in blood pressure through a negative feedback mechanism. Baroreceptors sensing elevations in blood pressure will communicate

with the brainstem to decrease heart rate and subsequently blood pressure. In addition to the pulmonary reflex response and the baroreceptor response, the body also utilizes a chemoreceptor response that senses changes in blood gases and leads to downstream responses to maintain homeostasis. It is important to note that the reflex responses in the lung communicate with the baroreflex and chemoreflex responses, and each pathway influences the other responses to coordinate the body's homeostatic control of breathing and heart rate at baseline and following exposure to a stimulus such as air pollution.

Pulmonary Neural Reflexes/Lung-Airway Sensory Receptors

There are three major types of sensory receptors in the lungs and airways: C-fibers, rapidly adapting pulmonary receptors (RARs or irritant receptors), and slowly adapting pulmonary receptors (SARs or stretch receptors) (Coleridge *et al.*, 1984). Inhalation of cigarette smoke, SO₂, and acrolein have been shown to activate pulmonary C fibers leading to reflex bronchospasm, apnea, and bradycardia (Lee *et al.*, 1987). These responses were inhibited with capsaicin treatment of the vagus nerve, the predominate parasympathetic signaling pathway, to block conduction of C fibers and with bilateral vagotomy (Wang *et al.*, 1996). It has been observed that an increase in inspiration occurs only after C fiber afferents are blocked, showing that the irritant response to air pollutants occurs through a combination of C fiber and RAR receptor activation with C fiber activation being the predominate driver of the response (Lee *et al.*, 1992). A variety of ion channels are responsible for the responses seen in the different pulmonary receptors. One of the most well characterized responses in the lung is ion channel activation following exposure to hypoxia, or low ambient oxygen. Acute hypoxia will lead to pulmonary vasoconstriction while chronic hypoxia will lead to

pulmonary vascular remodeling. Acute hypoxia increases cytosolic Ca^{2+} through inhibition of voltage gated K^+ (K_v) and tandem pore domain K^+ (K_T) channels, activation of voltage gated Ca^{2+} channels (VDCC), and alteration of Ca^{2+} storage in the sarcoplasmic reticulum (SR) in pulmonary artery smooth muscle cells (PASMCs) (Mauban *et al.*, 2005; Fig. 1.1). The complex pulmonary sensory and ion channel response to irritants and environmental stressors such as hypoxia also elicits a host of responses in the cardiovascular system.

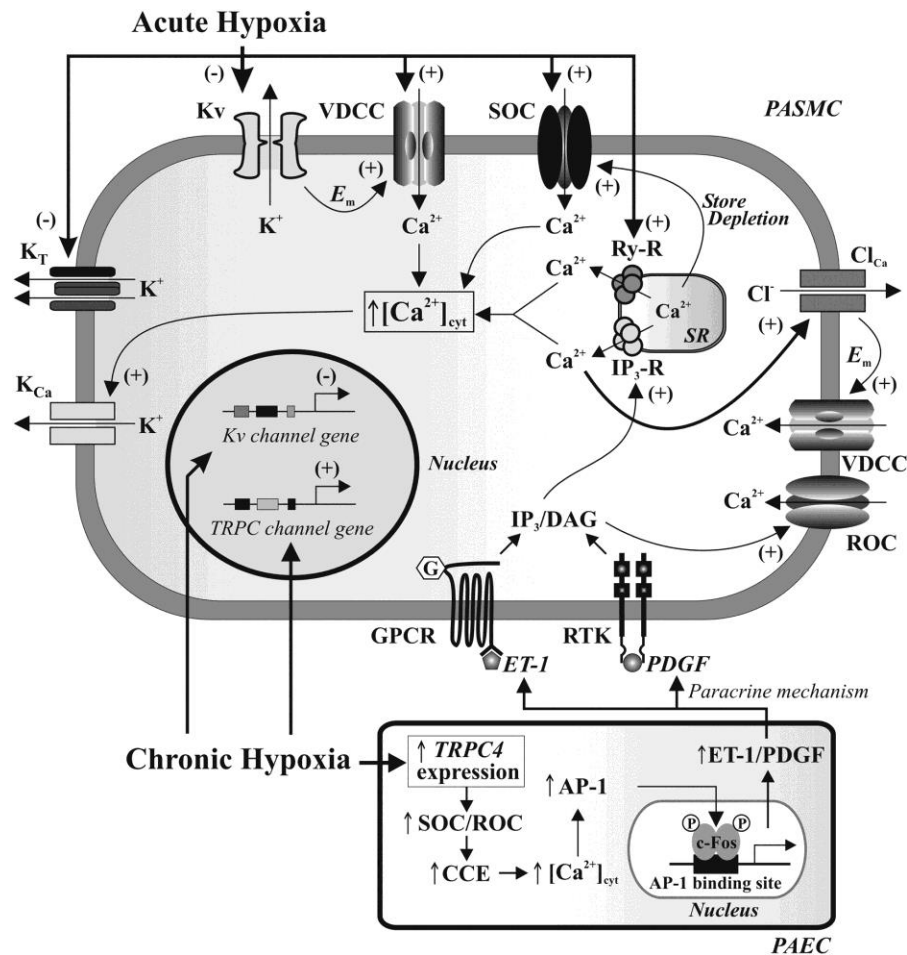


Figure 1.1. The role of ion channels in the response to hypoxia. (Reprinted with permission from Mauban *et al.*, 2005).

In addition to causing changes in tidal volume and breathing frequency, pulmonary reflex responses also influence cardiovascular responses to air pollutants. Inhalation of irritants in the upper airway tract causes reflex bradycardia coupled with increases or decreases in heart rate depending on the airway site (Widdicombe and Lee., 2001). Administration of atropine prevents bradycardia, showing that the response is predominately vagally driven (Widdicombe and Lee, 2001). In the lower airways, C fibers and RARs will respond strongly to inhaled irritants and lead to pronounced bradycardia and hypotension (Lee *et al.*, 2001). The pulmonary and cardiovascular systems are intimately related, and airway reflexes originating in the larynx have been shown to cause cardiac dysrhythmia and ST depression (Prys-Roberts *et al.*, 1971). The lung and heart are intimately connected, and physiologically, the two systems are always referred to as the cardiopulmonary system. Many of the responses in the lungs from sensory receptor activation will lead to a host of downstream events affecting heart rate and blood pressure. The mechanism by which the body responds to changes in blood pressure is through the baroreceptor response, and like the reflex pulmonary response, activation of baroreflex receptors will lead to downstream responses that are meant to maintain homeostasis and allow the body to respond to diverse stimuli.

Baroreceptor Reflex Responses

The baroreceptor reflex is the body's way of maintaining control of blood pressure. Baroreceptors are stretch sensitive mechanoreceptors located in the carotid sinus and aortic arch, and each location is innervated by the glossopharyngeal nerve and vagus nerve, respectively, which communicate downstream with the nucleus of the solitary tract (NTS) in

the brainstem (Williamson *et al.*, 2006). The baroreceptor reflex functions through a negative feedback mechanism where an increase in blood pressure will be sensed by the receptors and causes a reflex decrease in heart rate and blood pressure. The same is true for decreases in blood pressure eliciting a reflex increase in heart rate and subsequently blood pressure. Studies using isolated carotid sinuses found that when pressure was lowered in the sinus, it caused a reflex increase in systemic arterial blood pressure and heart rate (Walgenbach and Shepard, 1984). Upon receiving input from the baroreceptors, the NTS communicates with regions of the brain controlling the autonomic nervous system and leads to an inhibition of the sympathetic nervous system and an activation of the parasympathetic nervous system (Lohmeier and Iliescu, 2011). The baroreceptor reflex is crucial in day-to-day homeostatic control, but it has also been implicated in the response to air pollution exposure.

Multiple studies have reported significant changes in blood pressure and baroreflex sensitivity following air pollution exposure. In a cohort of German citizens, individuals living in close proximity to high traffic had higher arterial blood pressure and greater prevalence of hypertension than individuals living in low traffic areas (Fuks *et al.*, 2011). Exposure to particulate matter causes significant increases in diastolic blood pressure in healthy adults and this increase was coupled with decreases in heart rate variability (Brook *et al.*, 2009). The baroreceptor reflex has important influence over changes in heart rate variability through its communication with the brainstem. In addition to changes in blood pressure and autonomic control, healthy non-smokers exposed to sidestream cigarette smoke had significant increases in muscle sympathetic nerve activity (Hausberg *et al.*, 1997). Rats exposed to carbon nanotubes had alterations to baroreflex sequences (Legramante *et al.*, 2009), and exposure to concentrated ambient particles (CAPs) in dogs resulted in increases in

arterial blood pressure and significantly greater baroreflex sensitivity that was reversed with alpha-adrenergic blockade (Bartoli *et al.*, 2009). These studies demonstrate both the importance of the baroreceptor reflex in controlling blood pressure changes and the relationship between exposure to air pollution and baroreflex activation. The baroreceptor reflex is a mechano-reflex that responds to changes in blood pressure mainly through stretch receptors, but the body also utilized chemoreceptors that respond to stimuli such as air pollutants.

Chemoreceptor Reflex Response- Carotid Body Reflex Response to Hypoxia

Both the pulmonary and baroreceptor reflex responses are integrated in the peripheral chemoreflex receptors, including the carotid body. Hypoxia is defined as the lack of sufficient oxygen to tissues. In mammals, the response to systemic hypoxia is controlled by the carotid body. The carotid body is a cluster of chemo-sensitive cells located at the bifurcation of the carotid artery and serves as the major chemoreceptor for pO₂ levels in mammals (Conde *et al.*, 2004). In response to hypoxia, the carotid body releases excitatory neurotransmitters that elicit reflex hyperventilation to compensate for reduced oxygen levels through a HIF-1 mediated pathway (Roux *et al.*, 2000; Liu *et al.*, 2003; Lopez-Barneo *et al.*, 2008). Carotid body signaling then leads to increased sympathetic activation, which increases blood pressure, ventilation, heart rate, and arrhythmogenicity (Lopez-Barneo *et al.*, 2008). The carotid body response is also closely linked with baroreceptor response, and signals from both will be integrated in the brainstem (Fig. 1.2; McGraw Hill Companies). Chronic hypoxia is associated with increased myocyte apoptosis and is implicated in cardiac remodeling and progression to heart failure (Bao *et al.*, 2011). In addition, hypoxia has been

shown to cause atrial fibrillation, AV block, and ventricular tachycardia as well as changes in autonomic balance associated with ventricular arrhythmias (Davies *et al.*, 1993). Many of these responses occur after carotid body sensing of hypoxia and present a potential pathway to explain the role of hypoxia in air-pollutant induced cardiovascular function.

The body has a very specific response to hypoxic environments known as the hypoxia ventilatory response (HVR). The first step is a reflex increase in ventilation, which is followed by a gradual decrease or plateau if hypoxia is chronic. These responses are heavily influenced by the pulmonary reflex response discussed previously. The body is actually much more sensitive to changing levels of carbon dioxide than it is to oxygen, and the central chemoreceptor in the medulla will respond to hypercapnia and increase the HVR. Peripheral chemoreceptors, including the carotid bodies and aortic bodies, sense changes in oxygen and carbon dioxide levels and communicate with the nucleus tractus solitarius which has downstream communication with the respiratory network and can stimulate movement of the diaphragm (Richard and Koehle, 2012). This will also lead to increased minute volume and, in some cases, hypertrophy of the carotid body, especially in the event of chronic hypoxia. Individuals who suffer from obstructive sleep apnea (OSA) and chronic obstructive pulmonary disease (COPD) experience chronic intermittent hypoxia (CIH) characterized by short episodes of hypoxia followed by normoxia (Iturriaga *et al.*, 2005). In OSA, obstruction of the upper airway during sleep causes both hypoxia and hypercapnia, which stimulate the carotid body chemoreceptors. This causes a reflex increase in ventilation, sympathetic tone and arterial blood pressure. The stimulation of these pathways is accompanied by both chemoreceptor and pulmonary mechanoreceptor activation, restoring normal ventilation, often causing individuals to awake from sleep (Iturriaga *et al.*, 2005). In addition to

immediate reflex responses, approximately half of all people who suffer from OSA will develop arterial hypertension, and other individuals develop pulmonary hypertension and have effects on cardiac output (Quan and Gersh, 2004). Evidence of the cardiovascular effects in sleep apnea patients combined with pulse oximetry data from humans exposed to air pollutants suggests a potential link between pollutant exposure and acute hypoxia that warrants further study. It was recently found that cardiac arrhythmias in particulate-exposed heart failure mice were in part due to altered sensitivity of the carotid body (Wang *et al.*, 2012), demonstrating that the chemoreflex response of the carotid body may be a potential mechanism by which air pollution causes adverse responses.

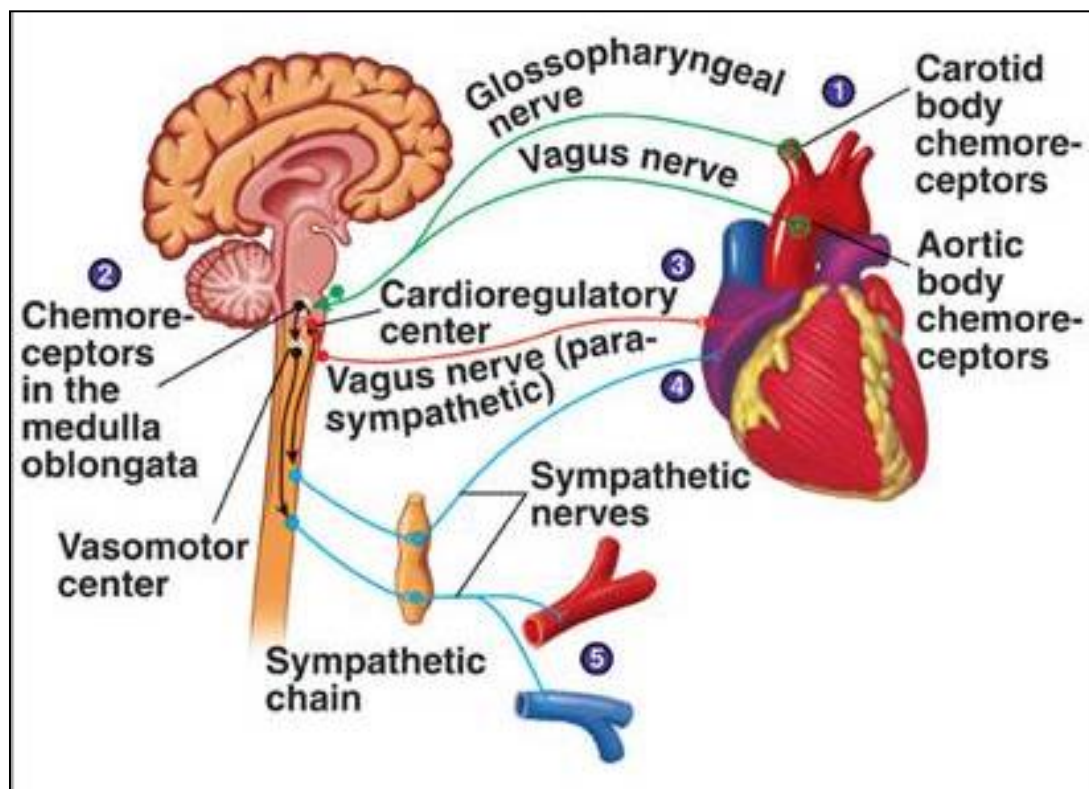


Figure 1.2. Chemoreceptor reflex control of blood pressure. (Reprinted with permission from McGraw Hill Companies).

Air Pollution and Normal Blood Oxygen Levels

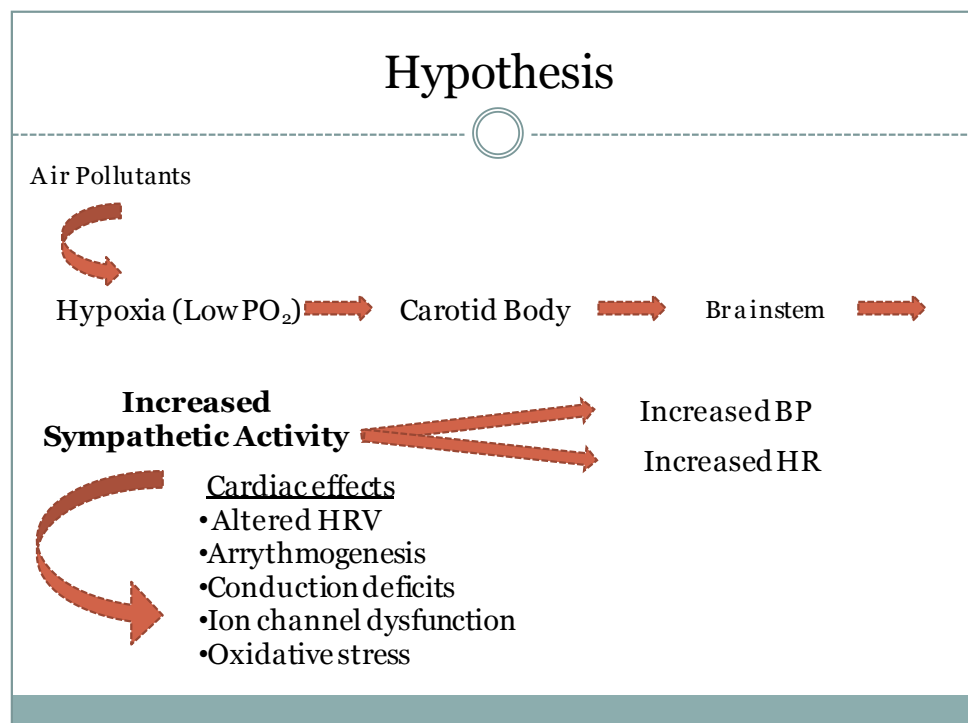
In humans, oxygen saturation is usually measured by pulse oximetry with a device placed on the finger that measures arterial blood oxygen saturation using light absorbance. While pulse oximetry devices exist for animal models, more reliable measurements are obtained by taking serial blood draws and measuring blood gas analytes with a specialized machine. Most modern blood gas analyzers measure a host of factors including oxygen saturation (SaO_2), partial pressure of oxygen (pO_2) and carbon dioxide (pCO_2), Na^+ , K^+ , and Ca^{++} . The body will respond to changes in blood oxygen levels almost immediately, and small changes in carbon dioxide and oxygen levels can also impact important ions that effect cellular repolarization. Many of these effects are mediated by the carotid body, and present an important but surprisingly unappreciated focus of air pollution studies.

Exposure to air pollutants causes a host of responses in the cardiovascular system including changes in heart rate, blood pressure, and increased arrhythmias. Episodes of hypoxia elicit similar responses, and studies with pulmonary irritants have reported apnea as a dominant reflex response to pollutant exposures (Hazari *et al.*, 2008). Several epidemiological studies have found significant decreases in oxygen saturation after exposure to air pollutants. Pulse oximetry recordings in adults exposed to concentrated ultrafine particles showed a 0.5% mean decrease in arterial O_2 saturation (Gong *et al.*, 2005). In addition, healthy elderly adults exposed to concentrated ambient particles had significant decreases in oxygen saturation during exposure (Gong *et al.*, 2004). Recent work by Wang *et al.* (2012) in heart failure mice demonstrated that cardiac arrhythmias with particulate-exposure were in part due to altered sensitivity of the carotid body. Furthermore, exposures to the air pollutants tobacco smoke (Adgent, 2006), sulfur dioxide and nitrogen dioxide

(Hoppenbrouwers *et al.*, 1981) have been linked to abnormal cardiopulmonary sensitivity responses to hypoxia. These studies demonstrate the potential for hypoxia to mediate the adverse cardiovascular events following air pollution exposure and establish a strong justification for research focusing on hypoxia as a potential mechanism for air-pollutant induced cardiovascular dysfunction.

Overarching Hypothesis/Purpose of Research

Despite documented decreases in oxygen saturation during pollutant exposure, a direct link between air pollution-induced hypoxia and adverse cardiovascular effects has not been established, and the exact mechanism leading to adverse cardiovascular events remains unclear. Preliminary results show that exposure to components of DE, such as acrolein, trigger immediate irritant responses characterized by apnea and bradycardia, suggesting that air pollution exposure may lead to hypoxia. Hypoxia has adverse effects on heart rate, blood pressure, and sympathetic activation in heart rate variability and can precipitate adverse cardiovascular responses. Thus, my overarching hypothesis for this dissertation project is that air pollutant-induced cardiovascular dysfunction (e.g., increased arrhythmias, autonomic imbalance, changes in cardiac function, and/or deficits in repolarization) is in part mediated by systemic hypoxia (i.e., low arterial blood oxygen levels). The primary purpose for this research effort is to better define mechanisms of actions of air pollutants to establish biological plausibility for the spikes in human cardiovascular morbidity and mortality associated with small fluctuations in ambient air pollution. This may help reduce uncertainty in standard setting and ultimately contribute to a reduction in the risk associated with exposure.



To achieve this goal, the following Specific Aims were carried out:

Specific Aim (SA) 1. Characterize the impacts of short-term exposure to DE on cardiovascular physiology in rats. DE was studied because it is a significant contributor to ambient PM and traffic-related adverse clinical outcomes. Thus, DE is an ideal model pollutant to examine the role of hypoxia in the mediation of cardiovascular dysfunction. The goal of this study was to perform a comprehensive assessment of the electrocardiographic response to DE exposure in the rat. SA 1 will contribute valuable knowledge to the field by providing a comprehensive characterization of the electrocardiographic impacts of exposure before, during, and after exposure to both whole and filtered diesel exhaust in hypertensive and normal rats. In addition, we will determine if DE exposure causes changes in heart rate variability as well as pulmonary and cardiac toxicity and inflammation. This study will enable the determination of the effects of the classes of components within DE (particulate versus gas), shedding light on the drivers of toxicity within DE and inform on the role of pre-

existing cardiovascular disease in sensitivity to the effects of air pollution. I hypothesized that DE exposure will cause concentration-dependent cardiac dysfunction in SH, but not normal rats, and that exposure to whole DE will elicit greater effects than particle-free DE.

Specific Aim 2. Characterize the cardiovascular response to exposure to the DE component acrolein, and determine if acrolein exposure increases the risk of adverse cardiovascular responses to a cardiac stressor (i.e, hypoxic atmosphere) in hypertensive rats. Acrolein is a component of DE that has been associated with reflex pulmonary irritation and changes in autonomic tone. The US EPA has listed acrolein as the number one non-cancer hazard driver in the United States, showing that the effects of acrolein exposure warrant additional research. Epidemiological studies indicate that the cardiovascular effects of air pollution inhalation are not immediate, usually manifesting some time after exposure. For example, patients with implanted cardioverter defibrillators had increased incidence of life-threatening arrhythmias up to 2 days after air pollution exposure (Peters *et al.*, 2000). These findings suggest that exposure to air pollution alters cardiovascular physiology and increases conditional susceptibility to triggers of thrombosis and arrhythmia. Our lab has previously found using other stress tests (e.g., exercise or exposure to an agent that triggers cardiac arrhythmia) that air pollutant exposure disrupts homeostasis such that individuals respond abnormally to normally tolerable stressors. Thus, sensitivity to hypoxia will reflect the degree to which homeostatic mechanisms have been compromised. The goal of Aim 2 is to determine if acrolein confers increased susceptibility to a cardiac stressor and determine if this response is exaggerated in hypertensive rats. Acrolein was selected as the model pollutant for this study because of the exaggerated effects observed with DE gases. I

hypothesized that acrolein exposure will modify the response to hypoxia in hypertensive but not normal rats.

Specific Aim 3. Determine if inhibition of the sensory response to hypoxia will attenuate air-pollutant-induced cardiac dysfunction. Although all of the mechanisms mediating the adverse cardiovascular health effects of air pollution are unclear, epidemiological evidence has linked exposure to air pollution to drops in blood oxygen saturation, suggesting that hypoxia may play a role. Acute and repeated hypoxia is associated with carotid body-mediated cardiovascular effects which overtime lead to hypertension and predisposition to cardiac arrhythmia. The goal of Aim 3 is two fold: 1) determine if acrolein exposure causes hypoxia by measuring oxygen levels in arterial blood and 2) determine if inhibition of the carotid body, the major sensory organ involved in hypoxia sensing and the triggering of reflex cardiorespiratory responses, leads to a reduction in cardiac abnormalities associated with acrolein exposure. I hypothesized that inhibition of hypoxic sensing by the carotid body will lead to the reduction and/or absence of air pollutant-induced cardiac dysfunction. ECG, HRV, blood pressure, and cardiac contractility were assessed in this study.

Chapter 2

Divergent Electrocardiographic Responses to Whole and Particle-Free Diesel Exhaust Inhalation in Spontaneously Hypertensive Rats

Diesel exhaust (DE) is a major contributor to traffic-related fine PM_{2.5}. While inroads have been made in understanding the mechanisms of PM related health effects, DE's complex mixture of PM, gases and volatile organics makes it difficult to determine how the constituents contribute to DE's effects. We hypothesized that exposure to particle-filtered DE (gases alone) will elicit less cardiac effects than whole DE (particles plus gases). In addition, we hypothesized that Spontaneously Hypertensive (SH) rats will be more sensitive to the electrocardiographic effects of DE exposure than Wistar Kyoto rats (WKY; background strain with normal blood pressure). SH and WKY rats, implanted with telemeters to monitor electrocardiogram (ECG) and heart rate (HR), were exposed once for 4 hrs to 150ug/m³ or 500ug/m³ of whole (wDE; gases plus PM) or filtered (fDE; gases alone) DE, or filtered air. Exposure to fDE, but not wDE, caused immediate electrocardiographic alterations in cardiac repolarization (ST depression) and atrioventricular conduction block (PR prolongation) as well as bradycardia in SH rats. Exposure to wDE, but not fDE, caused post-exposure ST depression and increased sensitivity to the pulmonary C fiber agonist capsaicin in SH rats. The only notable effect of DE exposure in WKY rats was a decrease in heart rate. Taken together, hypertension may predispose to the potential cardiac effects of DE and components of DE may have divergent effects with some eliciting immediate irritant effects (e.g., gases) while others (e.g., PM) trigger delayed effects potentially via separate mechanisms.

Introduction

Inhalation of fine particulate matter (PM) air pollution at concentrations frequently encountered in ambient air sheds increases cardiovascular morbidity and mortality (Brook *et al.*, 2010), especially in individuals with pre-existing cardiovascular diseases (Brook and Rajagopalan, 2009). Vehicular traffic is a dominant source of ambient PM particularly in urban environments (Zhu *et al.*, 2002) and studies have shown that proximity to traffic sources (e.g., highway or tunnel) is a major determinant of cardiovascular health outcomes (Hoek *et al.*, 2002; Hoffman *et al.*, 2006; Van Hee *et al.*, 2009). Diesel exhaust (DE), largely emanating from heavy duty diesel engines, is a significant source of fine (PM_{2.5}) and ultrafine PM air pollution (EPA/600/8-90/057F 2002), and is a major contributor to near roadway emissions and near road-related adverse clinical outcomes. Peters *et al.* (2004) found that exposure to traffic with high levels of DE was associated with onset of myocardial infarction. Similarly, a study in eight European countries attributed hospitalizations for acute coronary syndrome in older patients to exposure to DE (Le Tertre *et al.*, 2002). In a controlled human exposure study, Mills *et al.* (2007) found that DE exposure accentuated exercise-induced electrocardiographic ST depression in subjects with known coronary artery disease and exercise-induced ischemic ECG changes.

Several mechanisms of ambient PM effects have been postulated including pulmonary receptor mediated modulation of autonomic balance, systemic inflammation/oxidative stress leading to altered vasomotor regulation, and direct actions through particles entering systemic circulation (Brook *et al.*, 2010). DE is a chemically complex source of ambient PM and thus defining modes of action is challenging. In addition to PM, DE also consists of a mixture of gases including nitrogen oxides (NO_x), sulfur oxides

(SO_x), carbon monoxide (CO), and volatile organics including aldehydes, benzene, and polycyclic aromatic hydrocarbons. While DE PM has been linked to altered cardiovascular effects (Anselme *et al.*, 2007; Mills *et al.*, 2011a), other studies have shown that DE gases affect health adversely. In addition to noting the effects of PM, Berger *et al.* (2006) found that increased risk of supraventricular tachycardia in men with coronary heart disease was associated with NO₂, and Dockery *et al.* (2005) found similar responses associated with exposure to NO₂ and CO, gases known to originate from DE. In addition, atherosclerotic mice exposed to DE gases showed increased endothelin-1 induced vasoconstriction and altered T wave morphology (Campen *et al.*, 2005).

Abnormal impulse formation and conduction can lead to clinically important heart rhythm disorders and even sudden cardiac death. Despite clear associations with cardiac dysfunction, it is unclear what modifying effects DE components may have when in combination on heart rate and cardiac electrophysiology in individuals with pre-existing cardiovascular disease. We have previously shown that exposure to PM (Farraj *et al.*, 2009; Carll *et al.*, 2010) or the irritant acrolein (Hazari *et al.*, 2009) in rat models of hypertension or heart failure causes bradycardia, increased parasympathetic tone, ST depression, and arrhythmia. In addition, we have previously shown that air pollution exposure (acrolein) enhances sensitivity of pulmonary C fibers (Hazari *et al.*, 2008), suggesting that air pollution exposure may modify chemoreflex responses and potentially autonomic effects. Given the heightened sensitivity to air pollution in individuals with cardiovascular disease, we hypothesized that 1) Spontaneously Hypertensive (SH) rats will be more sensitive to the electrocardiographic effects of a single DE exposure than similarly exposed Wistar Kyoto rats (WKY; background strain with normal blood pressure), 2) exposure to particle-filtered

DE (gases alone) will elicit less cardiac effects than exposure to whole DE (particles plus gases), and 3) the pulmonary chemoreflex response to capsaicin (C fiber agonist) provocation will be potentiated 24 h after DE exposure. Physiological endpoints were monitored during and up to one day after exposure to two different concentrations of DE as studies have shown that exposure to traffic-related air pollution can trigger short term effects within hours after exposure (Peters *et al.*, 2004).

Materials and Methods

Animals

Twelve week-old male Spontaneously Hypertensive (SH) (n = 65) and Wistar Kyoto (WKY) normotensive (n = 15) rats (Charles River, Raleigh, NC) were housed in plastic cages (one per cage), maintained on a 12-hr light/dark cycle at approximately 22°C and 50% relative humidity in our Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, and held for a minimum of 1 week before implantation. The Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency approved all protocols. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum, and all rats were randomized by weight.

Radiotelemetry Implantation

Animals (SH rats; n = 7 per group; 35 total and WKY rats; n = 5 per group; 15 total) were anesthetized with a ketamine/xylazine solution (80 mg/ml ketamine HCL and 12 mg/ml xylazine HCL; 1 ml/kg i.p.; Sigma Chemical Co., St. Louis, MO), and were implanted with radiotelemeters as previously described (Watkinson *et al.*, 1995). Briefly, an aseptic surgical

technique was used to implant a radiotelemetry transmitter (Model TA11CTA-F40; Data Science International, Inc., St. Paul, MN) in the abdominal cavity. Electrode leads were guided through the abdominal musculature through stab wounds. Leads were tunneled subcutaneously and secured in a lead II configuration. Body heat was maintained during and after surgery using a heating pad. Animals recovered for two weeks after surgery before inhalation studies.

Diesel exhaust generation and exposure

Rats were assigned to exposure groups and acclimated to exposure chambers for 1 hour once per day beginning two days before exposure. On the exposure day, rats were allowed to acclimate to the chambers for 1 hour and then baseline data was recorded for the next hour. SH rats were then exposed once to filtered air, 150ug/m³ filtered diesel exhaust (fDE) or whole diesel exhaust (wDE), or 500 ug/m³ fDE or wDE for 4 hours in whole body exposure chambers. We hypothesized that the highest concentration of fDE and wDE would cause the most severe effects. Because of this, WKY rats were exposed to only 500 ug/m³ fDE or wDE for 4 hours in whole body exposure chambers. wDE for exposure experiments was generated using a 4.8 kW (6.4 hp) direct injection single-cylinder 0.320 L displacement Yanmar L70 V diesel generator operated at a constant 3600 rpm. Resistance heating elements provided a constant 3 kW load. Low sulfur diesel fuel (32 ppm), purchased from a local distributor was available from a large storage tank. Engine lubrication oil (Shell Rotella, 15W-40) was changed before each set of exposure tests. From the engine, the exhaust was mixed with particulate (HEPA) filtered room air. wDE concentrations were based on the fine particulate matter (PM_{2.5}; Mass Median Aerodynamic Diameter < 2.5 microns) fractions of

the diluted exhaust. Approximately 85 L/min of the exhaust was directed to a cone diluter and mixed with approximately 595 L/min (7:1 dilution) of high efficiency particulate air (HEPA) filtered room air. The diluted exhaust then traveled approximately 12 m through 7.1 cm diameter stainless steel tubing to a Hazelton 1000 (984 L) exposure chamber housed in an isolated animal exposure room. Target wDE concentration of the diluted exhaust was 500 μg of particulate matter (PM)/ m^3 (high) and 150 μg of PM/ m^3 (low) which was routed to filtered and unfiltered exposure chambers. Multiple human and rodent studies have performed DE studies at concentrations similar to and/or greater than the present study (Mills *et al.*, 2007; Harkema *et al.*, 2009). The filtered chamber was operated at the same pressure, temperature, flow-rate and gas concentrations as the whole particle chamber. The only difference was that the filtered chamber pulled its exposure gas through a Solberg (Itaska, IL) filter housing (model number is CSL-851-200HC) containing a HEPA canister filter. The housing has an inlet to the outside of the pleated canister filter and discharges through the core. The housing was equipped with 2 inch NPT thread inlet and exit ports. The HEPA canister filter (part number HE-851) had a height of 8.75 inches and an OD of 5.75 inches. This filter features a 99.97% removal efficiency standard to 0.3 micron and a temperature range from -15° F to 220° F. Although the filtered chamber had nearly no PM present, it still contained all the diluted combustion gases as the unfiltered chamber. The chamber concentrations were controlled by periodic adjustments of dilution air based on continuous mass concentrations determined by tapered element oscillating microbalance (TEOM, Rupprecht and Patashnick Co., series 1400, Albany, NY) instruments. These instruments include a heated (50 °C) chamber that could theoretically vaporize low temperature volatiles. Control animals were placed in a third chamber supplied with the same HEPA filtered room

air. The chambers were operated at the same flow rate (424 L/min) resulting in approximately 25 air exchanges per hour. Integrated 4 h filter samples (14.1 L/min) were collected daily from each chamber and analyzed gravimetrically to determine particle concentrations. Continuous emission monitors (CEMs) were used to measure chamber concentrations of PM by TEOM, oxygen (O₂, Beckman Corp., model 755, La Habra, CA), carbon monoxide (CO, Thermo Electron Corp., model 48, Franklin, MA), nitrogen oxides (NO and NO₂, Teledyne Technology Co., model 200A4, San Diego, CA), and sulfur dioxide (SO₂, Thermo Electron Corp, model 43c, Franklin, MA). Samples were extracted through fixed stainless steel probes in the exposure chambers. Gas samples were passed through a particulate filter prior to the individual gas analyzers. Dilution of air was adjusted periodically to maintain target PM concentrations as measured by the TEOM. Particle size distributions were characterized during each exposure using an engine exhaust particle sizer (EEEPS, TSI Inc., model 3090, St. Paul, MN). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges.

Radiotelemetry Data Acquisition

Radiotelemetry methodology (Data Sciences International, Inc.) allowed constant monitoring of electrocardiographic data in unrestrained, un-anesthetized rats from implantation until sacrifice. Electrocardiographic data was monitored by remote receivers (DataART3.01; Data Sciences International, Inc.) positioned under the home cages within the animal facility, and under the exposure cages within the exposure chambers. The exposure cages were modified with plastic siding to limit signal noise from metal interference and positioned away from other animals to prevent signal crosstalk. In home cages, sixty-second

segments of ECG waveforms were acquired and saved at 15-minute intervals from surgical recovery through sacrifice not including the exposure period. Pre-exposure baseline data was collected from home cages, as well as a 30 min baseline in exposure cages after a 1 hour acclimation period. During the 4 hr exposure, sixty-second segments were acquired and saved at 5-minute intervals. After exposure, rats were monitored in home cages until sacrifice, approximately 18 hrs after the end of exposure. Heart rate (HR) was automatically obtained from the ECG waveforms with data acquisition software (DataART3.01; Data Sciences International, Inc.).

Electrocardiogram, Arrhythmia Identification and Heart Rate Variability (HRV) Analysis

ECGAuto software (EMKA Technologies, Falls Church, VA) was used for automated analyses of ECG wave amplitudes and segment durations and areas, as well as for the visual identification and enumeration of cardiac arrhythmias. Several parameters were determined for each ECG waveform: PR interval; R amplitude and interval; QRS duration, amplitude, and area; ST interval, amplitude, and area; and T-wave amplitude and area; QT interval, heart rate-corrected QT interval (QTc). To account for potential effects of normal circadian rhythm, ECG parameters were quantified over four 6-hour periods for time-matched comparisons between pre-exposure and post-exposure periods while the rats were unrestrained in their home cages. The times analyzed included 12 AM–6 AM, 6 AM–12 PM, 12 PM–6 PM, and 6 PM–12 AM. ECG parameters during exposure were analyzed in terms of baseline (1-hr recordings while in the exposure chambers immediately before the beginning of exposure) and Hours 1–4 (constituting the entire exposure period between 7:00 AM and 11:00 AM). For magnitude change, exposure values were subtracted from the baseline

values. Magnitude changes were assessed to allow for comparisons between strains and exposures and are presented where appropriate. Most of the data are presented as baseline vs. exposure information and is separated by hour in some cases.

Cardiac arrhythmic events were identified in part by using the Lambeth conventions (Walker *et al.*, 1988) as a guideline for the identification of arrhythmias in rats. Arrhythmias were identified as atrial premature beats (APB), ventricular premature beats (VPB), sinoatrial blocks (SAB) atrioventricular blocks (AVB), or ventricular tachycardia (VT). Arrhythmias were quantified and totaled over an 18 hour period prior to exposure (this corresponded to the same times assessed after exposure), during the 4 hour exposure period, or during the 18 hour period beginning immediately after exposure. Total arrhythmia counts during exposure were quantified (total of 48 two-minute segments during 4 h exposure period). To arrive at counts per hour, the total amount of time sampled in minutes (96) was divided by the number of minutes per hour (60).

For the analysis of HRV, thorough visual inspection was conducted to identify and exclude arrhythmias, artifacts, and 1-min ECG waveforms lacking distinguishable R waves for more than 30 sec. The analysis of HRV generated heart rate (HR) and time-domain measures, including mean time between adjacent QRS complex peaks (the RR interval), a standard deviation of the RR interval (SDNN), SDNN normalized for the effects of heart rate [$SDNN/(RR \text{ interval} \times 100)$], and the square root of the mean of squared differences of adjacent RR intervals (RMSSD). The SDNN represents overall HRV, whereas RMSSD represents parasympathetic influence over HR. The analysis of HRV also calculated frequency domain parameters, particularly low frequency (LF) and high frequency (HF), and the ratio of these two frequency domains (LF/HF). LF is generally believed to represent a

combination of sympathetic and parasympathetic tones, whereas HF indicates cardiac vagal (parasympathetic) tone, and LF/HF serves as an index of sympathovagal balance.

Necropsy, Blood Collection, and Bronchoalveolar Lavage

Rats were deeply anesthetized with an intraperitoneal injection of Euthasol (200 mg/kg Na pentobarbital and 25 mg/kg phenytoin; Virbac Animal Health, Fort Worth, TX). The 35 SH rats and 15 WKY rats implanted with radiotelemeters were sacrificed 18 hrs after exposure. The remaining 30 SH rats were sacrificed 1 hr after exposure. Blood samples were collected from the abdominal aorta with a syringe. The trachea was cannulated, and the lungs were lavaged with a total volume of 35 ml/kg of Ca²¹, Mg²¹, and phenol red-free Dulbecco's PBS (SAFC Biosciences, Lenexa, MD), divided into two equal aliquots. Cytospins and cell differentials from lavaged cell samples, assays for total protein (Coomassie Plus Protein Reagent; Pierce, Rockford, IL), albumin (Diasorin, Inc., Stillwater, MN), lactate dehydrogenase (Thermo DMA, Louisville, CO), and N-acetyl-b-d-glucosaminidase (Roche Diagnostics, Mannheim, Germany) in lavage supernatants, and serum C-reactive protein and creatine kinase (kit from Diasorin, Inc.; standard from Kamiya Biomedical Co., Seattle, WA) were analyzed as previously described (Ghio et al. 2002).

Capsaicin challenge

A separate cohort of 15 male age-matched SH rats (not implanted with telemeters) were used for this challenge test. Intravenous capsaicin challenge was performed as described by Hazari *et al.* (2008). Capsaicin (Sigma-Aldrich, St. Louis, MO) was prepared as a stock solution (500µg/ml) in a vehicle of 10% Tween 80, 10% ethyl alcohol, and 80%

saline. On the day of the experiment solutions with incremental capsaicin concentrations were made by diluting the stock solution in saline based on individual animal body weights. Briefly, 24 hrs after exposure, animals were anesthetized with urethane (1.5g/kg, *i.p.*), supplemental doses of the anesthetic were administered intravenously as necessary to abolish pain reflex. Body temperature was maintained at $\sim 36^{\circ}\text{C}$ with a heating pad. The left jugular vein was cannulated with P.E. 50 polyethylene tubing for the administration of capsaicin. The cannula was exteriorized through an airtight port in the whole-body plethysmograph (Buxco Electronics, Inc, Wilmington, NC). The flow signal was integrated to give V_T , and f was computed from the amount of time it took for one breath. A flow threshold of approximately 60% of the peak inspiratory flow was programmed using Biosystems XA software (Buxco Electronics, Inc, Wilmington, NC) into the analyzing computer. To be registered the flow signal had to drop below this threshold, indicating an adequate inspiration. T_i was calculated as the time between the start of the breath, or where the flow last crossed zero flow, and the start of expiratory flow, or the point where the flow signal rose to zero. T_e was calculated as the difference between the length of the breath and the inspiratory time. A breath was “rejected” when the expired volume differed by more than 2/3 the inspired volume. The parameters were measured and recorded on a breath-by-breath basis and averaged over 10-sec intervals. For each dose, 0.1 ml of capsaicin was first injected into the catheter and then flushed into the animal with 0.2ml of physiological saline. The volume of the catheter was approximately 0.2 ml. Administration of capsaicin in this manner initiated the pulmonary chemoreflex apneic response (rapid decrease in f and increase in T_e). At least 15 min elapsed between doses to allow complete recovery. Data from ten breaths immediately preceding the capsaicin injection were pooled to represent the

baseline values. Animals were euthanized with an overdose of Na pentobarbital after the experiment.

Real-Time Polymerase Chain Reaction (PCR)

Total RNA was isolated from the left ventricle of the heart, using the TriZol (Invitrogen, Carlsbad, CA) method of RNA isolation. RNA concentration and integrity were confirmed using the NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and the Agilent 2100 Bioanalyzer (Thermo Scientific), respectively. RNA was converted to complementary DNA (cDNA) using the TaqMan Reverse Transcription Reagents (Roche; Applied Biosystems, New Jersey). Real-Time PCR was performed using the TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays: Hmox1 (Rn01536933_m1), IL-6 (Rn01410330_m1), and Gapdh housekeeping gene (Rn01462661_g1) (Roche; Applied Biosystems, New Jersey). Reaction plates (96 wells) were run on the Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems, New Jersey) with the following temperature cycle: RT 50°C for 2 min, activation at 95°C for 10 min, amplification for 40 cycles at 95°C for 15 sec and 60°C for 1 min. Results were analyzed using the Sequence Detection System 2.2.2 (Applied Biosystems, New Jersey).

Statistics

ECG and HRV data included 5 different treatment groups (Air, 150ug/m³ fDE or wDE, and 500 ug/m³ fDE or wDE). The statistical analyses of the ECG and HRV data in this study were performed using SAS version 9.2 software (SAS Institute Inc, Cary NC). We used PROC MIXED of SAS since it offers greater flexibility for the modeling of repeated

measures data than PROC GLM. It is also suitable for analysis of large, unbalanced data with missing data at random. A linear mixed model with restricted maximum-likelihood estimation analysis, least squares means and repeated measures ANOVA was used to determine which TIME*TRT interactions were statistically significant between baseline and exposure. Multiple comparison adjustment for the p values and confidence limits for the differences between the least squares means was done using adjust=Tukey HSD (Honest Significant Difference) test. All the biochemical, differential, lung physiology and molecular data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) with a one, two, or three-way analysis of variance (ANOVA) model examining the main effects of each model as well as the interactive effects of two- and three-way ANOVA models. $p < 0.05$ was considered statistically significant.

Results

Exposure Characterization

Table 2.1 lists average particle and gas exposure concentrations, particle size and number. Chamber temperature and humidity were 72.45 ± 0.69 °F and 43.58 ± 1.27 % respectively for all chambers.

Heart Rate

Heart rates decreased in SH rats during exposure to both $150 \mu\text{g}/\text{m}^3$ and $500 \mu\text{g}/\text{m}^3$ fDE when compared to heart rates before exposure (Fig. 2.1A-D). By the second hour of exposure, SH rats exposed to $150 \mu\text{g}/\text{m}^3$ fDE had a 16% decrease in heart rate, from 361 ± 8 beats/min to 311 ± 9 beats/min ($p < 0.05$; Fig. 2.1B). For SH rats exposed to $500 \mu\text{g}/\text{m}^3$ fDE,

heart rates decreased by 11.4% by the second hour of exposure, from 348 ± 6 beats/min to 312 ± 4 beats/min ($p < 0.05$; Fig. 2.1B). For WKY rats, exposure to $500 \mu\text{g}/\text{m}^3$ wDE caused a 14.9% decrease in heart rate by hour 4 from 353 ± 8 beats/min to 308 ± 4 beats/min ($p < 0.05$; Fig. 2.2D). No changes in heart rate were measured in SH or WKY rats exposed to filtered air.

Heart Rate Variability

There were no significant changes were in SDNN or RMSSD during exposure in SH (Table 2.3) or WKY rats (data not shown). All SH rats including those exposed to air had significant decreases in LF/HF during exposure, likely an effect due to chamber stress (Table 2.3).

Electrocardiogram and Arrhythmia

Exposure of SH rats to $500 \mu\text{g}/\text{m}^3$ fDE increased PR interval during hour 2 of exposure, an effect that persisted throughout hours 3 and 4 of exposure (Fig. 2.3). There were no significant changes in PR interval in SH rats exposed to filtered air, $150 \mu\text{g}/\text{m}^3$ fDE or wDE, $500 \mu\text{g}/\text{m}^3$, or WKY rats under any exposure condition.

Only SH rats exposed to $150 \mu\text{g}/\text{m}^3$ fDE exposed had a significant increase in negative amplitude during exposure (% change, p-value) (Figure 2.4A). Although still reduced after exposure (a decrease of 160%), this difference was not statistically significant (Figure 2.4B). There were no changes in the amplitude of the ST segment in any of the other exposure groups. No changes in ST segment amplitude were seen in WKY rats.

SH rats exposed to 150 $\mu\text{g}/\text{m}^3$ fDE had significant increase in negative ST area during exposure (Table 2.2), while only exposure to 500 $\mu\text{g}/\text{m}^3$ wDE caused significant increases in negative ST area 18 hours after exposure (data not shown). Negative ST area is an electrocardiographic parameter that measures the area under the curve between the S wave and the peak of the T wave and can be used to infer changes in repolarization. During exposure, 150 $\mu\text{g}/\text{m}^3$ fDE caused negative ST area to increase from -0.78 ± 0.02 mVsec to -0.94 ± 0.01 mVsec, a 17% decrease ($p < 0.05$). When 18 hours of post exposure data is averaged, 500 $\mu\text{g}/\text{m}^3$ wDE caused negative ST area to increase from -1.23 ± 0.01 mVsec to -1.32 ± 0.01 mVsec, a 6.8% decrease ($p < 0.05$).

There were no significant effects in any of the measured arrhythmias during or after exposure in any of the exposure groups in either strain.

Capsaicin Challenge

Capsaicin (1, 2, 4, 8 $\mu\text{g}/\text{kg}$, i.v.) caused a dose-dependent increase in Te in air exposed SH rats (Figure 2.5). A single exposure to 500 $\mu\text{g}/\text{m}^3$ wDE significantly potentiated the chemoreflex apneic response to capsaicin in SH rats (this assay was not performed in WKY rats); a greater than 60% increase in the apneic period at the two highest doses.

Heart Weight

SH rats had on average higher average heart weight than WKY rats (means, SEMs, and p values). There were no significant effects of exposure on heart weight in either strain (data not shown).

Oxidative Stress and Pulmonary Inflammation

There were no significant effects of exposure on the measured systemic and pulmonary markers of oxidative stress and inflammation in either strain at either 1 or 24 hr after exposure (data not shown).

Real-Time PCR

There were no significant gene expression changes in HO-1 or IL-6 in the left ventricles of SH and WKY rats both 1 hr and 24 hrs after exposure.

Discussion

The current study demonstrates that exposure to filtered and whole DE affects electrocardiographic parameters in SH rats, but not WKY rats, corroborating studies that demonstrate enhanced sensitivity to the effects of air pollution in susceptible clinical subgroups having underlying cardiovascular disease including hypertension (Brook and Rajagopalan, 2009). Only exposure to the gaseous components of DE (fDE) caused heart rate slowing, PR prolongation, and ST segment depression during exposure in SH rats. Conversely, exposure to the high concentration of whole DE (wDE, both particles and gases) caused ST depression after exposure in SH rats and was associated with elevated post-exposure sensitivity to the pulmonary C fiber agonist capsaicin, suggesting that the gaseous components of DE may activate separate pathways than the gas/particle mixture of wDE.

Exposure to fDE, but not wDE, in SH rats caused electrocardiographic alterations during exposure indicative of changes in the early phase of repolarization (ST depression) and atrioventricular conduction slowing (PR prolongation). In contrast, exposure to wDE

caused ST depression only after exposure. The exact reason for this disparity is unclear and is discussed below. Nonetheless, multiple studies have reported ST segment depression with both particulate and gaseous air pollution exposure. In a cohort of adults with stable coronary heart disease, PM levels two days before clinic visits were positively associated with increased risk of ST segment depression during an exercise test (Pekkanen *et al.*, 2002), and were attributed to the gaseous pollutants NO₂ and CO (Pekkanen *et al.*, 2002). Likewise, ST segment depression has also been reported after exercise in elderly patients exposed to black carbon (Gold *et al.*, 2005). Although the mechanism behind ST depression is unclear, previous studies have shown that combustion related gases in DE such as volatile organics/aldehydes and alkanes cause coronary vasoconstriction (Campen *et al.*, 2005). In addition, air pollution exposure causes variability in T wave morphology and repolarization and may lead to myocardial vulnerability and the potential for adverse myocardial events (Henneberger *et al.*, 2005). While the ST segment changes are suggestive of ischemia, measurements of biological indicators of ischemia were not carried out in this study and will be needed to confirm ischemia in future studies. Moreover, this is the first study to report DE induced PR prolongation. PR prolongation is fairly common irrespective of the presence of disease and is usually associated with increased vagal tone (Sapire *et al.*, 1979). These findings are similar to our previous findings with inhaled PM (Farraj *et al.*, 2009; Farraj *et al.*, 2011) and suggest that air pollution exposure secondarily impacts intra-myocardial conduction.

While cardiac responses were the focus of this study, there were also respiratory observations, some of which may have linkage to the cardiac responses and may explain the divergence in cardiac responses with whole DE and DE gases alone. Previous reports from

our laboratory have demonstrated that stimulation of irritant receptors in the airways with acrolein or DE can initiate cardiac responses. Capsaicin is an agonist for the transient receptor potential channel (TRP) V1 receptor found on pulmonary C fibers; it causes reflex bronchospasm and is useful as a challenge regimen to determine enhanced sensitivity to pulmonary irritation and injury (Hazari *et al.*, 2008). In this study, only SH rats exposed to high wDE had significant increases in Te, a measure of apnea, after i.v. injection of capsaicin 24 hrs after exposure, suggesting enhanced sensitivity to capsaicin induced respiratory dysfunction. The discrepancy in effects with whole and filtered DE suggests the activation of separate mechanisms. Traditionally, air pollution toxicity is believed to occur through three major pathways: 1) irritant or sensory receptor activation leading to changes in autonomic balance, 2) activation of systemic inflammatory pathways that impact vascular function, and 3) direct effects of translocated PM (Brook *et al.*, 2010). While there was no evidence of inflammatory changes in the heart, lung, and circulation, the immediacy of effects with fDE exposure (HR slowing, PR prolongation, and ST depression during exposure) suggest the activation of irritant pathways while the delayed effects with wDE exposure suggest non-irritant pathways that are potentially mediated by oxidative or inflammatory pathways. There were no significant HRV changes after exposure to DE. Interestingly, Mills *et al.* (2011b) had similar finding showing that exposure to 300ug/m³ DE did not affect HRV in human volunteers. Although there was no evidence of altered HRV with fDE during exposure, high fDE did cause bradycardia and accompanying PR prolongation that are associated with increased vagal tone. Conversely, the delayed response with the wDE (i.e., ST segment depression after exposure) was accompanied by exaggerated sensitivity to capsaicin, suggesting potential mediation by neurogenic/neuroimmune

mechanisms. Further work is needed to decipher the mechanisms that mediate these responses.

The divergence in responsiveness is likely due to the distinct composition of fDE and wDE. Several studies have shown that several components within DE, including particles, particle/organic compound mixtures, and organic compounds found in the gas phase are capable of eliciting adverse cardiovascular effects (Ris, 2007). For example, atherosclerotic mice exposed to gaseous components of DE had increased coronary vasoconstrictive responses (Campen *et al.*, 2005). Epidemiological studies have also shown that gaseous components of ambient air pollution also drive some of the adverse cardiovascular effects of air pollution. For example, pollutant models excluding the effects of PM_{2.5} found increased resting diastolic blood pressure due to ozone concentrations in patients with pre-existing cardiovascular disease (Zanobetti *et al.*, 2004). In addition, gaseous and particulate components of DE interact and these interactions may have driven some of the divergent responses seen in this study. The carbonaceous core of diesel particles creates a high surface area vehicle suitable for absorption of a number of organic compounds including volatile organic compounds (VOCs) and polyaromatic hydrocarbons (PAHs) that can then be transported more deeply into the lung (Sosnowski *et al.*, 2011). In addition, Kamm *et al.* (1999) found that the surface properties of diesel soot were significantly altered after interaction with ozone. Thus the composition of the exhaust affects particle and chemical characteristics, which in turn might affect both the nature of the cardiopulmonary responses and their time to onset.

While DE exposure decreased heart rate in both strains of rats, only the hypertensive rat had significant electrocardiographic changes during and after exposure to DE. The

mechanisms accounting for the elevated sensitivity of the SH rat are uncertain, but may relate to the structural, biochemical, and physiological characteristics of the cardiovascular system attendant to prolonged hypertension. Previous studies have shown that SH rats have, on average, 40 mmHg higher mean arterial pressure than background control rats with normal blood pressure at the stage of life used in the present study (El-Mas and Abdel-Rahman, 2005) as well as greater arterial wall thickness (Mulvany and Halpern, 1977). Over time hypertension leads to structural and biological remodeling of the left ventricle characterized by hypertrophy, fibrosis, and changes in membrane channels, cellular energetics and ion regulation that combine to heighten myocardial sensitivity (Bernardo *et al.*, 2010). Such remodeling has been demonstrated in SH rats (Goltz *et al.*, 2007) and may account for the differences in responsiveness to DE among the SH and WKY rat. The potential effects of the low DE concentration on electrocardiographic response in WKY rats are unknown and should be determined in future studies.

Collectively, the present findings demonstrate for the first time that whole DE and particulate-free DE from the same diesel engine source trigger divergent electrocardiographic responses. As illustrated with DE in this study, the specific composition of ambient pollutants at any one location likely exerts unique physicochemical characteristics that influence both the quality and magnitude of toxicity. The complex composition of DE makes these divergent responses likely and also displays the challenge in determining mechanisms controlling responses to multipollutant environments. The mixing of particles and gases likely results in the generation of multiple new byproducts not found in particles or gases alone. Thus, as evident from the data, traditional dose-response relationships may not readily apply. DE exhaust exposure also caused little to no effect in healthy rats, stressing the

necessity of modeling susceptible human populations. Future studies should model additional susceptible populations such as the elderly or individuals suffering from asthma. Taken together, these findings highlight the need for additional studies that focus on the effects of multi-pollutant exposures to more accurately assess the comparative cardiopulmonary toxicity of different air sheds.

Table 2.1. Summary of concentrations and characteristics of the diesel exhaust particles and gases within the animal exposure chambers.^a

Constituent	Units					
		Filtered Air Control	150µg/m ³ Filtered DE	500µg/m ³ Filtered DE	150µg/m ³ Whole DE	500µg/m ³ Whole DE
Particle mass concentration (filter) ^b	µg/m ³	32.5 ± 8.5	15 ± 2	21 ± 5	168 ± 32	425 ± 31
Oxygen (O ₂)	%	21 ± 0	20.7 ± 0	20.6 ± 0	20.8 ± 0.0	20.6 ± 0
Carbon monoxide (CO)	ppm	<1	6 ± 0	18 ± 0	6 ± 0	19 ± 0
Nitrogen oxide (NO)	ppm	<0.5	4 ± 0	13 ± 0	5 ± 0	13 ± 0
Nitrogen dioxide (NO ₂)	ppm	<0.5	<0.5	<0.5	<0.5	<0.5
Sulfur dioxide (SO ₂)	ppm	<0.5	<0.5	<0.5	<0.5	<0.5
Particle number concentration ^c	#/cm ³	1441 ± 400	N/A ^c	1428 ± 182	459333 ± 53446	2.1 x10 ⁶ ± 696 x10 ³
Number median Particle Diameter ^c	nm	26 ± 6	N/A ^c	25 ± 6	60 ± 0	69 ± 9
Volume Median Particle Diameter ^c	nm	141 ± 34	N/A ^c	133 ± 30	98 ± 30	113 ± 21

^aTapered element oscillating microbalance (TEOM), O₂, CO, NO, NO₂, and SO₂ data represent mean values from continuous measurements taken over the exposure periods ± standard error (SE). TEOM was not taken at control or filtered chambers since the particulate matter was not adjustable.

^bFilter data represent mean values from one measurement from all chambers per day taken over the exposure periods ± SE.

^cParticle number count and size was determined using a TSI Engine Exhaust Particle Sizer Model 3090. Instrument was operated during another study utilizing the same conditions. No data was available to the 150 µg/m³ filtered diesel exhaust because the instrument was on loan during this study. Based upon a number of studies, particle size and count is almost equivalent to the value for 500µg/m³ fDE.

Table 2.2. 4 hour average of electrocardiographic parameters in Spontaneously Hypertensive rats during exposure. * denotes a significant change from baseline ($p < 0.05$). Values represent means \pm standard error.

Exposure	Timing	QRS (msec)	QTc (msec)	Negative ST Area (mV x sec)	T Wave Area (mV x sec)
Air	Baseline	36.04 \pm 0.69	91.45 \pm 0.61	-0.88 \pm 0.04	1.59 \pm 0.10
	Exposure	34.69 \pm 0.32	90.77 \pm 0.22	-0.86 \pm 0.01	1.83 \pm 0.04
fDE 150μg/m³	Baseline	30.10 \pm 0.96	93.38 \pm 0.57	-0.78 \pm 0.02	3.37 \pm 0.14
	Exposure	32.71 \pm 0.28	93.16 \pm 0.16	-0.94 \pm 0.01*	2.04 \pm 0.04*
fDE 500μg/m³	Baseline	30.27 \pm 0.60	90.96 \pm 0.41	-1.03 \pm 0.04	1.84 \pm 0.07
	Exposure	29.79 \pm 0.16	87.74 \pm 0.15*	-1.05 \pm 0.01	1.89 \pm 0.03
wDE 150μg/m³	Baseline	38.49 \pm 1.15	88.20 \pm 0.57	-0.74 \pm 0.04	1.94 \pm 0.10
	Exposure	40.66 \pm 0.40	90.75 \pm 0.19*	-0.82 \pm 0.01	1.87 \pm 0.03
wDE 500μg/m³	Baseline	40.96 \pm 1.08	94.00 \pm 0.57	-1.12 \pm 0.04	1.66 \pm 0.07
	Exposure	41.38 \pm 0.36	93.23 \pm 0.19	-1.10 \pm 0.02	1.44 \pm 0.03

Table 2.3. 4 hour average of heart rate variability parameters in Spontaneously Hypertensive rats during exposure. * denotes a significant change from baseline ($p < 0.05$). Values represent means \pm standard error.

Exposure	Timing	SDNN	RMSSD	LF	HF	LF/HF
Air	Baseline	6.14 \pm 0.38	3.76 \pm 0.48	1.45 \pm 0.52	0.74 \pm 0.91	2.26 \pm 0.37
	Exposure	8.74 \pm 0.75*	3.97 \pm 0.47	1.15 \pm 0.26	0.90 \pm 0.14	1.28 \pm 0.14*
fDE 150μg/m³	Baseline	8.50 \pm 0.38	4.00 \pm 0.98	2.10 \pm 0.46	0.60 \pm 0.065	2.73 \pm 0.47
	Exposure	8.50 \pm 1.00	4.20 \pm 0.96	1.00 \pm 0.29*	1.15 \pm 0.25*	1.5 \pm 0.32*
fDE 500μg/m³	Baseline	7.70 \pm 0.77	3.49 \pm 0.72	0.84 \pm 0.18	0.50 \pm 0.09	2.54 \pm 0.58
	Exposure	7.93 \pm 0.56	3.19 \pm 0.24	0.73 \pm 0.12	0.84 \pm 0.10	1.07 \pm 0.16*
wDE 150μg/m³	Baseline	8.38 \pm 0.69	4.78 \pm 0.62	1.26 \pm 0.27	0.76 \pm 0.11	1.72 \pm 0.13
	Exposure	8.09 \pm 0.65	3.89 \pm 0.46	0.89 \pm 0.16	1.08 \pm 0.24	1.14 \pm 0.14
wDE 500μg/m³	Baseline	7.82 \pm 0.64	4.34 \pm 0.84	2.35 \pm 0.83	0.95 \pm 0.28	2.33 \pm 0.28
	Exposure	7.75 \pm 0.57	3.64 \pm 0.42	0.87 \pm 0.20*	1.01 \pm 0.24	1.10 \pm 0.12*

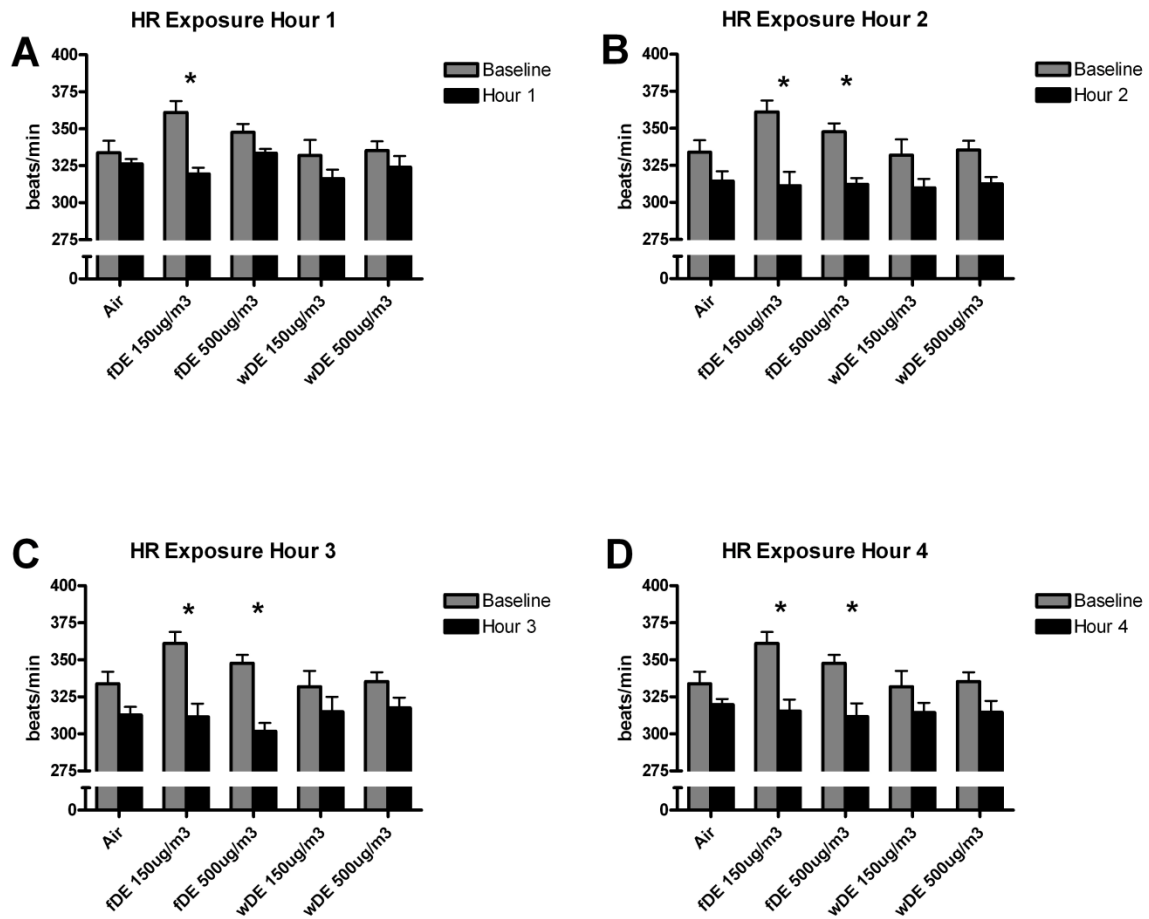


Figure 2.1. fDE causes decreases in heart rate (HR) during exposure in SH rats. Panels A, B, C and D relate to heart rate responses measured 1, 2, 3 and 4 hours after exposure, respectively. Baseline HR is compared to the average HR value during each hour of exposure. Means and standard errors are reported. Significant differences ($p < 0.05$) are denoted with a *.

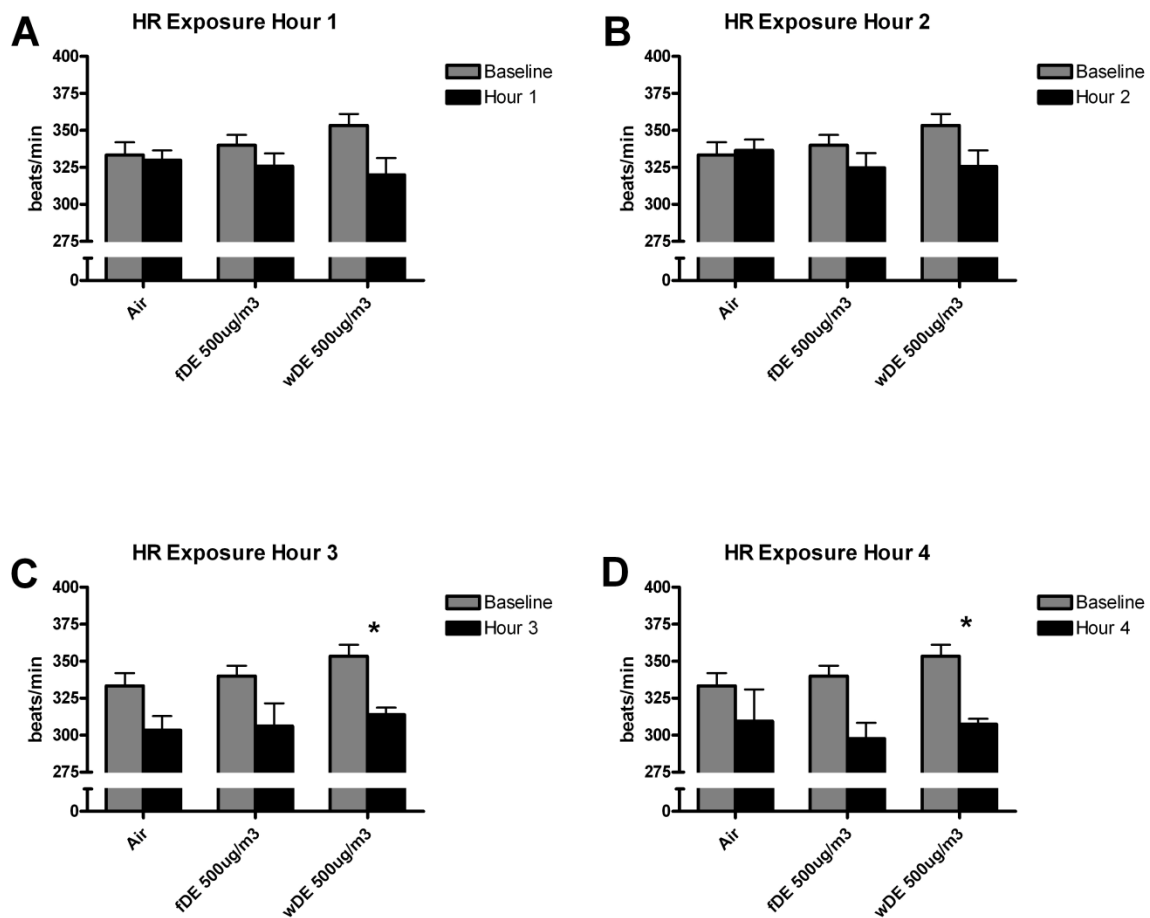


Figure 2.2. wDE causes decreases in heart rate (HR) during exposure in WKY rats. Panels A, B, C and D relate to heart rate responses measured 1, 2, 3 and 4 hours after exposure, respectively. Baseline HR is compared to the average HR value during each hour of exposure. Means and standard errors are reported. Significant differences ($p < 0.05$) are denoted with a *.

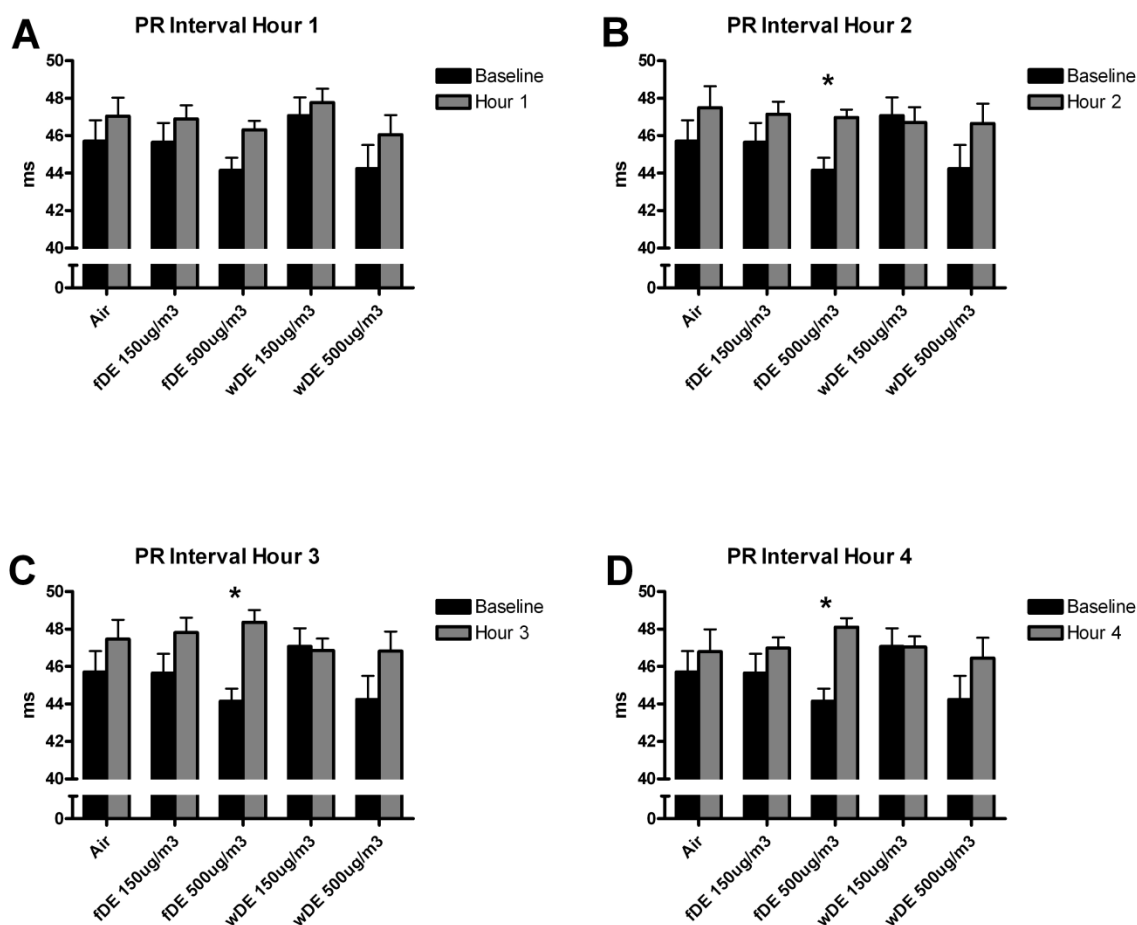


Figure 2.3. SH rats exposed to fDE have increased PR interval during exposure. Panels A, B, C and D relate to PR responses measured 1, 2, 3 and 4 hours after exposure, respectively. Baseline PR interval is compared to the average PR interval value during each hour of exposure. Means and standard errors are reported. Significant differences ($p < 0.05$) are denoted with a *.

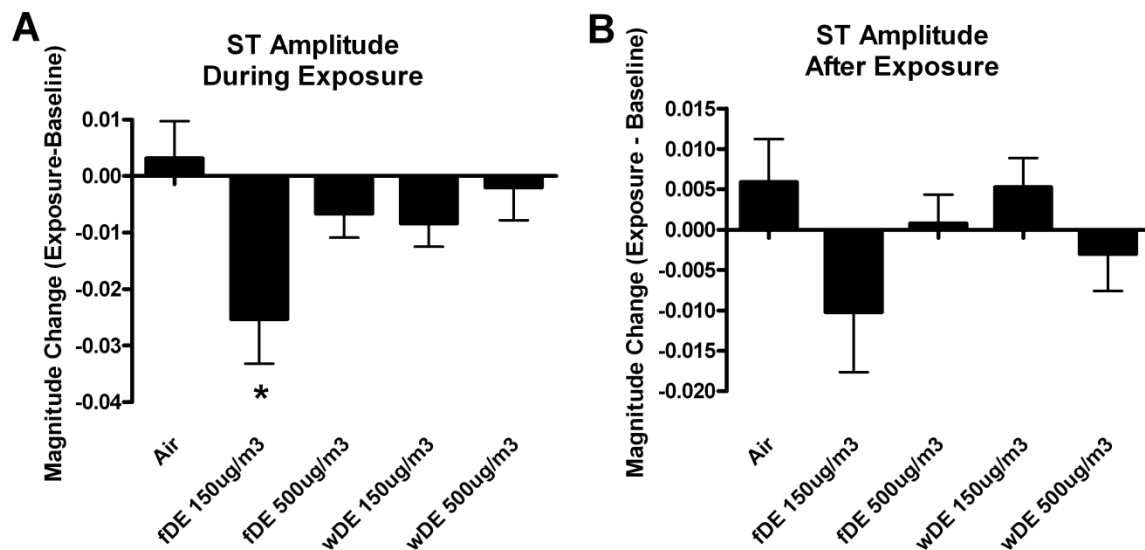


Figure 2.4. SH rats exposed to fDE had increased negative ST amplitude during and after exposure. Magnitude of ST segment shift compared to baseline (A) during exposure, and (B) 18 hrs after exposure to low and high dose whole diesel exhaust (wDE) and PM filtered diesel exhaust (fDE). SH rats exposed to 150ug/m³ fDE had significant ST depression during exposure. Significant differences ($p < 0.05$) are denoted with a *.

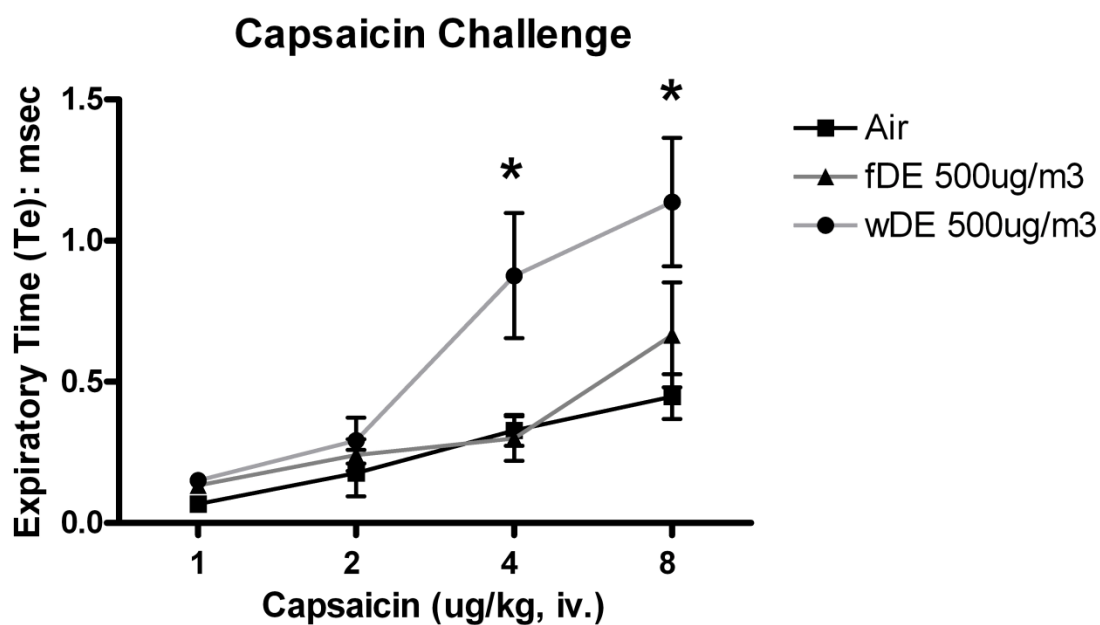


Figure 2.5. SH rats exposed to wDE have increased susceptibility to apnea after capsaicin challenge. SH rats were exposed to air, 500 $\mu\text{g/m}^3$ fDE, or 500 $\mu\text{g/m}^3$ wDE and then i.v. injected with 1, 2, 4, and 8 $\mu\text{g/kg}$ capsaicin 24 hours after exposure. Means and standard errors are reported. Significant differences ($p < 0.05$) are denoted with a *.

Chapter 3

Hypoxia Stress Test Reveals Exaggerated Cardiovascular Effects in Hypertensive Rats after Exposure to the Air Pollutant Acrolein

Exposure to air pollution increases the risk of cardiovascular morbidity and mortality, especially in susceptible populations with cardiovascular disease. Despite increased risk, adverse responses are often delayed and require additional stress tests to reveal latent effects of exposure. The goal of this study was to use an episode of “transient hypoxia” as an extrinsic stressor to uncover latent susceptibility to environmental pollutants in a rodent model of hypertension. We hypothesized that exposure to acrolein, an unsaturated aldehyde and mucosal irritant found in cigarette smoke, diesel exhaust, and power plant emissions would increase cardiopulmonary sensitivity to hypoxia, particularly in hypertensive rats. Spontaneously hypertensive (SH) and Wistar Kyoto (WKY; normotensive) rats, implanted with radiotelemeters, were exposed once for 3 hours to 3 ppm acrolein gas or filtered air in whole body plethysmograph chambers and challenged with a 10% oxygen atmosphere (10 minutes) 24 hours later. Acrolein exposure increased heart rate, blood pressure, breathing frequency, and minute volume in hypertensive rats and also increased the heart rate variability parameter LF, suggesting a potential role for increased sympathetic tone. Normotensive rats only had increased blood pressure during acrolein exposure. The hypoxia stress test after acrolein exposure revealed increased diastolic blood pressure only in hypertensive rats and increased minute volume and expiratory time only in normotensive rats. These results suggest that hypertension confers exaggerated sensitivity to air pollution

and that the hypoxia stress test is a novel tool to reveal the potential latent effects of air pollution exposure.

Introduction

Exposure to air pollution increases cardiovascular morbidity and mortality, especially in individuals with pre-existing cardiovascular diseases (Brook *et al.*, 2010). Epidemiological studies indicate that these effects are not immediate, usually manifesting some time after exposure. For example, Peters *et al.* (2004) showed that humans exposed to traffic were more susceptible to myocardial infarction up to 24 hours after exposure. In addition, patients with implanted cardioverter defibrillators had increased incidence of life-threatening arrhythmias up to 2 days after air pollution exposure (Peters *et al.*, 2000). These findings suggest that exposure to air pollution alters cardiovascular physiology and increases conditional susceptibility to triggers of thrombosis and arrhythmia. Moreover, the fact that these effects happen at lower exposure concentrations than those shown to elicit effects in animal studies indicates that the responses are more complicated than the standard monotonic dose-response relationship of traditional toxicology. The reduced capacity to compensate to daily stressors may account for the “lagged” responses associated with air pollution exposure. The study of the factors that determine the capacity to compensate fully presents a potentially informative approach when seeking to uncover and explain the latent effects of air pollution exposure.

A stress test is a common way to reveal enhanced sensitivity or the potential for adverse responses because increased cardiovascular effort causes reduced oxygen availability to tissues and may provoke autonomic imbalance (Goldberger *et al.*, 2006). Numerous

studies have shown that stress tests trigger many adverse cardiovascular effects including increased cardiac arrhythmias and deleterious changes in heart rate (HR), heart rate variability (HRV), an indirect indicator of cardiac autonomic tone, and ECG parameters (Watanabe *et al.*, 2001). Recently, exercise stress was used to demonstrate that exposure to diesel exhaust during exercise in men with coronary artery disease caused myocardial ischemia (Mills *et al.*, 2007). Analogous effects, including increases in cardiac arrhythmia and myocardial ischemia, were demonstrated in rats infused with the sympathomimetic dobutamine one day after exposure to diesel exhaust (Hazari *et al.*, 2012). In addition, rats exposed to particulate matter or acrolein had exaggerated sensitivity to aconitine, an agent that causes myocardial calcium loading (Hazari *et al.*, 2009). These results are consistent with epidemiological evidence for a time lag in responses and highlight the value of stress tests in unmasking latent responses.

We have previously demonstrated that exposure to both particulate or gaseous air pollutants, including acrolein, causes exaggerated cardiovascular effects in rat models of hypertension and heart failure (Carll *et al.*, 2012; Farraj *et al.*, 2011; Hazari *et al.*, 2009; Lamb *et al.*, 2012). While the mechanisms responsible for these effects are unclear, recent work by Wang *et al.* (2012) demonstrated that cardiac arrhythmias in particulate-exposed heart failure mice were in part due to altered sensitivity of the carotid body, a key organ involved in oxygen sensing and initiating reflex cardiopulmonary changes to maintain homeostasis. Furthermore, exposures to the air pollutants tobacco smoke (Adgent, 2006), sulfur dioxide and nitrogen dioxide (Hoppenbrouwers *et al.*, 1981) have been linked to abnormal cardiopulmonary sensitivity responses to hypoxia. These findings suggest that the response to hypoxia may be useful in unmasking the latent effects of air pollution. The

purpose of this study was to: 1) examine the utility of a novel hypoxia stress test in revealing the potential latent effects of air pollution, and 2) given that hypertensive individuals have exaggerated sensitivity to low atmospheric oxygen (Yu *et al.*, 1999), test the hypothesis that acrolein exposure will modify the response to hypoxia in hypertensive, but not normal rats. HR, blood pressure, ECG, and HRV were measured during acrolein exposure and one day later during hypoxia challenge.

Materials and Methods

Animals

Twelve week-old male Spontaneously Hypertensive (SH) (n = 6) and Wistar Kyoto (WKY) normotensive (n = 6) rats (Charles River, Raleigh, NC) were housed in plastic cages (one per cage), maintained on a 12-hr light/dark cycle at approximately 22°C and 50% relative humidity in our Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, and held for a minimum of 1 week before exposure. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided *ad libitum*, and all rats were randomized by weight. After all experiments were performed, rats were deeply anesthetized with an intraperitoneal injection of Euthasol (200 mg/kg Na pentobarbital and 25 mg/kg phenytoin; Virbac Animal Health, Fort Worth, TX) and euthanized by exsanguination. The Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency (US EPA) approved all protocols.

Telemetry Implantation

Animals were implanted with radiotelemetry transmitters to monitor heart rate (HR), blood pressure (BP), and heart rhythms and electrocardiographic intervals (Model TL11M2-C50-PXT; Data Science International, Inc., St. Paul, MN). Charles River Laboratories performed all telemetry implantation surgeries in accordance with methods specified by the vendor (Charles River Laboratories, 2005). Animals were shipped to the US EPA 10 days after implantation surgeries, and animals were allowed to recover for 1 week at the US EPA before exposures began.

A separate cohort of SH (n = 5) and WKY (n = 5) rats were implanted with femoral artery catheters in accordance with methods specified by the vendor (Charles River Laboratories, 2005). Animals were shipped to the US EPA within 1 week of surgery. The catheters were flushed with saline, locked with heparin and plugged immediately upon arrival and every two days thereafter until exposure began.

Acrolein Exposure and Hypoxia Stress Test

A paired design was utilized to control for baseline differences in HR, BP, and ECG rhythms between rats. SH and WKY rats were first exposed to air control for 3 hours and then underwent a hypoxia stress test 24 hours later. The hypoxia stress test was performed 24 hours after acrolein exposure to uncover latent responses consistent with the delay between exposure and adverse health effect observed in some epidemiological studies (Peters *et al.*, 2000; Peters *et al.*, 2004). Exposure to subatmospheric oxygen has been used as a stress test in humans with hypertension to demonstrate exaggerated cardiorespiratory responses to hypoxia (Ledderhos *et al.*, 2002). After 5 days, these same rats were exposed to 3 ppm

acrolein for 3 hours and then underwent a second hypoxia stress test 24 hours later (Please refer to Figure 3.3A). We assumed that a period of 5 days between the initial hypoxia stress test and the subsequent exposures would allow residual effects, if any, of the initial hypoxia stress test to lapse. This was confirmed by the absence of any differences in air-exposed rats at baseline relative to the previous air and hypoxia exposures.

All exposures (air and acrolein) as well as the hypoxia stress test took place in whole body plethysmography chambers (WBP; Model PLY3213, Buxco Electronics, Inc, Wilmington, NC), which continuously and non-invasively monitor ventilatory parameters in conscious animals. All rats were acclimated to the chambers for 1 hour on the two days prior to both air and acrolein exposure. On exposure days, rats were allowed to acclimate to exposure chambers for 30 minutes, and then baseline data were recorded for the next 30 minutes. For air exposure, rats were exposed to filtered air in whole-body plethysmography chambers for 3 hours. HR, systolic and diastolic BP, ECG waveforms, and ventilatory data were collected during the entire exposure period. After exposure, animals were returned to their home cages. Twenty-four hours after air exposure, animals underwent a hypoxia stress test. Animals were placed in whole-body plethysmography chambers and allowed to acclimate for 30 minutes. A 30 minute baseline was then recorded. After baseline measurements, animals were kept at 20% oxygen concentration (ambient O₂ concentration) for 5 minutes before the hypoxia stress test. To reduce the PO₂ in the chamber, 100% nitrogen (N₂) gas was delivered to a glass mixing chamber where the gas was mixed with dry filtered air to achieve a fraction of inspired O₂ (FIO₂) of 10% at a flow rate of 2 L/min. Oxygen levels were monitored continuously with an O₂ sensor attached directly to the chamber, and an FIO₂ of 10% was maintained for 10 minutes. Nitrogen was then turned off,

and the FIO₂ oxygen saturation rose to 20% (Figure 3.3B). Rats were then kept in the chambers for an additional 15 minutes. HR, systolic and diastolic BP, ECG waveforms, and ventilatory data were collected continuously during the stress test.

Five days after air exposure and the hypoxia stress test when all latent preconditioning was absent, these same rats were exposed to 3 ppm acrolein for 3 hours in the same whole-body plethysmography chambers. The concentration of acrolein (3 ppm) is higher than ambient levels, but it may represent concentrations in high combustion areas (Hazari *et al.*, 2008). In addition, cigarette smoke contains up to 90 ppm acrolein (Esterbauer *et al.*, 1991), and acrolein levels in sidestream tobacco smoke are as high as 10 ppm (Esterbauer *et al.*, 1991). Acrolein gas was metered from a 1000 ppm cylinder into a glass mixing chamber where the gas was mixed with dry filtered dilution air to achieve a final concentration of 3 ppm of acrolein with a total flow of 6 L/min. The actual chamber concentration was measured using an HP5890 gas chromatograph (GMI Inc., Ramsey, MN) equipped with manual injection, a flame ionization detector and a DB-VRX capillary column. The plethysmograph pressure was monitored using Biosystems XA software (Buxco Electronics, Inc, Wilmington, NC). Using respiratory-induced fluctuations in ambient pressure, respiratory parameters including tidal volume, breathing frequency, inspiratory time and expiratory time were calculated and recorded on a breath-by-breath basis and averaged over 10 second intervals. HR, systolic and diastolic BP, ECG waveforms, and ventilatory data were collected during the exposure, and animals were returned to their home cages after exposure. Rats underwent a hypoxia stress test 24 hours later as previously described. Because the data collected were repeated measures, the data during acrolein

exposure and the subsequent hypoxia stress test are presented as % change from air exposure and the initial hypoxia stress test, respectively.

Validation of Hypoxia Stress Test

The hypoxia stress test was validated by measuring arterial blood O₂ saturation (SaO₂) during hypoxia challenge in a separate cohort of rats. Rats implanted with femoral artery catheters were acclimated to whole body plethysmography chambers (WBP; Model PLY3213, Buxco Electronics, Inc, Wilmington, NC), for 1 hour on the two days prior to hypoxia validation. On the day of validation, the exteriorized catheter was extended with a 1 ml PE50 catheter attached to a 23 gauge adapter and run through a pre-measured hole in the wall of the plethysmograph to allow for blood draws outside of the sealed exposure chamber. Rats were acclimated to the exposure chambers for 30 minutes and then underwent a 250µl blood draw while conscious and unrestrained within the chamber. This served as the baseline blood sample. Nitrogen was metered into the chamber to reduce the FIO₂ to 10%, and a second 250µl blood sample was taken after the FIO₂ stabilized at 10%. The addition of N₂ was discontinued, and a final 250µl blood sample was taken 15 minutes after the oxygen content of the chamber stabilized at an FIO₂ of 20%. For each blood draw, the pin was removed from the tip of the catheter, and the heparin was allowed to exit the catheter. When only blood remained, a 23-gauge adapter attached to a 1 ml syringe was placed on the end of the catheter and used to draw a 250µl sample. The blood sample was immediately read on an OPTI CCA-TS Blood Analyzer (OPTI Medical Systems, Inc.) using the OPTI Cassette E-Ca which measures blood pH, PCO₂, PO₂, Na⁺, K⁺, Ca²⁺, total hemoglobin (tHb),

oxygen saturation (SaO₂), and hematocrit (Hct). The catheter was flushed with saline and locked with heparin after each blood draw.

Whole-body Plethysmograph Data Acquisition and Analysis

All exposures were performed in whole-body plethysmography chambers (Buxco Electronics, Sharon, CT). The plethysmography methodology permitted continuous monitoring of breathing frequency, tidal volume, minute volume, inspiratory time and expiratory time. Animals were acclimated to plethysmographs for 1 hour each day on the two days prior to exposure before both air and acrolein exposure. Plethysmography chambers (model PLY3213; Buxco Electronics) were calibrated each day before every animal loading. A bias flow regulator delivered fresh air (1.8 L/min) to each cylindrical chamber, preventing CO₂ buildup within the chamber. Unrestrained animals were placed in individual cylindrical plethysmograph boxes containing a built-in reference chamber for measuring respiration-induced pressure fluctuations. Data were channeled to computer software (BioSystem XA; Buxco Electronics) that calculated respiratory parameters. Data were collected continuously for each parameter, and automated breath-by-breath analyses were performed using a rejection algorithm to eliminate breaths that were outside a given range. On exposure day, animals were acclimated to the chambers for 30 minutes before any readings were taken. After acclimation, 30 minutes of baseline data were taken, and animals were exposed to either control air or 3ppm acrolein for 3 hours. After exposure, rats were removed and returned to home-cages.

Radiotelemetry Data Acquisition

Radiotelemetry methodology (Data Sciences International, Inc.) enabled constant monitoring of ECG data in unrestrained, un-anesthetized rats from implantation until euthanasia. Remote receivers (DataART3.01; Data Sciences International, Inc.) positioned under the home cages, and under the plethysmographs during exposure collected the ECG data and transferred it to the computer for storage. In home cages, sixty-second segments of ECG waveforms were acquired and saved at 15-minute intervals from surgical recovery through euthanasia not including the exposure period. Pre-exposure baseline data were collected from home cages, as well as a 30 min baseline in exposure cages after a 30 min acclimation period. During the 3 hr exposure, sixty-second segments were acquired and saved at 5-minute intervals. After exposure, rats were monitored in home cages until euthanasia, approximately 18 hrs after the end of the final hypoxia challenge. HR and BP were automatically obtained from the ECG and BP waveforms, respectively, with data acquisition software (DataART3.01; Data Sciences International, Inc.).

Electrocardiogram, Arrhythmia Identification and Heart Rate Variability (HRV) Analysis

ECGAuto software (EMKA Technologies, Falls Church, VA) was used for automated analyses of ECG wave amplitudes and segment durations and areas, as well as for the visual identification and enumeration of cardiac arrhythmias. Several parameters were determined for each ECG waveform: PR interval; R amplitude and duration; QRS area; ST interval, amplitude, and area; and T-wave amplitude and area; QT interval, and heart rate-corrected QT interval (QTc). ECG parameters during exposure were analyzed in terms of baseline (30 min

recordings while in the exposure chambers immediately before the beginning of exposure) and Hours 1–3 (constituting the entire exposure period between 8:00 AM and 11:00 AM).

Cardiac arrhythmic events were identified in part by using the Lambeth conventions (Walker *et al.*, 1988) as a guideline for the identification of arrhythmias in rats. Arrhythmias were identified as atrial premature beats (APB), ventricular premature beats (VPB), sinoatrial blocks (SAB), atrioventricular blocks (AVB), or ventricular tachycardia (VT). Arrhythmias were quantified, counted and totaled over an 18 hour period prior to exposure (this corresponded to the same times assessed after exposure), during the 4 hour exposure period, or during the 18 hour period beginning immediately after exposure. Total arrhythmia counts during exposure were quantified (total of 48 two-minute segments during 3 h exposure period) and expressed as counts per minute.

For the analysis of HRV, thorough visual inspection was conducted to identify and exclude arrhythmias, artifacts, and 1-min ECG waveforms lacking distinguishable R waves for more than 30 sec. The analysis of HRV generated heart rate (HR) and time-domain measures, including mean time between adjacent QRS complex peaks (the RR interval), a standard deviation of the RR interval (SDNN), SDNN normalized for the effects of heart rate [$\text{SDNN}/(\text{RR interval} \times 100)$], and the square root of the mean of squared differences of adjacent RR intervals (RMSSD). The SDNN represents overall HRV, whereas RMSSD represents parasympathetic influence over HR. The analysis of HRV also calculated frequency domain parameters, particularly low frequency (LF) and high frequency (HF), and the ratio of these two frequency domains (LF/HF). LF is generally believed to represent a mixture of sympathetic and parasympathetic tone, whereas HF indicates cardiac parasympathetic (vagal) tone, and LF/HF serves as an index of sympathovagal balance.

Statistics

The statistical analyses of the Buxco, ECG and HRV data in this study used SAS version 9.2 software (SAS Institute Inc, Cary NC). We used PROC MIXED of SAS since it offers greater flexibility for the modeling of repeated measures data than PROC GLM. It is also suitable for analysis of large, unbalanced data with missing data at random. A linear mixed model with restricted maximum-likelihood estimation analysis, least squares means and repeated measures ANOVA was used to determine which TIME*TRT interactions were statistically significant between baseline and exposure. Multiple comparison adjustment for the *p* values and confidence limits for the differences between the least squares means was done using adjust=Tukey HSD (Honest Significant Difference) test. All blood gas data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) with a one-way analysis of variance (ANOVA) model examining the main effects of each model. All strain comparisons were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) with a student t-test. Trend analysis of SDNN data during exposure and HR data during hypoxia challenge was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) with a one-way ANOVA followed by a post-hoc test for linear trend between mean and column number. $P < 0.05$ was considered statistically significant.

Results

Effect of Acrolein on Heart Rate, Blood Pressure, and Heart Rate Variability

Heart rates increased in SH rats during all three hours of acrolein exposure (Fig. 3.1A). At hour 1, mean heart rates increased from baseline levels of 294.4 ± 5.4 beats per

minute (bpm) to 313.3 ± 15.3 bpm. At hour 2, mean heart rates increased from baseline levels of 269.2 ± 3.9 bpm to 297.7 ± 16.0 bpm. At hour 3, mean heart rates increased from baseline levels of 274.1 ± 6.7 bpm to 305 ± 16.7 bpm. When each acrolein exposed rat is compared to itself during air exposure these changes constituted a $10.3 \pm 4.22\%$ increase from air at hour 1, a $10.8 \pm 4.57\%$ increase from air at hour 2 and a $14.6 \pm 3.17\%$ increase from air at hour 3 ($p < 0.05$; Fig. 3.1A). Heart rate did not change in WKY rats during acrolein exposure.

Before exposures commenced, the baseline BP values for all rats were calculated and it was determined that SH rats had an average of 44 mmHg higher baseline mean arterial blood pressure level than WKY rats (162.46 ± 3.71 mmHg v. 118.57 ± 4.26 mmHg, respectively), consistent with their hypertensive phenotype. Mean arterial blood pressure increased during hour 2 of acrolein exposure in SH rats and during hour 3 of acrolein exposure in both SH and WKY rats (Fig. 3.1B). In SH rats, mean arterial blood pressure increased from baseline levels of 150.20 ± 2.35 mmHg to 169.65 ± 4.04 mmHg at hour 2, constituting a $15.19 \pm 2.51\%$ increase from air ($p < 0.05$; Fig. 3.1B). At hour 3, mean arterial blood pressure increased from baseline levels of 154.14 ± 2.07 mmHg to 189.32 ± 4.06 mmHg, constituting a $23.67 \pm 2.79\%$ increase from air in SH rats ($p < 0.05$; Fig. 3.1B). The increases in mean arterial blood pressure at hour 3 were accompanied by $22.86 \pm 3.38\%$ and $23.31 \pm 3.30\%$ increases in systolic and diastolic blood pressure, respectively. In WKY rats, blood pressure significantly increased from baseline levels of 120.15 ± 2.74 mmHg to 130.24 ± 5.38 mmHg at hour 3, constituting a $14.44 \pm 2.55\%$ increase from air ($p < 0.05$; Fig. 3.1B).

SH rats also had a significant increase in the low frequency (LF) heart rate variability parameter during hour 3 of acrolein exposure (Fig. 1C). LF increased from baseline levels of

$1.55 \pm 0.37 \text{ ms}^2$ to $3.07 \pm 0.51 \text{ ms}^2$, constituting a $109.39 \pm 29.74\%$ increase from air ($p < 0.05$; Fig. 3.1C). In addition, SH rats had a non-significant decrease in SDNN during all hours of acrolein exposure, but there was a significant trend in the data ($p = 0.0275$; $r^2 = 0.2076$) (Fig. 3.1D). There were no significant changes in RMSSD, HF, and LF/HF in SH rats, and there were no significant changes in any HRV parameter in WKY rats.

Ventilatory Changes during Acrolein Exposure

SH rats had a significant increase in breathing frequency and minute volume in the third hour of acrolein exposure (Fig. 3.2A,B). Breathing frequency increased from baseline levels of 101.33 ± 8.86 breaths per minute at baseline to 122.95 ± 12.96 breaths per minute at hour 3, constituting a $34 \pm 8.61\%$ increase from air ($p < 0.05$; Fig. 3.2A). Minute volume increased from 149.87 ± 11.4 ml at baseline to 181.4 ± 20.85 ml at hour 3 of acrolein exposure, constituting an increase of $25 \pm 4.27\%$ from air ($p < 0.05$; Fig. 3.2B). There were no significant changes in ventilatory parameters during acrolein exposure in WKY rats.

Electrocardiographic Changes and Arrhythmogenesis during Acrolein Exposure

There were no significant changes in PR interval, QRS interval or area, ST or T wave area in SH and WKY rats exposed to acrolein (Supplementary Table 3.1). WKY rats did have a significant increase in the ST segment, but this change occurred during exposure to air and is not related to acrolein exposure (Supplementary Table 3.1). There were no significant changes in ECG, HRV or arrhythmia after either hypoxia challenge. There were no significant changes in the frequency of spontaneous arrhythmia during or after acrolein exposure.

Validation of the Hypoxia Stress Test

All data for hypoxia validation is presented in Table 3.1. A separate cohort of SH and WKY rats implanted with femoral artery catheters underwent the hypoxia stress test and blood samples were analyzed before exposure to the 10% chamber O₂ atmosphere, during exposure to the 10% chamber O₂ atmosphere, and after the return to normal 20% chamber O₂ concentration. Exposure to 10% chamber O₂ atmosphere caused decreases in pO₂, pCO₂, and arterial blood O₂ saturation in both strains of rats. In SH rats, pO₂ levels decreased from 98.6 ± 2.42 mmHg at baseline to 50.20 ± 1.71 mmHg during exposure to 10% chamber O₂ and increased again to 94.60 ± 4.53 mmHg after O₂ levels were returned to 20% ($p < 0.05$; Table 3.1). In WKY rats, pO₂ levels decreased from 99.80 ± 5.16 mmHg at baseline to 54.80 ± 2.60 mmHg during exposure to 10% chamber O₂ and increased again to 95.40 ± 1.89 mmHg after O₂ levels were returned to 20% ($p < 0.05$; Table 3.1).

In SH rats, pCO₂ levels decreased from 37.00 ± 1.00 mmHg at baseline to 31.00 ± 0.84 mmHg during exposure to 10% chamber O₂ and increased again to 38.20 ± 1.80 mmHg after O₂ levels were returned to 20% ($p < 0.05$; Table 3.1). In WKY rats, pCO₂ levels decreased from 41.40 ± 2.86 mmHg at baseline to 28.60 ± 1.25 mmHg during exposure to 10% chamber O₂ and increased again to 41.80 ± 1.80 mmHg after oxygen levels were returned to 20% ($p < 0.05$; Table 3.1).

In SH rats, SaO₂ levels decreased from $93.60 \pm 0.51\%$ at baseline to $79.40 \pm 0.68\%$ during exposure to 10% chamber O₂ and increased again to $94.20 \pm 0.37\%$ after O₂ levels were returned to 20% ($p < 0.05$; Table 3.1). In WKY rats, O₂ saturation levels decreased from

94.60 \pm 0.68% at baseline to 82.40 \pm 0.68% during exposure to 10% chamber O₂ and increased again to 93.40 \pm 0.40% after O₂ levels were returned to 20% (p<0.05; Table 3.1).

Blood Pressure and Heart Rate Changes during Hypoxia Stress Test

Hypoxia stress test was initially performed 24 hours after a 3 hr exposure to air. The 10% chamber O₂ concentration caused similar increases in heart rate in both the SH and WKY rats during the hypoxia period but did not affect systolic blood pressure in either strain (data not shown).

Hypoxia stress test after acrolein exposure caused a significant increase in diastolic blood pressure during exposure to 10% chamber O₂ in SH rats (p<0.05; Fig. 3.4A). Diastolic blood pressure increased from 124.99 \pm 5.16 mmHg at baseline to 135.99 \pm 2.51 mmHg during exposure to 10% chamber O₂, constituting a 12.11 \pm 2.23% increase from air (p<0.05; Fig. 3.4A). The hypoxia stress test caused a non-significant decrease in heart rate during the descend period, the time during which chamber O₂ saturation decreases from 20% to 10%, in the SH rat and a non-significant increase in heart rate during exposure to 10% chamber O₂ in WKY rats (Fig. 3.4B). In addition, SH rats displayed a higher baseline blood pressure than WKY rats during the entire hypoxia stress test.

Respiratory Changes during Hypoxia Stress Test

Hypoxia stress test following air exposure increased breathing frequency and minute volume in SH and WKY rats during the descent and hypoxia period of the stress test and decreased expiratory time in SH and WKY rats during the descend period (data not shown). The magnitudes of these changes were similar between the two strains.

WKY rats, but not SH rats, showed significant increases in minute volume and expiratory time during hypoxia stress test performed after acrolein exposure. Minute volume increased from baseline levels of 125.00 ± 11.74 ml to 187.36 ± 9.49 ml, constituting a $57.93 \pm 23.8\%$ increase from air ($p < 0.05$; Fig. 3.5A). Expiratory time increased from baseline levels of 0.30 ± 0.02 sec to 0.72 ± 0.03 sec, constituting a $130.02 \pm 31.60\%$ change from air during the ascend period ($p < 0.05$; Fig. 3.5B).

Arrhythmogenesis, Electrocardiographic and Heart Rate Variability Changes during Hypoxia Stress Test

There were no significant changes in frequency of spontaneous arrhythmia, electrocardiographic or heart rate variability parameters during the hypoxia stress test after acrolein exposure in SH or WKY rats (Supplementary Table 3.2 and 3.3, respectively).

Discussion

The current study demonstrates that acrolein exposure alone increases heart rate, mean arterial blood pressure, breathing frequency, and minute volume in the hypertensive SH rat, with limited effects in the normotensive WKY rat. The cardiovascular responses in the SH rat during acrolein exposure were coupled with increases in the heart rate variability (HRV) parameter LF indicative of altered autonomic tone. The hypoxia stress test performed after acrolein exposure revealed additional strain-dependent cardiopulmonary responses including increased diastolic blood pressure only in the SH rat and increased minute volume and expiratory time only in WKY rats.

The increases in HR and BP during acrolein exposure in the hypertensive rat may be related to altered autonomic tone during exposure. While chronic exposure to acrolein in mice has been linked to left ventricular dilation (Ismahil *et al.*, 2011) and progression of atherosclerosis (Srivastava *et al.*, 2011), acute exposure to 3 ppm acrolein in this study caused significant increases in HR in SH rats during all 3 hours of exposure. This was accompanied by increases in BP and the LF component of HRV and, although not statistically significant, a decrease in SDNN. These findings are consistent with elevated sympathetic tone. Increased sympathetic tone coupled with decreased heart rate variability is associated with an elevated risk of heart disease (Pope *et al.*, 2001). While the implications of these changes in rats are unclear, similar changes in humans are consistent with elevated sympathetic tone. In addition, intravenous injection of acrolein causes significant increases in blood pressure that are reversed with guanethidine, a sympatholytic drug (Green and Egle, 1983). Despite these findings, there were no significant changes in LF/HF, a measure of sympathetic and parasympathetic balance. Furthermore, LF alone may not be a reliable indicator of sympathetic tone and may instead reflect an interaction of the sympathetic and parasympathetic nervous systems (Houle and Billman, 1999). Thus, further work is required to confirm linkages with altered autonomic tone. Although WKY rats had a significant increase in BP in the third hour of acrolein exposure, this response was not accompanied by increases in HR or any significant changes in HRV.

The hypoxia stress test revealed exaggerated cardiovascular sensitivity, characterized by increased diastolic blood pressure, only in acrolein-exposed SH rats. This response may be due to increased vascular resistance, as *in vivo* acrolein exposure in SH rats has been shown to increase *ex vivo* aortic reactivity (Conklin *et al.*, 2006). Hypoxia has been shown to

heighten sympathetic activation in healthy men during periods of submaximal exercise (Al Haddad *et al.*, 2012). In addition, chronic intermittent hypoxia has been shown to increase arterial blood pressure and impair vasodilation through increased levels of angiotensin II (Marcus *et al.*, 2012). Carotid body-mediated sensation of low blood oxygen saturation levels triggers reflex cardiovascular responses including tachycardia, hypertension, and increased cardiac output (Downing *et al.*, 1962). SH rats have larger carotid bodies (Habeck *et al.*, 1985) and are more sensitive to the effects of hypoxia as evidenced by increased carotid sinus nerve activity and intracellular Ca^{2+} changes (Weil *et al.*, 1998). Thus, differential carotid body sensitivity may in part underlie the differences in responses to acrolein and hypoxia in normotensive and hypertensive rats. Recent work by Wang *et al.* (2012) demonstrated that particulate matter exposure-induced cardiac arrhythmias in mice with heart failure were in part due to dysregulation of carotid body sensitivity. Further research is required to examine the impact of carotid body sensitivity and the potential effects of air pollutant exposure on carotid body-mediated cardiovascular responses. Although the ventilatory responses to hypoxia after the mock air exposure were similar in SH and WKY rats, only WKY rats exposed to acrolein had significant increases in minute volume and expiratory time during the hypoxia stress test. It is unclear exactly why these ventilatory responses occurred only in WKY and not SH rats, but it may relate to the increased respiratory sensitivity to air pollutant inhalation in the SH rat compared to the WKY rat. Previous studies have shown that SH rats respond with greater airway inflammation and injury after air pollution exposure than WKY rats, and underlying susceptibility in the SH rat may result in the nerve fibers being overloaded and unable to illicit a normal response to

acrolein. An acrolein-induced airway lesion in the SH rat may have precluded a normal ventilatory response to hypoxia during the hypoxia stress test (Leikauf *et al.*, 1989).

These findings demonstrate that the hypoxia stress test is a novel approach to reveal latent responses to air pollutants. Researchers are becoming increasingly interested in latent responses to air pollution exposure, and stress tests provide a useful model to study latent effects that would be missed in an acute exposure setting. Traditionally, epidemiological studies have estimated morbidity and mortality within 24 hours of air pollution events. Although this time frame reveals significant increases in cardiovascular events after air pollution exposure, it may miss equally severe delayed responses that can occur up to 3 days (Faustini *et al.*, 2011) and even 2 weeks after exposure (Goodman *et al.*, 2004). Several stress tests have been used to determine enhanced sensitivity to air pollutants including exercise stress test in humans (Gold *et al.*, 2005), dobutamine stress test in rats (Hazari *et al.*, 2012) and isoproterenol cardiac stress test in rats (Andre *et al.*, 2011). Intermittent hypoxia has been used to model nocturnal desaturation in sleep apnea patients (Norton *et al.*, 2011). We designed a new hypoxia stress test with one prolonged (10 min) acute session of hypoxia followed by a return to normoxic conditions. The effects of the 10% O₂ atmosphere were validated by measuring blood O₂ saturation in arterial blood drawn before, during and after hypoxia challenge. Arterial O₂ saturation, pO₂ and pCO₂ decreased significantly during hypoxia in both strains of rats. In addition, pH was not significantly affected by hypoxia challenge, demonstrating that the rat completely compensates for the drop in pCO₂, suggesting that the effects are not due to metabolic acidosis. Normal pO₂ levels in humans range from 80-100 mmHg, and a drop in partial pressure below 60 mmHg is required for hypoxemia. Normal pCO₂ levels in the human range from 35-45 mmHg and the hypoxia

stress test reduced pCO₂ levels well below this level. While the stress test was used in this context to examine susceptibility after air pollution exposure, the data suggest that it can easily be applied to different models to examine overall cardiovascular sensitivity.

The exaggerated effects of acrolein in the SH rats in this study are consistent with our previous studies with other air pollutants indicating enhanced sensitivity in this strain (Farraj *et al.*, 2011; Lamb *et al.*, 2012). In this study, SH rats had an average of 44 mmHg higher blood pressure than WKY rats. It has long been known that hypertension increases the risk of cardiovascular disease and stroke (Brook *et al.*, 2010). In addition to higher baseline blood pressure, SH rats have increased arterial wall thickness (Mulvany and Halpern, 1977) and undergo left ventricular remodeling characterized by hypertrophy, fibrosis, and changes in membrane channels, cellular energetics and ion regulation that combine to heighten myocardial sensitivity (Bernardo *et al.*, 2010). SH rats were not only more susceptible to HR and BP increases during acrolein exposure, they also had significant increases in breathing frequency and minute volume in the third hour of exposure. Previous studies have found that acrolein, like other pulmonary irritants, causes decreases in respiratory rate (Hazari *et al.*, 2008). It is unclear why SH rats had increased breathing frequency at hour 3 of acrolein exposure, but it's possible that these responses were secondary to the significant increases in HR and BP that were initiated during hours 1 and 2 of acrolein exposure. Minute volume tends to increase with exercise or stress, and it has been shown to increase with acrolein exposures (Linhart *et al.*, 1996). Taken together, the results demonstrate that the SH rat has increased susceptibility to the adverse effects of acrolein exposure including cardiovascular effects that appear directly and immediately as well as a priming effects through homeostatic

control pathways perhaps involving autonomic reflexes that set the stage for latent responsiveness to various stimuli.

The present findings demonstrate that acrolein-induced cardiovascular responses may result from modulation of autonomic tone, specifically increased sympathetic input to the heart. These findings also highlight the utility of the hypoxia stress test as a tool to unmask the potential for latent effects of air pollution via less appreciated mechanisms that mediate cardiovascular dysfunction associated with exposure to air pollution. To further examine the utility of this hypoxia stress test, we will assess the effects of exposure to other gaseous and particulate air pollutants (e.g., ozone and diesel exhaust) in future studies. Finally, the results further show that pre-existing cardiovascular disease confers exaggerated sensitivity to the effects of a model irritant, acrolein, that are consistent with the temporal relationship between exposure and clinical outcomes observed in epidemiologic studies showing “delayed or latent” outcomes of air pollutant exposures

Supplementary Data Description

Supplementary data includes the average of electrocardiographic parameters in SH and WKY rats during acrolein exposure (Supplementary Table 3.1) and the hypoxia stress test (Supplementary Table 3.2) as well as the average of heart rate variability parameters in during the hypoxia stress test (Supplementary Table 3.3).

Table 3.1. Arterial blood parameters during the hypoxia stress test. The hypoxia stress test successfully decreases pO₂, pCO₂ and blood oxygen saturation (SaO₂) in arterial blood in SH and WKY rats. pH and K⁺ concentrations are also reported. Means and standard errors are reported. Significant differences (p < 0.05) are denoted with a *.

Exposure	Timing	pO ₂ (mmHg)	pCO ₂ (mmHg)	SaO ₂ (%)	pH	K ⁺ (mmol/L)
SH Rat	Baseline	98.60 ± 2.42	37.00 ± 1.00	93.60 ± 0.51	7.31 ± 0.033	7.66 ± 1.10
	10% Chamber O ₂	50.20 ± 1.71*	31.00 ± 0.84*	79.40 ± 0.68*	7.47 ± 0.044	7.36 ± 0.81
	20% Chamber O ₂	94.60 ± 4.53	38.20 ± 1.80	94.20 ± 0.37	7.39 ± 0.048	6.70 ± 0.72
WKY Rat	Baseline	99.80 ± 5.16	41.40 ± 2.86	94.60 ± 0.68	7.41 ± 0.045	5.70 ± 0.49
	10% Chamber O ₂	54.80 ± 2.60*	28.60 ± 1.25*	82.40 ± 0.68*	7.43 ± 0.070	5.80 ± 0.87
	20% Chamber O ₂	95.40 ± 1.89	41.80 ± 1.80	93.40 ± 0.40	7.40 ± 0.023	5.76 ± 0.69

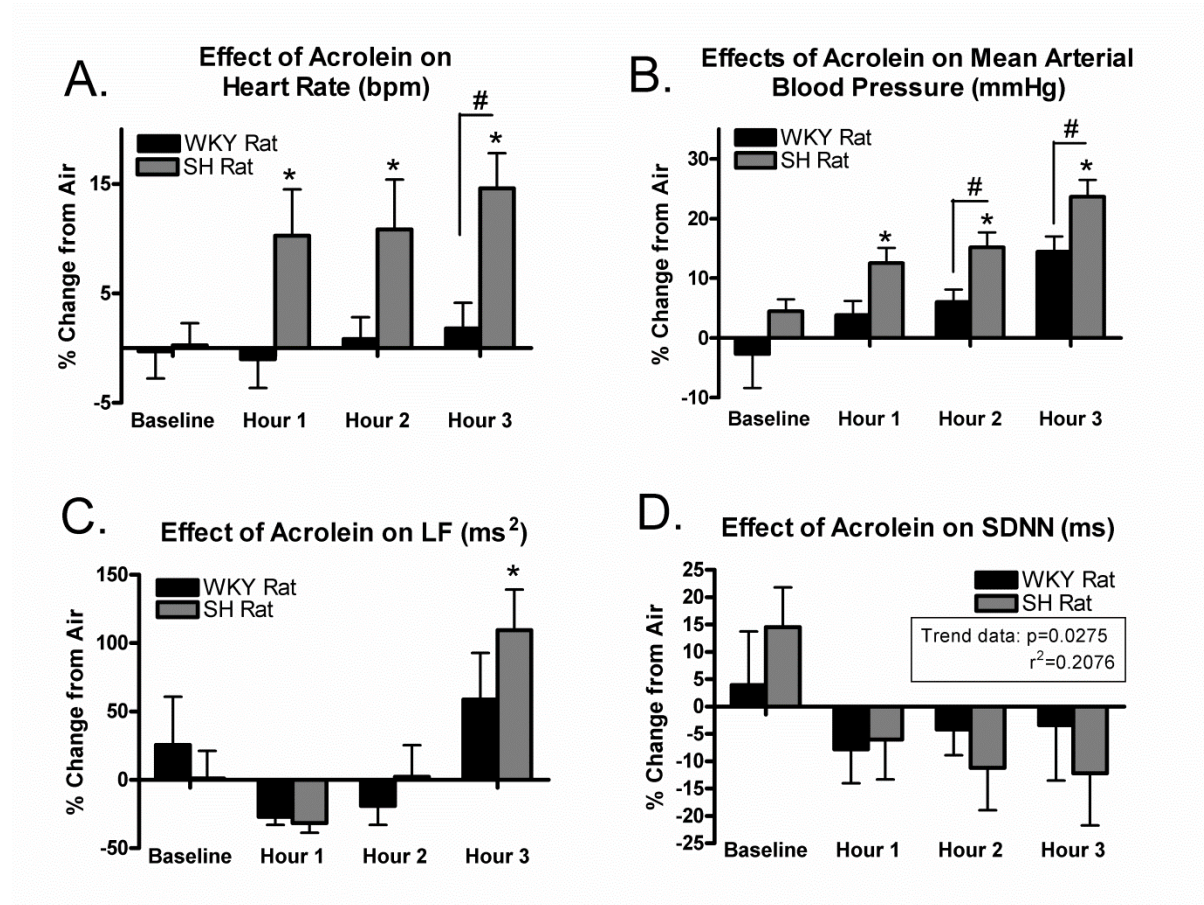


Figure 3.1. Acrolein exposure causes increases in heart rate, mean arterial blood pressure, and the low frequency component of heart rate variability in SH rats. Panels A, B, C and D relate to heart rate, blood pressure, low frequency and SDNN values at baseline and all three hours of acrolein exposure, respectively. Panels A, B, C and D are presented as % change from air. Percent change baseline values are compared to the average % change values during each hour of exposure. Means and standard errors are reported. Significant differences from baseline percent change values ($p < 0.05$) are denoted with a *. Significant differences ($p < 0.05$) between SH and WKY strains at each hour are denoted with a #. There was a significant trend in the SDNN data ($p = 0.0275$; $r^2 = 0.2076$)

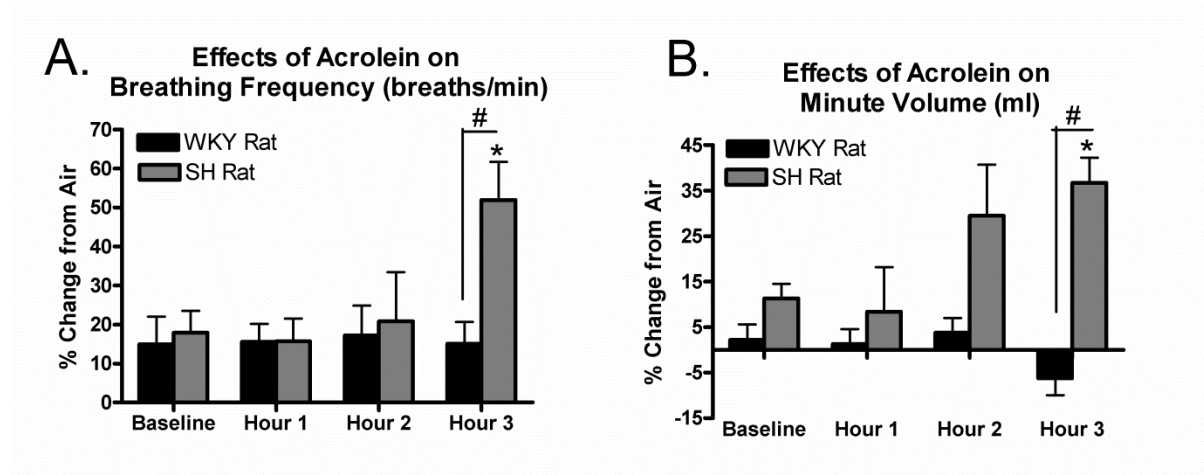


Figure 3.2. Acrolein exposure causes significant increases in breathing frequency and minute volume in SH rats. Panels A and B relate to breathing frequency and minute volume, respectively, measured at baseline, 1, 2 and 3 hours during acrolein exposure. All values are presented as % change from air. Percent change baseline values are compared to the average % change values during each hour of exposure. Significant differences from baseline percent change values ($p < 0.05$) are denoted with a *. Significant differences ($p < 0.05$) between SH and WKY strains at each hour are denoted with a #.

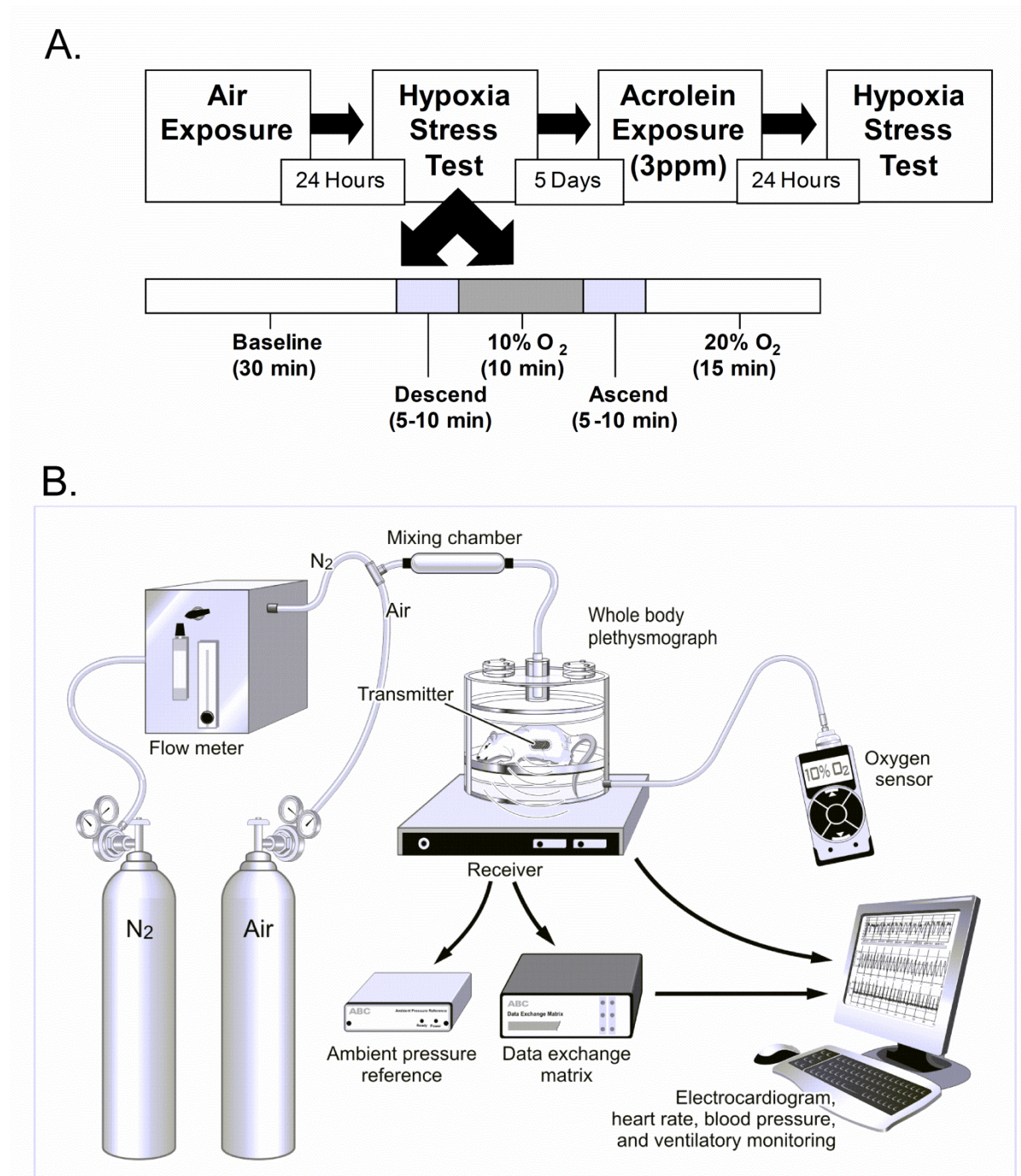


Figure 3.3. A novel hypoxia stress test may reveal latent sensitivity to a gaseous pollutant. Panel A describes the experimental design for the study. Rats were exposed to air for 3 hours on day 1 and underwent a hypoxia stress test 24 hours after air exposure. These same rats were given a 5 day wash out period and then exposed to 3ppm acrolein for 3 hours followed

by a hypoxia stress test 24 hours after acrolein exposure. The hypoxia stress test consists of a 30 min baseline, the descend period (time it takes for oxygen saturation to decrease from 20% to 10%), a 10 min hypoxia period (10% oxygen saturation), an ascend period (time it takes for oxygen saturation to increase from 10% to 20%) and a 15 min period after the return to normal oxygen saturation (20%). Panel B represent the hypoxia stress test experimental setup. The rat is placed in a Buxco plethysmograph that sits over a radiotelemetry receiver. Ventilatory, ECG, HR, and BP data is captured during the entire stress test. For hypoxia, nitrogen is metered into the plethysmograph and an oxygen sensor attached to the plethysmograph records oxygen saturation in real-time.

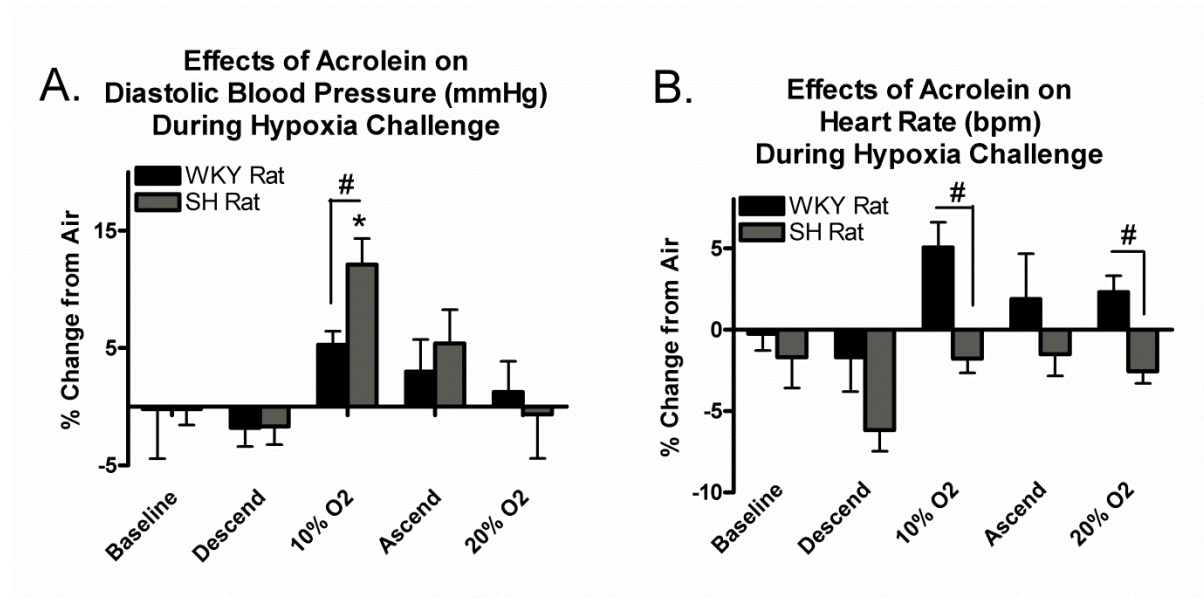


Figure 3.4. Hypoxia stress test after acrolein exposure causes significant increases in diastolic blood pressure in SH rats. Panel A and B refer to diastolic blood pressure and heart rate, respectively, measured at baseline, the descend period, 10% hypoxia, the ascend period and the return to 20% oxygen saturation. All values are presented as % change from hypoxia challenge after air exposure. Percent change baseline values are compared to the average % change values during the different segments of the hypoxia challenge. Means and standard errors are reported. Significant differences from baseline percent change values ($p < 0.05$) are denoted with a *. Significant differences ($p < 0.05$) between SH and WKY strains at each hour are denoted with an #.

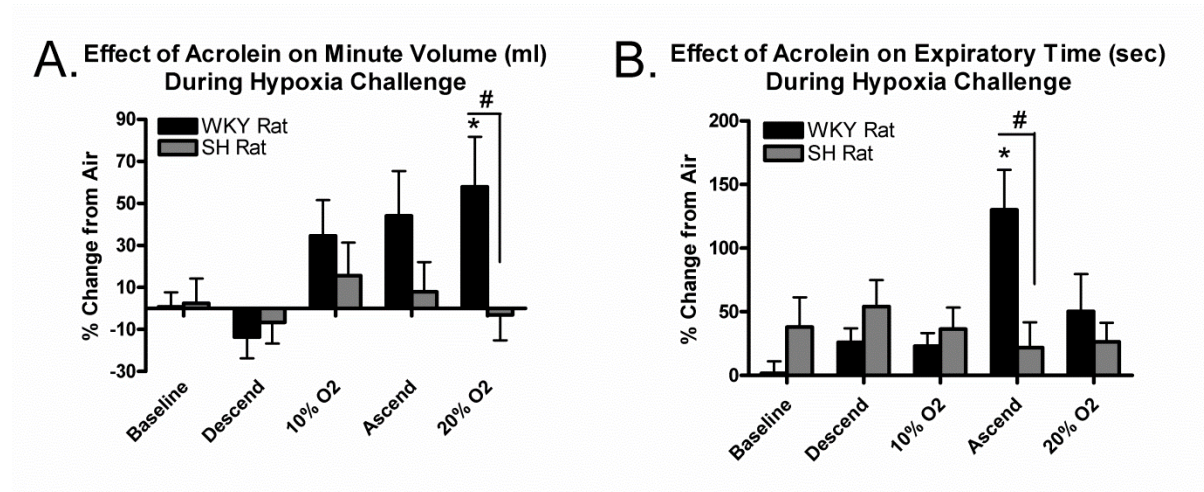


Figure 3.5. Hypoxia stress test after acrolein exposure causes significant increases in minute volume and expiratory time in WKY rats. Panels A and B relate to minute volume and expiratory time, respectively, measured at baseline, the descend period, 10% hypoxia, the ascend period and the return to 20% oxygen saturation. All values are presented as % change from hypoxia challenge after air exposure. Percent change baseline values are compared to the average % change values during the different segments of the hypoxia challenge. Means and standard errors are reported. Significant differences from baseline percent change values ($p < 0.05$) are denoted with a *. Significant differences ($p < 0.05$) between SH and WKY strains at each hour are denoted with a #.

Supplementary Data

Supplementary Table 3.1. 3 hour average of electrocardiographic parameters in Spontaneously Hypertensive and Wistar Kyoto rats during acrolein exposure. * denotes a significant change from baseline ($p < 0.05$). Values represent means \pm standard error.

Exposure	Timing	PR Interval (ms)	QRS Interval (ms)	QTcB (ms)	ST Level (mV)	ST Element (mV)	QRS Area (mV x Time)	Negative ST Area (mV x sec)	T Wave Area (mV x sec)
SH Rat Air	Baseline	47.53 \pm 0.44	32.27 \pm 1.63	85.62 \pm 2.20	- 0.055 \pm 0.01	-0.031 \pm 0.01	1.67 \pm 0.27	-0.82 \pm 0.12	0.98 \pm 0.15
	Exposure	47.97 \pm 0.49	37.04 \pm 2.68	80.81 \pm 0.83	- 0.052 \pm 0.01	-0.031 \pm 0.01	1.93 \pm 0.33	-0.82 \pm 0.12	0.97 \pm 0.12
SH Rat Acrolein	Baseline	47.13 \pm 0.67	36.41 \pm 2.60	88.06 \pm 0.71	-0.06 \pm 0.01	-0.04 \pm 0.01	1.93 \pm 0.30	-0.90 \pm 0.13	0.93 \pm 0.30
	Exposure	48.09 \pm 0.90	39.34 \pm 3.79	88.84 \pm 2.41	-0.05 \pm 0.28	-0.03 \pm 0.01	2.27 \pm 0.43	-0.82 \pm 0.09	1.17 \pm 0.08
WKY Rat Air	Baseline	46.46 \pm 1.57	26.78 \pm 3.98	64.57 \pm 1.85	-0.03 \pm 0.02	0.0002 \pm 0.02	1.28 \pm 0.27	-0.63 \pm 0.19	2.50 \pm 0.60
	Exposure	47.99 \pm 1.35	25.08 \pm 1.67	63.45 \pm 1.56	-0.02 \pm 0.01	0.01 \pm 0.01*	1.11 \pm 0.20	-0.46 \pm 0.13	2.39 \pm 0.37
WKY Rat Acrolein	Baseline	45.86 \pm 1.38	23.84 \pm 0.77	63.79 \pm 2.70	- 0.027 \pm 0.01	0.01 \pm 0.01	1.21 \pm 0.23	-0.46 \pm 0.11	2.41 \pm 0.55
	Exposure	47.79 \pm 1.38	27.83 \pm 3.96	63.45 \pm 1.49	-0.02 \pm 0.01	0.01 \pm 0.01	1.40 \pm 0.20	-0.43 \pm 0.11	2.17 \pm 0.38

Supplementary Table 3.2. Average of electrocardiographic parameters in Spontaneously Hypertensive and Wistar Kyoto rats during the hypoxia stress test. * denotes a significant change from baseline ($p < 0.05$). Values represent means \pm standard error.

Exposure	Timing	PR Interval (ms)	QRS Interval (ms)	QTcB (ms)	ST Level (mV)	ST Amplitude (mV)	QRS Area (mV x Time)	Negative ST Area (mV x sec)	T Wave Area (mV x sec)
SH Rat Air	Baseline	46.54 \pm 0.31	33.33 \pm 1.75	83.25 \pm 1.04	-0.05 \pm 0.01	-0.03 \pm 0.01	1.59 \pm 0.23	-0.72 \pm 0.10	1.13 \pm 0.15
	Descend	47.35 \pm 0.56	33.08 \pm 1.41	89.17 \pm 2.23	-0.05 \pm 0.01	-0.03 \pm 0.01	1.76 \pm 0.30	-0.80 \pm 0.12	1.11 \pm 0.17
	10% O ₂	48.67 \pm 1.00	33.05 \pm 1.85	87.97 \pm 2.37	-0.06 \pm 0.01	-0.03 \pm 0.01	1.67 \pm 0.27	-0.83 \pm 0.12	1.23 \pm 0.17
	Ascend	48.43 \pm 0.80	31.24 \pm 1.70	83.91 \pm 2.55	-0.04 \pm 0.01	-0.02 \pm 0.004	1.44 \pm 0.29	-0.63 \pm 0.08	0.95 \pm 0.11
	20% O ₂	47.48 \pm 0.54	33.13 \pm 3.32	82.81 \pm 2.53	-0.05 \pm 0.01	-0.02 \pm 0.01	1.79 \pm 0.38	-0.69 \pm 0.10	1.24 \pm 0.30
SH Rat Acrolein	Baseline	47.41 \pm 0.83	34.78 \pm 1.55	88.27 \pm 1.13	-0.07 \pm 0.01	-0.04 \pm 0.01	2.25 \pm 0.43	2.40 \pm 0.56	1.12 \pm 0.17
	Descend	47.70 \pm 0.53	36.88 \pm 1.67	92.26 \pm 1.79	-0.07 \pm 0.01	-0.05 \pm 0.01	2.46 \pm 0.33	1.99 \pm 0.38	0.87 \pm 0.08
	10% O ₂	48.73 \pm 0.97	36.49 \pm 2.85	92.26 \pm 2.58	-0.06 \pm 0.01	-0.04 \pm 0.01	2.36 \pm 0.54	2.40 \pm 0.52	1.07 \pm 0.12
	Ascend	49.40 \pm 0.94	35.08 \pm 2.20	90.10 \pm 2.39	-0.05 \pm 0.01	-0.03 \pm 0.01	2.04 \pm 0.38	2.54 \pm 0.65	1.29 \pm 0.09
	20% O ₂	48.41 \pm 0.71	37.43 \pm 3.83	86.18 \pm 1.41	-0.06 \pm 0.01	-0.03 \pm 0.01	2.22 \pm 0.45	2.73 \pm 0.60	1.30 \pm 0.20
WKY Rat Air	Baseline	47.25 \pm 1.20	24.92 \pm 1.06	65.76 \pm 1.50	-0.03 \pm 0.01	-0.003 \pm 0.01	1.18 \pm 0.20	-0.54 \pm 0.10	1.80 \pm 0.42
	Descend	47.79 \pm 1.06	23.18 \pm 1.13	67.30 \pm 1.97	-0.02 \pm 0.01	0.01 \pm 0.01	0.91 \pm 0.24	-0.45 \pm 0.16	2.14 \pm 0.30
	10% O ₂	47.87 \pm 1.46	24.64 \pm 1.17	68.37 \pm 2.21	-0.02 \pm 0.01	0.01 \pm 0.01	1.12 \pm 0.19	-0.49 \pm 0.11	2.42 \pm 0.60
	Ascend	48.05 \pm 1.46	23.40 \pm 0.78	63.57 \pm 2.37	-0.02 \pm 0.01	0.01 \pm 0.01	0.99 \pm 0.25	-0.44 \pm 0.14	2.47 \pm 0.65
	20% O ₂	47.92 \pm 1.52	22.53 \pm 1.02	63.58 \pm 2.98	-0.02 \pm 0.01	0.01 \pm 0.01	1.17 \pm 0.31	-0.49 \pm 0.15	2.69 \pm 0.63
WKY Rat Acrolein	Baseline	46.67 \pm 1.51	23.99 \pm 0.85	65.34 \pm 1.34	-0.027 \pm 0.01	0.01 \pm 0.01	1.13 \pm 0.18	-0.44 \pm 0.12	2.40 \pm 0.56
	Descend	47.29 \pm 1.38	23.32 \pm 1.39	70.38 \pm 1.79	-0.02 \pm 0.01	0.01 \pm 0.01	0.80 \pm 0.12	-0.40 \pm 0.10	1.99 \pm 0.38
	10% O ₂	48.13 \pm 1.28	22.42 \pm 0.69	68.15 \pm 1.92	-0.02 \pm 0.01	0.02 \pm 0.01	0.83 \pm 0.19	-0.39 \pm 0.12	2.40 \pm 0.52
	Ascend	48.10 \pm 1.64	23.04 \pm 0.59	66.28 \pm 2.37	-0.02 \pm 0.01	0.01 \pm 0.01	0.99 \pm 0.25	-0.43 \pm 0.13	2.47 \pm 0.65
	20% O ₂	46.68 \pm 1.40	23.40 \pm 0.56	63.06 \pm 2.93	-0.02 \pm 0.01	0.01 \pm 0.01	1.39 \pm 0.28	-0.49 \pm 0.13	2.69 \pm 0.63

Supplementary Table 3.3. Average of heart rate variability parameters in Spontaneously Hypertensive and Wistar Kyoto rats during the hypoxia stress test. * denotes a significant change from baseline ($p < 0.05$). Values represent means \pm standard error.

Exposure	Timing	SDNN (ms)	RMSSD (ms)	LF (ms ²)	HF (ms ²)	LF/HF
SH Rat Air	Baseline	8.19 \pm 3.34	4.01 \pm 0.31	0.94 \pm 0.27	2.08 \pm 0.38	0.55 \pm 0.06
	Descend	6.52 \pm 2.66	3.37 \pm 1.38	0.78 \pm 0.33	1.83 \pm 0.76	0.67 \pm 0.04
	10% O ₂	6.07 \pm 2.48	4.87 \pm 0.39	0.94 \pm 0.31	2.82 \pm 1.02	0.51 \pm 0.04
	Ascend	7.30 \pm 2.98	5.21 \pm 2.13	1.97 \pm 0.54	2.80 \pm 0.69	0.52 \pm 0.06
	20% O ₂	7.65 \pm 3.12	4.31 \pm 1.76	0.74 \pm 0.15	1.96 \pm 0.49	0.50 \pm 0.03
SH Rat Acrolein	Baseline	7.25 \pm 0.49	3.52 \pm 0.15	0.78 \pm 0.19	1.51 \pm 0.25	0.49 \pm 0.05
	Descend	5.69 \pm 0.57	2.94 \pm 0.13	0.59 \pm 0.22	1.01 \pm 0.32	0.54 \pm 0.06
	10% O ₂	5.43 \pm 0.65	3.98 \pm 0.44	0.60 \pm 0.23	1.78 \pm 0.72	0.52 \pm 0.04
	Ascend	6.56 \pm 0.96	3.91 \pm 0.34	0.84 \pm 0.39	2.15 \pm 0.66	0.48 \pm 0.06
	20% O ₂	7.82 \pm 0.67	3.95 \pm 0.31	0.92 \pm 0.15	1.82 \pm 0.28	0.50 \pm 0.02
WKY Rat Air	Baseline	9.90 \pm 4.04	4.33 \pm 1.77	0.81 \pm 0.23	2.04 \pm 0.65	0.43 \pm 0.04
	Descend	7.72 \pm 1.16	3.32 \pm 0.30	0.85 \pm 0.20	1.44 \pm 0.24	0.52 \pm 0.07
	10% O ₂	7.46 \pm 0.73	4.65 \pm 0.85	1.16 \pm 0.33	3.04 \pm 1.12	0.45 \pm 0.08
	Ascend	6.96 \pm 0.44	3.92 \pm 0.49	0.78 \pm 0.24	1.78 \pm 0.54	0.47 \pm 0.06
	20% O ₂	10.41 \pm 4.25	5.37 \pm 2.19	1.40 \pm 0.53	3.48 \pm 1.13	0.44 \pm 0.02
WKY Rat Acrolein	Baseline	10.17 \pm 0.86	4.34 \pm 0.53	1.03 \pm 0.23	2.20 \pm 0.45	0.45 \pm 0.03
	Descend	8.92 \pm 0.85	3.14 \pm 0.28	0.43 \pm 0.08	0.98 \pm 0.19	0.45 \pm 0.02
	10% O ₂	7.16 \pm 0.83	3.46 \pm 0.45	0.76 \pm 0.21	1.40 \pm 0.33	0.53 \pm 0.03
	Ascend	9.29 \pm 0.77	5.25 \pm 0.71	1.49 \pm 0.43	3.50 \pm 0.88	0.50 \pm 0.04
	20% O ₂	10.26 \pm 0.75	5.42 \pm 0.70	1.10 \pm 0.28	3.52 \pm 1.09	0.45 \pm 0.02

Chapter 4

Acrolein Induced Cardiovascular Dysfunction in Rats is Associated with Reduced Arterial Blood Oxygen and is Mediated by the Carotid Body

Exposure to air pollution increases the risk of cardiovascular morbidity and mortality, especially in susceptible populations with underlying cardiopulmonary disease. There are several postulated mechanisms accounting for adverse responses, including decreases in blood oxygen levels following pollutant exposure. Despite several epidemiological studies reporting decreases in oxygen saturation following exposure to different environmental pollutants, the direct link between air pollution-induced hypoxia and adverse cardiovascular effects is not well accepted, and the exact mechanism leading to adverse cardiovascular events remains unclear. We hypothesized that exposure to acrolein, an unsaturated aldehyde and mucosal irritant found in cigarette smoke, diesel exhaust, and power plant emissions, would decrease blood oxygen levels and exert cardiovascular effects through a carotid body mediated mechanism. A cohort of Spontaneously Hypertensive (SH) rats implanted with radiotelemeters were exposed once for 3 hours to 3 ppm acrolein gas or filtered air with or without pharmacological inhibition of the carotid body in whole body plethysmograph chambers while another identical cohort implanted with femoral artery catheters underwent blood gas analysis before, during, and after acrolein exposure. Acrolein exposure caused significant decreases in pO_2 and significant increases in pCO_2 during exposure. In addition, acrolein exposure caused significant increases in mean arterial, systolic, and diastolic blood

pressure that were eliminated with carotid body inhibition. These results suggest that acrolein-induced cardiac events may be mediated by the carotid body.

Introduction

Multiple lines of evidence demonstrate that exposure to air pollution increases cardiovascular morbidity and mortality (Brook *et al.*, 2010). Several mechanisms have been postulated that overlap among gaseous and particulate pollutants including vascular oxidative stress, endothelial/vascular dysfunction, inflammation, and altered autonomic tone (Brook *et al.*, 2010; Srebot *et al.*, 2009). Recent epidemiological evidence suggests that a relatively unexplored mechanism, systemic hypoxia, may drive some of the adverse cardiovascular effects associated with exposure to air pollution. DeMeo *et al.* (2004) found associations between increased PM_{2.5} levels and decreased oxygen saturation in older individuals with cardiopulmonary disease. Pope *et al.* (1999) found negative associations between PM₁₀ levels and blood oxygen saturation in 80-year-old males. In clinical exposure studies, Gong *et al.* (2004; 2005) found that exposure to concentrated ambient particulates in healthy elderly volunteers also caused a drop in blood oxygen saturation. Despite these associations, a direct link between hypoxia and air pollution-induced adverse cardiovascular effects has not been established.

In mammals, the response to systemic hypoxia is controlled by the carotid body, a major sensory organ located at the bifurcation of the carotid artery that is responsible for sensing changes in arterial blood oxygen levels and initiating reflex cardiopulmonary changes to maintain homeostasis. In response to hypoxia, the carotid body triggers reflex increases in sympathetic tone that in turn trigger increases in blood pressure (BP),

ventilation, heart rate (HR), and arrhythmogenic risk (Roux *et al.*, 2000; Liu *et al.*, 2003; Lopez-Barneo *et al.*, 2008). Repeated cycles of hypoxia, as happens in individuals with obstructive sleep apnea, have been shown to cause hypertension (Freet *et al.*, 2013). Hypoxia has also been shown to cause atrial fibrillation, AV block, ventricular tachycardia, and changes in autonomic balance associated with ventricular arrhythmias (Davies and Wedzicha, 1993). Parallels between carotid body-mediated effects and the cardiovascular responses associated with air pollution exposure suggest a linkage is plausible.

We have previously demonstrated that exposure to particulate or gaseous air pollutants causes exaggerated cardiovascular effects in rat models of hypertension and heart failure (Carll *et al.*, 2012; Farraj *et al.*, 2011; Hazari *et al.*, 2009; Lamb *et al.*, 2012). Recently, we found that exposure to acrolein, an unsaturated aldehyde and mucosal irritant found in cigarette smoke, diesel exhaust, and power plant emissions, causes apnea (Hazari *et al.*, 2008), increases blood pressure (BP) and modifies responses to a physiologic stressor in rats (Perez *et al.*, 2013). In addition, Wang *et al.* (2012) demonstrated that cardiac arrhythmias in particulate-exposed heart failure mice were associated with altered carotid body sensitivity. These findings suggest that exposure to air pollution may trigger adverse cardiovascular responses through a hypoxia-driven mechanism. Hypoxia sensing is dependent on the activity of the H₂S-generating enzyme cystathionine γ -lyase (CSE) within the carotid body (Peng *et al.*, 2010). The purpose of this study was to determine if the cardiovascular effects of acrolein inhalation were mediated by hypoxia. We hypothesized that 1) exposure to acrolein will cause decreases in blood oxygen levels in hypertensive rats, a strain with exaggerated sensitivity to acrolein (Perez *et al.*, 2013) and 2) inhibition of carotid body sensing of hypoxia via CSE inhibition will prevent the cardiovascular responses

to acrolein exposure. HR, BP, the electrocardiogram, and heart rate variability, an indicator of autonomic tone, were monitored before, during, and after exposure. Arterial blood was obtained during acrolein exposure to measure oxygen content and saturation among other parameters. In addition, left ventricular pressure, cardiac contractility, and lusitropy were assessed one day after acrolein exposure.

Materials and Methods

Drugs

Rats in exposure groups including carotid body inhibition were treated with the cystathionine γ -lyase enzyme (CSE) inhibitor DL-propargylglycine (PAG) (Sigma; 200 mg/kg) administered intraperitoneally immediately before exposures. All solutions with drugs were prepared fresh before every experiment. Rats not treated with DL-propargylglycine (PAG) were treated with an equivalent volume of saline administered intraperitoneally immediately before exposures.

Animals

Twelve week-old male Spontaneously Hypertensive (SH) (n = 28) (Charles River, Raleigh, NC) were housed in plastic cages (one per cage), maintained on a 12-hr light/dark cycle at approximately 22°C and 50% relative humidity in our Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, and held for a minimum of 1 week before exposure. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided *ad libitum*, and all rats were randomized by weight. All rats were assigned to one of four treatments groups: air exposure and saline injection; acrolein

exposure and saline injection; air exposure and PAG injection; and acrolein exposure and PAG injection. After all experiments were performed, rats were deeply anesthetized with an intraperitoneal injection of Euthasol (200 mg/kg Na pentobarbital and 25 mg/kg phenytoin; Virbac Animal Health, Fort Worth, TX) and euthanized by exsanguination. The Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency (US EPA) approved all protocols.

Telemeter Implantation

Animals were implanted with radiotelemetry transmitters to monitor heart rate (HR), blood pressure (BP), and heart rhythms and electrocardiographic intervals (Model TL11M2-C50-PXT; Data Science International, Inc., St. Paul, MN). Charles River Laboratories performed all telemetry implantation surgeries in accordance with methods specified by the vendor (Charles River Laboratories, 2005). Animals were shipped to the US EPA 10 days after implantation surgeries, and animals were allowed to recover for 1 week at the US EPA before exposures began.

A separate cohort of SH (n = 20) and WKY (n = 5) rats were implanted with femoral artery catheters in accordance with methods specified by the vendor (Charles River Laboratories, 2005). Animals were shipped to the US EPA within 1 week of surgery. The catheters were flushed with saline, locked with heparin and plugged immediately upon arrival and every two days thereafter until exposure began.

Acrolein Exposure

Rats were assigned to exposure groups (air exposure and saline injection; acrolein exposure and saline injection; air exposure and PAG injection; acrolein exposure and PAG injection) and acclimated to exposure chambers for 1 hour once per day beginning two days before exposure. On the exposure day, rats received an intraperitoneal injection of saline or PAG and were allowed to acclimate to the chambers for 30 minutes and then baseline data was recorded for the next 30 minutes. All exposures (air and acrolein) took place in whole body plethysmography chambers (WBP; Model PLY3213, Buxco Electronics, Inc, Wilmington, NC), which continuously and non-invasively monitor ventilatory parameters in conscious animals. SH rats were then exposed to filtered air or 3 ppm acrolein for 3 hours. The concentration of acrolein (3 ppm) is higher than ambient levels, but it may represent concentrations in high combustion areas (Hazari *et al.*, 2008). In addition, cigarette smoke contains up to 90 ppm acrolein (Esterbauer *et al.*, 1991), and acrolein levels in sidestream tobacco smoke are as high as 10 ppm (Esterbauer *et al.*, 1991). Acrolein gas was metered from a 1000 ppm cylinder into a glass mixing chamber where the gas was mixed with dry filtered dilution air to achieve a final concentration of 3 ppm of acrolein with a total flow of 6 L/min. The actual chamber concentration was measured using an HP5890 gas chromatograph (GMI Inc., Ramsey, MN) equipped with manual injection, a flame ionization detector and a DB-VRX capillary column. The plethysmograph pressure was monitored using Biosystems XA software (Buxco Electronics, Inc, Wilmington, NC). Using respiratory-induced fluctuations in ambient pressure, respiratory parameters including tidal volume, breathing frequency, inspiratory time and expiratory time were calculated and recorded on a breath-by-breath basis and averaged over 10 second intervals. HR, systolic and

diastolic BP, ECG waveforms, and ventilatory data were collected during the exposure, and animals were returned to their home cages after exposure. Rats underwent left ventricular pressure readings 24 hours after exposure.

Left Ventricular Pressure Readings 24 Hours Post Exposure

All rats exposed to air or acrolein with or without PAG injection underwent left ventricular pressure (LVP) readings to measure cardiac function 24 hours after exposure. Left ventricular pressure readings were performed as previously described (Carll *et al.*, to be submitted). Briefly, rats were anesthetized with urethane (1.5 mg/kg *i.p.*, Sigma) and prepared for LVP measurement by right carotid arterial catheterization with a 2-French transducer (SPR-320, Millar Instruments). The LV probe was connected via a Pressure Control Unit (Model 2000, Millar Instruments) to a receiver (Powerlab 4/30, ADInstruments) and a computer acquiring data at 1000 Hz. The probe was advanced into the LV for a 5 min baseline. Rats exposed to air with saline injection and acrolein with saline injection then underwent a dobutamine stress test followed by vagotomy which will be described in the next section. Rats exposed to air with PAG injection and acrolein and PAG injection were then euthanized by exsanguination. Acquisition software (LabChart Pro version 7.3.2, ADInstruments) generated pressure at end diastole and end systole (EDP and ESP) and the maximum and minimum pressure slopes (dP/dt_{\max} and dP/dt_{\min} , respectively) per beat, indicative of contractility and relaxation rate (lusitropy), respectively.

Left Ventricular Pressure Readings with Dobutamine Stress Test, and Vagotomy

Rats exposed to air with saline injection and acrolein with saline injection were prepared for left ventricular pressure readings as described above, and the left jugular vein was cannulated for cardiac stress test by sympathomimetic infusion (dobutamine) before the probe was inserted into the carotid artery. The probe was advanced into the LV, and after a 5 min baseline, freshly diluted dobutamine hydrochloride (dissolved in 0.9% NaCl saline at 20 $\mu\text{g}/\text{ml}$ and infused at a dose of 10 $\mu\text{g}/\text{kg}/\text{min}$ *i.v.*) was infused for 2 min. Rats were observed for 10 min after infusion cessation, which allowed adequate time for recovery to resting heart rate and dP/dt_{max} . Animals then received bilateral vagotomy by suture occlusion followed by a stabilization period (3 min), another 2-min dobutamine infusion at the same dose and a post-infusion observation period (2.5 min). Rats were then euthanized by exsanguination. Acquisition software (LabChart Pro version 7.3.2, ADInstruments) generated pressure at end diastole and end systole (EDP and ESP) and the maximum and minimum pressure slopes (dP/dt_{max} and dP/dt_{min} , respectively) per beat, indicative of contractility and relaxation rate (lusitropy), respectively.

Blood Gas Parameter Measurements During Acrolein Exposure

Changes in blood gas parameters during acrolein exposure were determined in a separate cohort of rats. All rats were assigned to one of four treatments groups: air exposure and saline injection; acrolein exposure and saline injection; air exposure and PAG injection; and acrolein exposure and PAG injection. Rats implanted with femoral artery catheters were acclimated to whole body plethysmography chambers (WBP; Model PLY3213, Buxco Electronics, Inc, Wilmington, NC), for 1 hour on the two days prior to exposure. On the day

of exposure, the rats were received an intraperitoneal injection of either saline or PAG. The exteriorized catheter was extended with a 1 ml PE50 catheter attached to a 23 gauge adapter and run through a pre-measured hole in the wall of the plethysmograph to allow for blood draws outside of the sealed exposure chamber. Rats were acclimated to the exposure chambers for 30 minutes and then were exposed to either filtered air or acrolein as described previously. For all exposure groups, a baseline 250µl blood sample was taken after acclimation. For air exposed rats, additional 250 µl blood samples were taken 30 minutes after baseline, 1 hour after baseline, and 1 hour 15 min after baseline. For acrolein exposed rats, additional 250 µl blood samples were taken 30 minutes after the start of acrolein, 1 hour after the start of acrolein, and 15 minutes after the end of acrolein exposure. For each blood draw, the pin was removed from the tip of the catheter, and the heparin was allowed to exit the catheter. When only blood remained, a 23-gauge adapter attached to a 1 ml syringe was placed on the end of the catheter and used to draw a 250µl sample. The blood sample was immediately read on an OPTI CCA-TS Blood Analyzer (OPTI Medical Systems, Inc.) using the OPTI Cassette E-Ca which measures blood pH, PCO₂, PO₂, Na⁺, K⁺, Ca²⁺, total hemoglobin (tHb), oxygen saturation (SaO₂), and hematocrit (Hct). The catheter was flushed with saline and locked with heparin after each blood draw.

Whole-body Plethysmograph Data Acquisition and Analysis

All exposures were performed in whole-body plethysmography chambers (Buxco Electronics, Sharon, CT). The plethysmography methodology permitted continuous monitoring of breathing frequency, tidal volume, minute volume, inspiratory time and expiratory time. Animals were acclimated to plethysmographs for 1 hour each day on the two

days prior to exposure before both air and acrolein exposure. Plethysmography chambers (model PLY3213; Buxco Electronics) were calibrated each day before every animal loading. A bias flow regulator delivered fresh air (1.8 L/min) to each cylindrical chamber, preventing CO₂ buildup within the chamber. Unrestrained animals were placed in individual cylindrical plethysmograph boxes containing a built-in reference chamber for measuring respiration-induced pressure fluctuations. Data were channeled to computer software (BioSystem XA; Buxco Electronics) that calculated respiratory parameters. Data were collected continuously for each parameter, and automated breath-by-breath analyses were performed using a rejection algorithm to eliminate breaths that were outside a given range. On exposure day, animals were acclimated to the chambers for 30 minutes before any readings were taken. After acclimation, 30 minutes of baseline data were taken, and animals were exposed to either filtered air or 3 ppm acrolein for 3 hours. After exposure, rats were removed and returned to home-cages.

Radiotelemetry Data Acquisition

Radiotelemetry methodology (Data Sciences International, Inc.) enabled constant monitoring of ECG data in unrestrained, un-anesthetized rats from implantation until euthanasia. Remote receivers (DataART3.01; Data Sciences International, Inc.) positioned under the home cages, and under the plethysmographs during exposure collected the ECG data and transferred it to the computer for storage. In home cages, sixty-second segments of ECG waveforms were acquired and saved at 15 minute intervals from surgical recovery through euthanasia not including the exposure period. Pre-exposure baseline data were collected from home cages, as well as a 30 minute baseline in exposure cages after a 30

minute acclimation period. During the 3 hour exposure, sixty-second segments were acquired and saved at 5 minute intervals. After exposure, rats were monitored in home cages until euthanasia, approximately 12 hours after the end of the final hypoxia challenge. HR and BP were automatically obtained from the ECG and BP waveforms, respectively, with data acquisition software (DataART3.01; Data Sciences International, Inc.).

Electrocardiogram, Arrhythmia Identification and Heart Rate Variability (HRV) Analysis

ECGAuto software (EMKA Technologies, Falls Church, VA) was used for automated analyses of ECG wave amplitudes and segment durations and areas, as well as for the visual identification and enumeration of cardiac arrhythmias. Several parameters were determined for each ECG waveform: PR interval; R amplitude and duration; QRS area; ST interval, amplitude, and area; and T-wave amplitude and area; QT interval, and heart rate-corrected QT interval (QTc). ECG parameters during exposure were analyzed in terms of baseline (30 minute recordings while in the exposure chambers immediately before the beginning of exposure) and Hours 1–3 (constituting the entire exposure period between 8:00 AM and 11:00 AM).

Cardiac arrhythmic events were identified in part by using the Lambeth conventions (Walker *et al.*, 1988) as a guideline for the identification of arrhythmias in rats. Arrhythmias were identified as atrial premature beats (APB), ventricular premature beats (VPB), sinoatrial blocks (SAB), atrioventricular blocks (AVB), or ventricular tachycardia (VT). Arrhythmias were quantified, counted and totaled over an 18 hour period prior to exposure (this corresponded to the same times assessed after exposure), during the 4 hour exposure period, or during the 18 hour period beginning immediately after exposure. Total arrhythmia counts

during exposure were quantified (total of 48 two-minute segments during 3 hour exposure period) and expressed as counts per minute.

For the analysis of HRV, thorough visual inspection was conducted to identify and exclude arrhythmias, artifacts, and 1 minute ECG waveforms lacking distinguishable R waves for more than 30 seconds. The analysis of HRV generated heart rate (HR) and time-domain measures, including mean time between adjacent QRS complex peaks (the RR interval), a standard deviation of the RR interval (SDNN), SDNN normalized for the effects of heart rate [$SDNN/(RR \text{ interval} \times 100)$], and the square root of the mean of squared differences of adjacent RR intervals (RMSSD). The SDNN represents overall HRV, whereas RMSSD represents parasympathetic influence over HR. The analysis of HRV also calculated frequency domain parameters, particularly low frequency (LF) and high frequency (HF), and the ratio of these two frequency domains (LF/HF). LF is generally believed to represent a mixture of sympathetic and parasympathetic tone, whereas HF indicates cardiac parasympathetic (vagal) tone, and LF/HF serves as an index of sympathovagal balance.

Statistics

The statistical analyses of the Buxco, ECG and HRV data in this study used SAS version 9.2 software (SAS Institute Inc, Cary NC). We used PROC MIXED of SAS since it offers greater flexibility for the modeling of repeated measures data than PROC GLM. It is also suitable for analysis of large, unbalanced data with missing data at random. A linear mixed model with restricted maximum-likelihood estimation analysis, least squares means and repeated measures ANOVA was used to determine which TIME*TRT interactions were statistically significant between baseline and exposure. Multiple comparison adjustment for

the p values and confidence limits for the differences between the least squares means was done using adjust=Tukey HSD (Honest Significant Difference) test. All blood gas data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) with a one-way analysis of variance (ANOVA) model examining the main effects of each model. All strain comparisons were performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) with a student t-test. Trend analysis of SDNN data during exposure and HR data during hypoxia challenge was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) with a one-way ANOVA followed by a post-hoc test for linear trend between mean and column number. $P < 0.05$ was considered statistically significant.

Results

Effects of Acrolein on Blood Parameters

In rats exposed to acrolein with saline injection, pO_2 decreased from baseline levels of 101.43 ± 1.67 mmHg to 91.00 ± 3.59 mmHg 30 minutes into exposure, to 90.20 ± 1.16 mmHg one hour into exposure, and continued to decrease to 89.80 mmHg after exposure ended ($p < 0.05$; Fig. 4.1A). Rats exposed to acrolein with saline injection and acrolein with PAG injection had significant increases in pCO_2 during acrolein exposure. For rats exposed to acrolein with saline injection, pCO_2 increased from baseline levels of 38.57 ± 1.15 mmHg to 41.85 ± 1.40 mmHg 30 minutes into exposure, to 42.00 ± 1.63 mmHg one hour into exposure, and remained high at 41.80 ± 1.39 mmHg after exposure ended ($p < 0.05$; Fig. 4.1B). For rats exposed to acrolein with PAG injection, pCO_2 increased from baseline levels of 36.50 ± 1.06 mmHg to 41.25 ± 0.43 mmHg 30 minutes into exposure, to 40.20 ± 0.58 mmHg one hour into exposure ($p < 0.05$; Fig. 4.1B). Rats exposed to acrolein with saline

injection had significant decreases in K^+ during and after exposure. Baseline K^+ levels of 6.64 ± 0.74 mmol/L decreased to 4.37 ± 0.24 30 minutes into exposure, to 4.63 ± 0.38 one hour into exposure, and increased slightly to 5.46 ± 0.54 mmol/L after exposure ended ($p < 0.05$; Fig. 4.1C).

pH, SO_2 , and Na^+ were not affected by acrolein exposure or PAG injection (Data not shown). WKY rats exposed to air or acrolein had no significant changes in any blood parameters measured (Table 4.1).

Effects of Acrolein on Ventilatory Parameters

Rats exposed to acrolein had significant decreases in breathing frequency in the first 30 seconds of exposure. Breathing frequency decreased significantly from 288.70 ± 23.22 breaths/min at baseline to 153.63 ± 14.99 breaths/min 20 seconds into acrolein exposure (Figure 4.2A). In addition, acrolein exposed rats had significant increases in expiratory time (T_e), a measure of apnea within the first 20 seconds of exposure. T_e increased from baseline levels of 0.16 ± 0.038 seconds to 0.28 ± 0.037 seconds at 10 seconds into acrolein exposure and 0.28 ± 0.023 seconds 20 seconds into acrolein exposure (Figure 4.2B). Decreased breathing frequency and increased expiratory time were not seen in PAG treated rats (Data not shown). There were no significant changes in minute volume, tidal volume, or inspiratory time in rats treated with saline of PAG.

Effects of Carotid Body Inhibition on Blood Pressure, Heart Rate, and Temperature during Acrolein Exposure

Rats exposed to acrolein with saline injection had significant increases in mean arterial blood pressure (MAP), systolic blood pressure, and diastolic blood pressure during acrolein exposure compared to the other exposure groups (Fig. 4.3).

For MAP at hour 2, rats exposed to acrolein with saline injection had a 3.94 ± 1.26 % decrease from baseline compared to rats exposed to air with saline injection (13.11 ± 1.80 % decrease from baseline) ($p < 0.05$; Data not shown). At hour 3, rats exposed to acrolein with saline injection had a 13.40 ± 2.53 % increase from baseline compared to rats exposed to air with saline injection (11.33 ± 3.37 % decrease from baseline) and rats exposed to air with PAG injection (3.75 ± 3.54 % decrease from baseline) ($p < 0.05$; Data not shown).

For systolic blood pressure at hour 2, rats exposed to acrolein with saline injection had a 3.28 ± 1.97 % decrease from baseline compared to rats exposed to air with saline injection (12.03 ± 1.74 % decrease from baseline) ($p < 0.05$; Fig. 4.3A). At hour 3, rats exposed to acrolein with saline injection had a 13.80 ± 2.24 % increase from baseline compared to rats exposed to air with saline injection (9.85 ± 2.78 % decrease from baseline), rats exposed to air with PAG injection (2.65 ± 3.33 % decrease from baseline), and rats exposed to acrolein with PAG injection (1.73 ± 2.34 % increase from baseline) ($p < 0.05$; Fig. 4.3A).

For diastolic blood pressure at hour 3, rats exposed to acrolein with saline injection had a 13.10 ± 2.61 % increase from baseline compared to rats exposed to air with saline injection (13.98 ± 3.72 % decrease from baseline), rats exposed to air with PAG injection

(5.57 ± 4.02 % decrease from baseline), and rats exposed to acrolein with PAG injection (1.09 ± 3.74 % increase from baseline) ($p < 0.05$; Fig. 4.3B).

Rats had no significant changes in heart rate during exposure regardless of exposure group or PAG injection (Fig. 4.3C).

Rats exposed to acrolein with saline injection had a significantly smaller decrease in temperature during hour 2 of acrolein exposure (Fig. 4.3D). Temperature decreased from baseline levels of 38.45 ± 0.13 °C to 37.62 ± 0.19 °C at hour 2 in rats exposed to acrolein with saline injection compared to rats exposed to air with saline injection (38.59 ± 0.24 °C at baseline to 37.33 ± 0.18 °C at hour 2), rats exposed to air with PAG injection (37.93 ± 0.10 °C at baseline to 36.95 ± 0.14 °C at hour 2), and rats exposed to acrolein with PAG injection (37.88 ± 0.28 °C at baseline to 36.59 ± 0.29 °C at hour 2). For rats exposed to acrolein with saline injection, this constituted a 0.019 ± 0.004 % decrease from baseline at hour 2 compared to a 0.041 ± 0.005 % decrease from baseline in rats exposed to air with saline injection ($p < 0.05$; Fig. 4.3D).

Differences in Measurements of Contractility 24 Hours after Acrolein Exposure

Rats exposed to acrolein with saline injection had a significant decrease in dp/dT_{\max} , a measure of cardiac contractility, 24 hours after exposure compared to the control air exposure with saline injection. Rats exposed to acrolein with saline injection had dp/dT_{\max} levels of 5672.56 ± 630.63 mmHg/s compared to rats exposed to air with saline injection (8992.40 ± 971.39 mmHg/s) ($p < 0.05$; Fig. 4.4). This decrease was eliminated with PAG injection (Fig. 4.4).

The Effect of Acrolein Exposure on Measurements of Cardiac Function

Rats exposed to acrolein had significant decreases in baseline dP/dT_{\max} 24 hours after exposure that were reversed with bilateral vagotomy. At baseline, rats exposed to acrolein with saline injection had average dP/dT_{\max} values of 5672.56 ± 630.63 mmHg/s compared to 8992.40 ± 971.39 mmHg/s in air exposed rats with saline injection ($p < 0.05$; Fig. 4.5A). After bilateral vagotomy, acrolein exposed rats with saline injection had baseline dP/dT_{\max} values of 14365.65 ± 1243.72 mmHg/s compared to 11588.65 ± 1305.35 mmHg/s in air exposed rats with saline injection (Fig. 4.5A). Rats exposed to acrolein had significant increases in dP/dT_{\max} during dobutmaine infusion I. Acrolein exposed rats had a 111.37 ± 16.19 % increase from baseline compared to a 58.68 ± 12.66 % increase from baseline in rats exposed to air ($p < 0.05$; Fig. 4.5). This increase in dP/dT_{\max} was eliminated after vagotomy (Fig. 4.5B)

Discussion

The current study demonstrates four major points: 1) that acrolein exposure-induced apnea is associated with decreases in arterial pO_2 and increases in pCO_2 in SH rats, suggesting hypoxemia, 2) hypoxemia was only evident in acrolein-exposed SH rats, but not rats with normal blood pressure, 3) acrolein exposure-induced cardiovascular responses including increased blood pressure and diminished contractility and elevated core body temperature are prevented by pharmacologic blockade of the carotid body, the hypoxia sensing organ, and 4) carotid body-mediated cardiovascular responses are associated with an oscillating autonomic pattern characterized by an early increase in sympathetic tone followed by a rebound increase in parasympathetic tone. Decreases in pO_2 during acrolein exposure

demonstrate the potential for hypoxemia and downstream peripheral chemoreceptor-mediated responses to hypoxia. Acrolein exposure alone caused significant increases in mean arterial, systolic, and diastolic blood pressure that were prevented with carotid body inhibition. Acrolein exposure caused significant reductions in baseline dP/dT_{\max} , a measure of cardiac contractility, which was reversed with carotid body inhibition. Bilateral vagotomy one day after exposure reversed significant decreases in baseline contractility, demonstrating that the reduction in contractility was likely mediated by increases in parasympathetic tone.

Acrolein exposure significantly decreased pO_2 in hypertensive rats, suggesting that the animals were hypoxic. Acrolein, like other environmental pollutants, stimulates airway irritant sensory nerves (Lee *et al.*, 1992; Oortgiesen *et al.*, 2000), especially chemosensitive bronchopulmonary C-fibers, in part by activation of transient receptor potential vanilloid 1 cation channels (TRPV1) present on the peripheral terminals of these fibers (Hazari *et al.*, 2008). Upon activation of these fibers, a chemoreflex is elicited that is characterized by apnea and bronchospasm (Coleridge and Coleridge, 1994), which in part may explain the responses observed. We found significant decreases in breathing frequency in SH rats immediately after the start of acrolein as well as significant increases in expiratory time, a marker of apnea, consistent with our previous findings (Hazari *et al.*, 2008). While this may explain the initial drop in pO_2 , the apnic response was transient, lasting only 30 seconds. Thus, apnea alone likely does not explain the decrease in pO_2 that was detected up to an hour into exposure and that persisted for 30 minutes after a 1-hour exposure to acrolein. Other potential explanations include inflammatory and edematous responses, although such responses are common at higher concentrations. Janssens *et al.* (1994) reported decreases in arterial pO_2 after acrolein smoke exposure in sheep and attributed the responses to acrolein-

induced airway resistance and edema. Acrolein exposure has been shown to increase lung lymph flow and extracellular lung water coupled with airway damage and pulmonary edema (Hales *et al.*, 1992). While acrolein exposure decreased pO_2 in SH rats, no such change was evident in similarly exposed WKY rats and may be related to underlying differences in sensitivity to air pollution. Previous studies have shown that the SH rat responds with greater airway inflammation, edema, and injury after air pollution exposure than WKY rats (Farraj *et al.*, 2009; Kodavanti and Costa, 2001). In addition, SH rats have larger carotid bodies (Habeck *et al.*, 1985) and are more sensitive to the effects of hypoxia as evidenced by increased carotid sinus nerve activity and intracellular Ca^{2+} changes (Weil *et al.*, 1998).

Although there were significant reductions in pO_2 with acrolein exposure in SH rats, there were no significant changes in blood oxygen saturation (i.e. SaO_2). pO_2 is a measure of dissolved oxygen in plasma, while SaO_2 measures the percentage of hemoglobin sites bound with oxygen. The discrepancy in pO_2 and SaO_2 is likely due to the fact that SaO_2 is well buffered from rapid decreases due to the sigmoidal nature of the oxy-hemoglobin dissociation curve (Urbano and Mohsenin, 2006). Because a large drop in pO_2 is required to alter SaO_2 , reductions in pO_2 may be a more sensitive indicator of a pollutant's effect on blood oxygen levels. Additionally, epidemiological studies linking reduction in SaO_2 and pollutant exposure are based on pulse oximetry recordings (Gong *et al.*, 2004; Gong *et al.*, 2005) derived from light absorbance readings of a sensor usually placed on a finger. This measurement is not as sensitive as direct analysis of arterial blood, and demonstrates the increased sensitivity of the system used in this study. Decreases in arterial pO_2 were coupled with significant increases in pCO_2 . Importantly, the body is significantly more sensitive to systemic changes in pCO_2 and the magnitude of the change in pCO_2 with acrolein exposure

in this study has been demonstrated to be sufficient to trigger activation of the carotid body (Iturriaga *et al.*, 2005). Thus, the small but significant decrease in pO₂ and the increase in pCO₂ during acrolein exposure suggest that carotid body activation took place.

In addition to changes in arterial blood gas parameters, acrolein exposure significantly decreased K⁺ in hypertensive rats. Hypokalemia, defined as less than 3.5mmol/L K⁺ in arterial blood, may occur in diseases with high aldosterone levels such as hypertension and is a result of abnormal renal regulation of K⁺ (Halperin and Kamel, 1998). SH rats are known to have kidney dysfunction (Hulstrom, 2012), but there are no reports of acrolein causing hypokalemia in humans or rodent models. Although it is unclear why acrolein exposure causes decreases in K⁺, acute hypokalemia has been shown to sensitize the heart to hypoxic injury (Shapiro *et al.*, 1998). We did not see reductions in K⁺ to the level of hypoxemia, but reduced K⁺ has been shown to have multiple adverse effects of cardiovascular function including cardiac arrhythmias, acceleration of kidney disease progression, and exacerbated hypertension (Asmar *et al.*, 2012). Hypokalemia can lead to elevated aldosterone levels and increased blood pressure (Abad-Cardiel *et al.*, 2013), and rats fed a high salt diet had increased sensitivity of the carotid body that was attributed to low potassium levels (Elias *et al.*, 2004). Reductions in K⁺ during acrolein exposure may be a potential driver of increased blood pressure and may influence the carotid body response to hypoxia following acrolein exposure.

Acrolein-induced blood pressure and temperature changes may be related to early decreases in arterial pO₂ and increases in pCO₂, as has been demonstrated in other studies. For example, acute exposure to high altitudes with low oxygen tension in humans increases systemic arterial blood pressure and precipitates acute mountain sickness (Rhodes *et al.*,

2011). Also, cats exposed to short term anoxia had decreased pO_2 and increased pCO_2 coupled with significant increases in arterial blood pressure (Yang *et al.*, 1994). In addition, acrolein exposure-induced decreases in pO_2 in sheep were accompanied by increases in pulmonary arterial pressure and pulmonary vascular resistance (Janssens *et al.*, 1994). Such blood pressure increases may have been triggered by increased sympathetic tone mediated by the carotid body in response to hypoxia. Although HRV assessments have not yet been analyzed, acrolein exposure in our previous study caused an increase in LF, the low frequency domain parameter indicative of sympathetic tone (Perez *et al.*, 2013). On the other hand, blood pO_2 levels below 60 mm Hg are required for significant activation of the carotid body in humans suggesting that carotid body sensing of pO_2 may not be the driver for these responses. While the changes in pO_2 may not have reached threshold levels necessary for activation of the carotid body, the carotid body may have been impacted to a greater extent by acrolein-induced increases in pCO_2 . The carotid body is much more sensitive to changes in pCO_2 and increases in pCO_2 as small as 5 mmHg have been shown to activate the carotid body (Whipp, 1994). In addition to blood pressure increases, acrolein exposure resulted in an increase in body temperature compared to control animals. Although the reason for this finding is unclear, sympathetic activation has been shown to increase body temperature by stimulating vasoconstriction (Constanzo, 2006). In addition, Loyola *et al.* (1991) reported that increases in core body temperature activate chemosensory discharges from the carotid nerve, suggesting that acrolein induced increases in body temperature may be related to carotid body signaling during exposure. In mammals, the response to systemic hypoxia is controlled by the carotid body, and blood pressure and temperature responses following decreases in arterial pO_2 may be controlled by the carotid body chemoreceptor response.

Pharmacological inhibition of carotid body sensing prevented acrolein-induced increases in MAP, systolic, and diastolic blood pressure during exposure and the reduction in cardiac contractility one day after exposure. These responses suggest that carotid body signaling may be influencing the cardiovascular response to acrolein. Carotid body signal transduction is dependent on hydrogen sulfide production by the cystathionine γ -lyase (CSE) enzyme that generates hydrogen sulfide from cysteine (Peng *et al.*, 2010). PAG pharmacologically inhibits the carotid body through inhibition of the CSE enzyme, and it has been shown to inhibit responses to hypoxia in the carotid body of rats (Peng *et al.*, 2010). The prevention of acrolein-induced blood pressure increases in PAG-treated rats is of great interest because hypoxia is associated with increased myocyte apoptosis and is implicated in cardiac remodeling and progression to heart failure (Bao *et al.*, 2011). In addition, hypoxia has been shown to cause atrial fibrillation, AV block, and ventricular tachycardia as well as changes in autonomic balance associated with ventricular arrhythmias (Davies and Wedzicha, 1993). Oral exposure to acrolein in mice has been shown to cause inflammation and dilated cardiomyopathy characterized by increased left ventricular dilation (increased end diastolic volume), contractile dysfunction (decreased dp/dT_{max}), and impaired relaxation (Ismahil *et al.*, 2011). Recent work by Wang *et al.* (2012) demonstrated that cardiac arrhythmias in particulate-exposed heart failure mice were in part due to altered sensitivity of the carotid body. Furthermore, exposures to the air pollutants tobacco smoke (Adgent, 2006), sulfur dioxide and nitrogen dioxide (Hoppenbrouwers *et al.*, 1981) have been linked to abnormal cardiopulmonary sensitivity responses to hypoxia. Collectively, these finds provide plausibility for a role for hypoxia in the mediation of some of the adverse cardiovascular effects of air pollution.

Acrolein exposure caused a decrease in cardiac contractility one day after exposure as determined by assessments of left ventricular pressure, which may have stemmed from a shift in autonomic tone. We have previously determined (Carll *et al.*, to be submitted) that early increases in sympathetic tone with diesel exhaust exposure are followed by rebound increases in parasympathetic tone after exposure. An increase in contractility during exposure to acrolein, although not directly measured, is possible and would be consistent with the increase in blood pressure in this study and previous demonstrations of elevated sympathetic tone during acrolein exposure. Forthcoming assessments of QA interval, an indirect measure of cardiac contractility derived from ECG and blood pressure signals, and HRV will shed light on contractility responses during exposure and potential associations with altered autonomic tone. Acrolein-induced increases in contractility are plausible given that other acrolein-induced cardiovascular responses were prevented by carotid body inhibition in this study and carotid body activation in dogs has been shown to increase cardiac contractility (Vatner and Rutherford, 1978). The baseline decrease in contractility was likely parasympathetically controlled, as bilateral vagotomy reversed significant decreases in baseline contractility one day after exposure. The prevention of the acrolein-induced decrease in contractility with PAG pretreatment suggests that the impaired contractility response one day after exposure with acrolein was a compensatory mechanism characterized by rebound increases in parasympathetic tone mediated by carotid body chemoreceptor signaling.

Acrolein exposed rats showed enhanced sensitivity to dobutamine, a sympathetic agonist, one day after exposure. With an intact autonomic nervous system, rats exposed to acrolein had a markedly significant increase in dP/dT_{max} , a measure of contractility, in response to dobutamine infusion one day after exposure. It is unclear whether this is due to

effects by the autonomic nervous system, or if the increases in contractility are the result of other factors. This response may be due to increased vascular resistance, as has been found with exposure to acrolein (Green and Egle, 1983), and may not be the result of exaggerated sympathetic tone. Alternatively, acrolein activates TRP fibers/receptors (Hazari *et al.*, 2008), which are plentiful in the heart, suggesting that the enhanced sensitivity may be occurring only at the level of the heart. Increases in contractility during dobutamine infusion were eliminated with bilateral vagotomy. Although the mechanism causing the responses one day after exposure are difficult to decipher, the body has many ways to respond to air pollutants, and it is likely that more than one mechanism is activated upon inhalation of different pollutants. Future studies will include pharmacological block of parasympathetic nerve activity to the heart with atropine to fully gauge the role of the parasympathetic response. It is conceivable that the carotid body mediated physiologic responses to hypoxia trigger sympathetic-driven cardiovascular responses during exposure followed by parasympathetic-driven responses after acrolein exposure (Figure 4.6).

The present findings demonstrate for the first time that acrolein exposure may cause changes in blood pressure and cardiac function through carotid body chemoreceptor signaling. In addition, exposure to irritants known to illicit apnea can affect blood gas concentrations and stimulate chemoreceptor activation with downstream cardiovascular consequences. These findings also highlight the previously underexplored role of hypoxia signaling during air pollution exposure and reveal the complex interplay between chemoreceptor signaling and the autonomic nervous system.

Table 4.1. Arterial blood parameters during acrolein exposure in WKY rats. Means and standard errors are reported.

Exposure	pO ₂ (mmHg)	pCO ₂ (mmHg)	SaO ₂ (%)	pH	K ⁺ (mmol/L)	Na ⁺ (mmol/L)
Baseline	109 ± 2.77	36.6 ± 1.86	94.8 ± 0.66	7.27 ± 0.05	7.66 ± 1.10	149.8 ± 2.96
30 minutes	101.4 ± 4.53	36.7 ± 2.09	94.4 ± 0.24	7.28 ± 0.04	7.36 ± 0.81	147.2 ± 1.98
1 Hour	102.6 ± 4.71	35.4 ± 1.47	93.40 ± 0.68	7.27 ± 0.05	6.70 ± 0.72	152.2 ± 1.47
Post Exposure	98 ± 2.19	36.7 ± 0.68	93.4 ± 0.40	7.41 ± 0.02	5.70 ± 0.49	143.6 ± 0.51

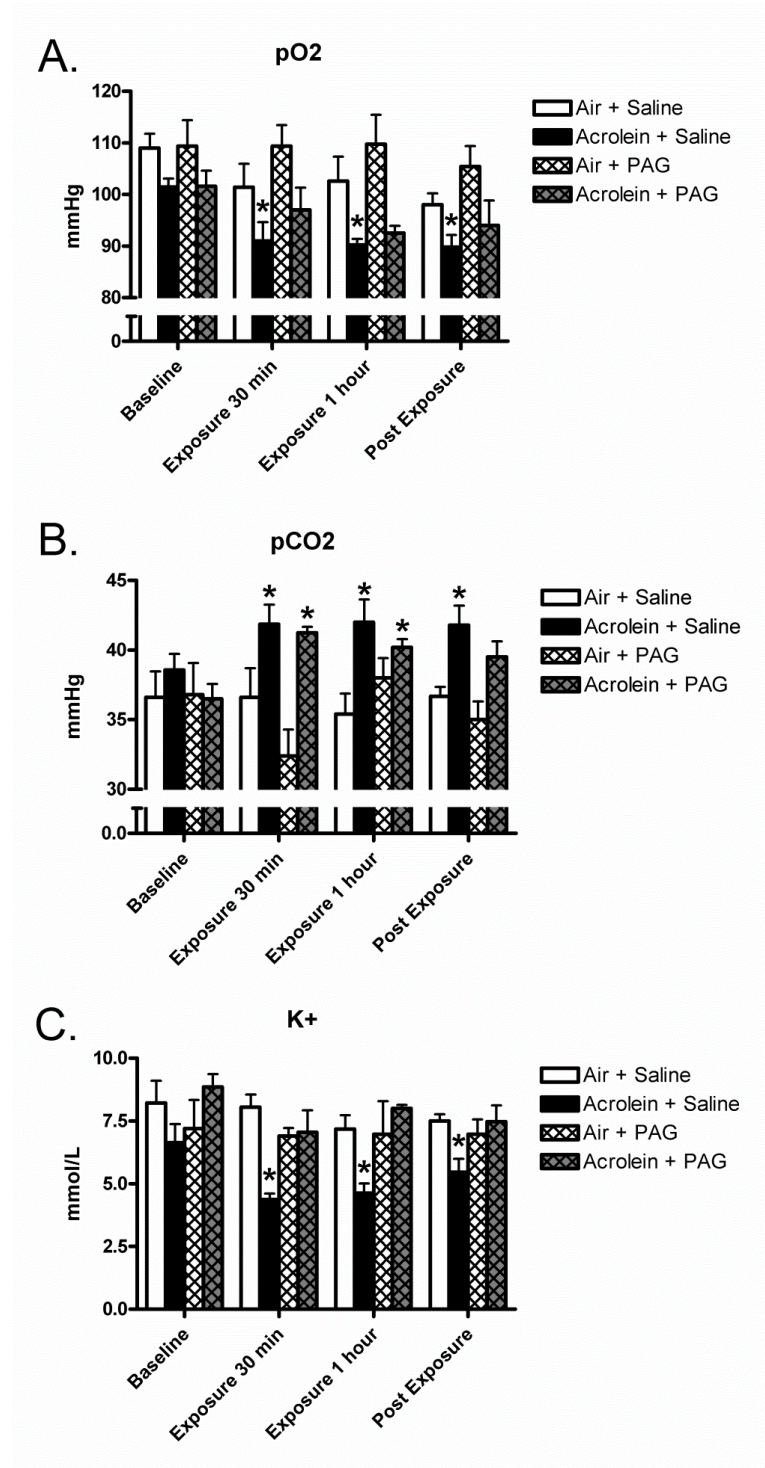


Figure 4.1. Acrolein exposure causes significant decreases in arterial blood oxygen and potassium and significant increases in arterial carbon dioxide. SH rats implanted with femoral artery catheters were injected with saline or PAG carotid body inhibitor and exposed

to 3 ppm acrolein or air control. Panel A, B, and C refer to pO_2 , pCO_2 , and K^+ , respectively, measured at baseline, 30 minutes into acrolein exposure, 1 hour into acrolein exposure, and 15 minutes post exposure. Means and standard errors are reported. Significant differences from baseline values ($p < 0.05$) are denoted with a *.

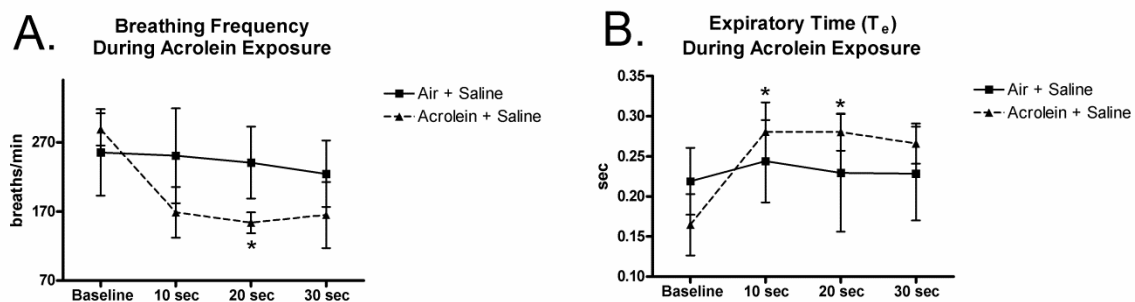


Figure 4.2. Acrolein exposure causes significant decreases in breathing frequency and significant increases in expiratory time immediately after the beginning of exposure. Panels A and B refer to breathing frequency and expiratory time, respectively. Means and standard errors are reported. Significant differences from baseline ($p < 0.05$) are denoted with a *.

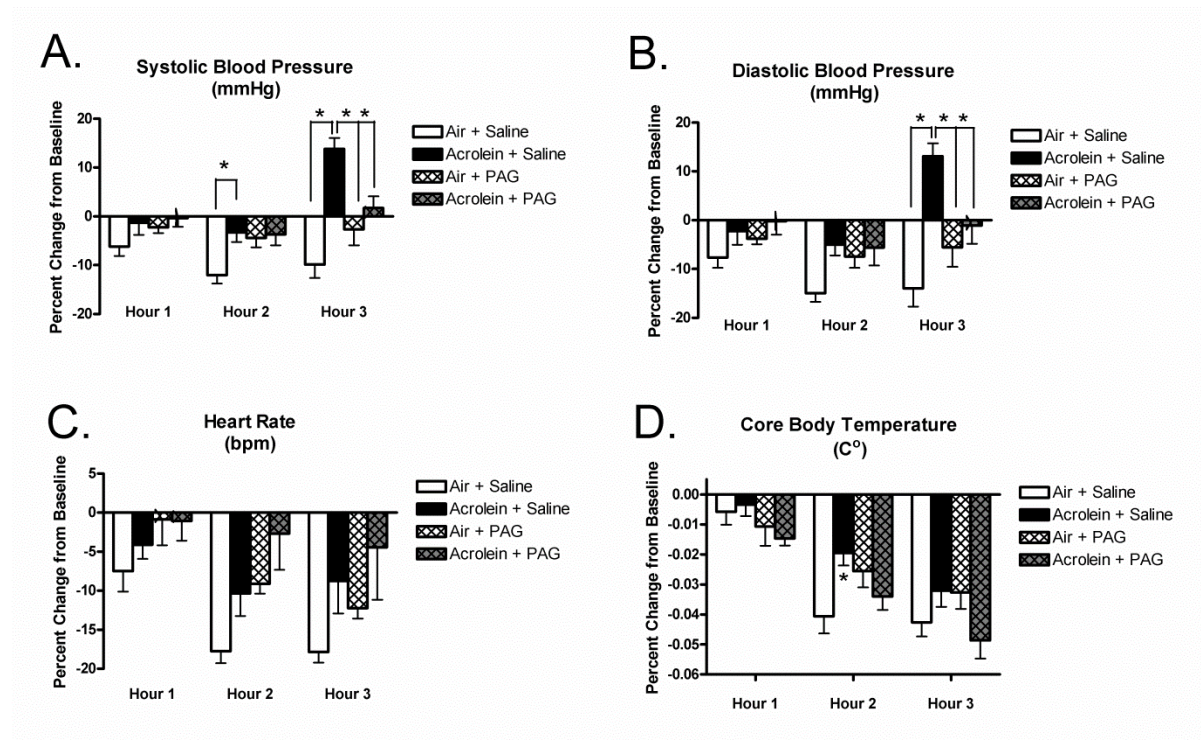


Figure 4.3. Carotid body inhibition attenuates blood pressure and temperature increases during acrolein exposure. Panel A, B, C, and D refer to systolic blood pressure, diastolic blood pressure, heart rate, and core body temperature respectively. Means and standard errors are reported. Significant differences between groups ($p < 0.05$) are denoted with a *.

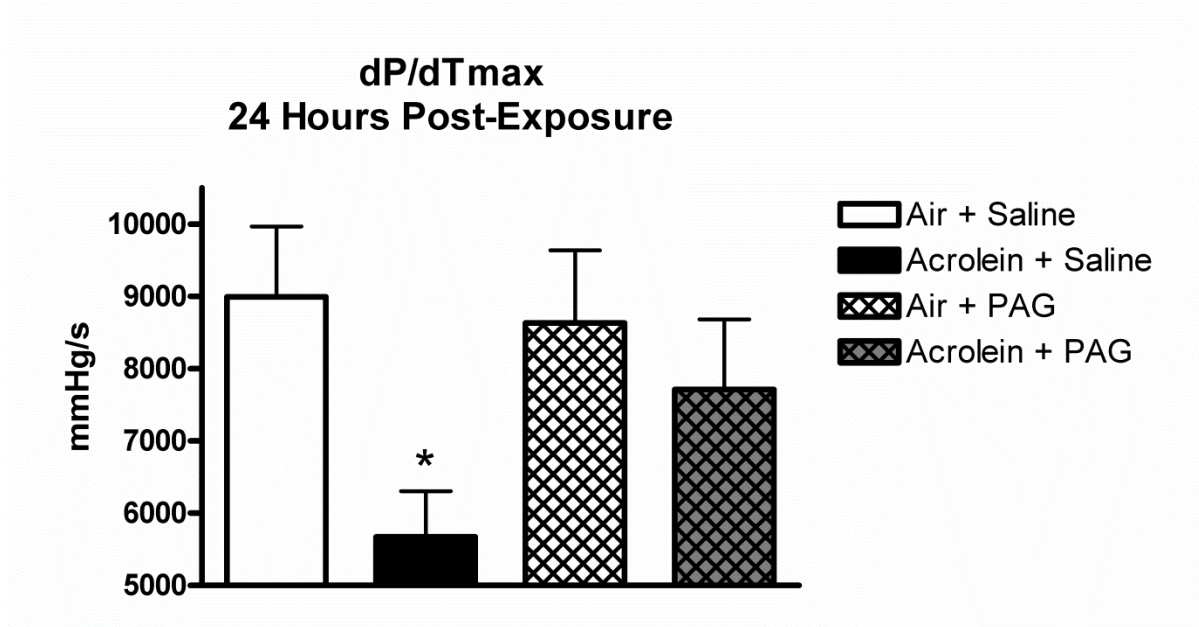


Figure 4.4. Carotid body inhibition reverses decreases in dP/dT_{max} , a measure of contractility, 24 hours post acrolein exposure. Means and standard errors are reported. Significant differences between groups ($p < 0.05$) are denoted with a *.

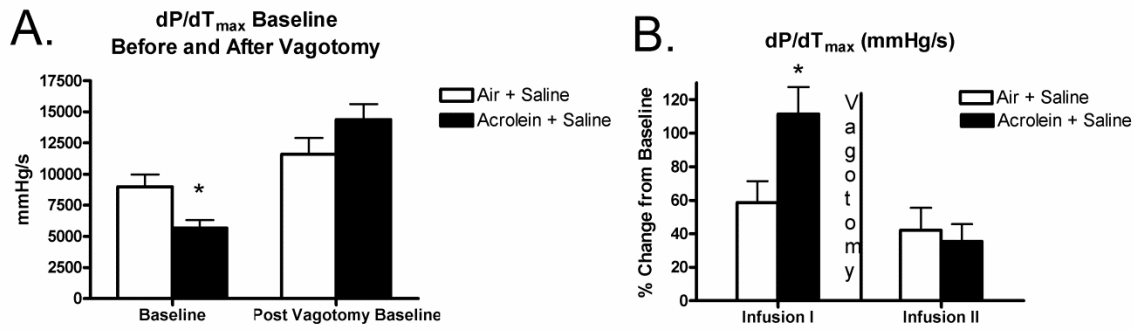


Figure 4.5. Decrease contractility one day after exposure is likely mediated by the parasympathetic nervous system, while dobutamine infusion reveals significant increases in contractility that is reversed by vagotomy. Panel A refers to baseline values of dP/dT_{max} 24 hours post exposure. Means and SEMs are reported. Significant differences ($p < 0.05$) from air exposed rats with saline injection are denoted with a *. Panel B refers to dP/dT_{max} values 24 hours post exposure during 2 minute dobutamine infusion. Percent change values from baseline and SEMs are reported. Significant differences between groups ($p < 0.05$) are denoted with a *.

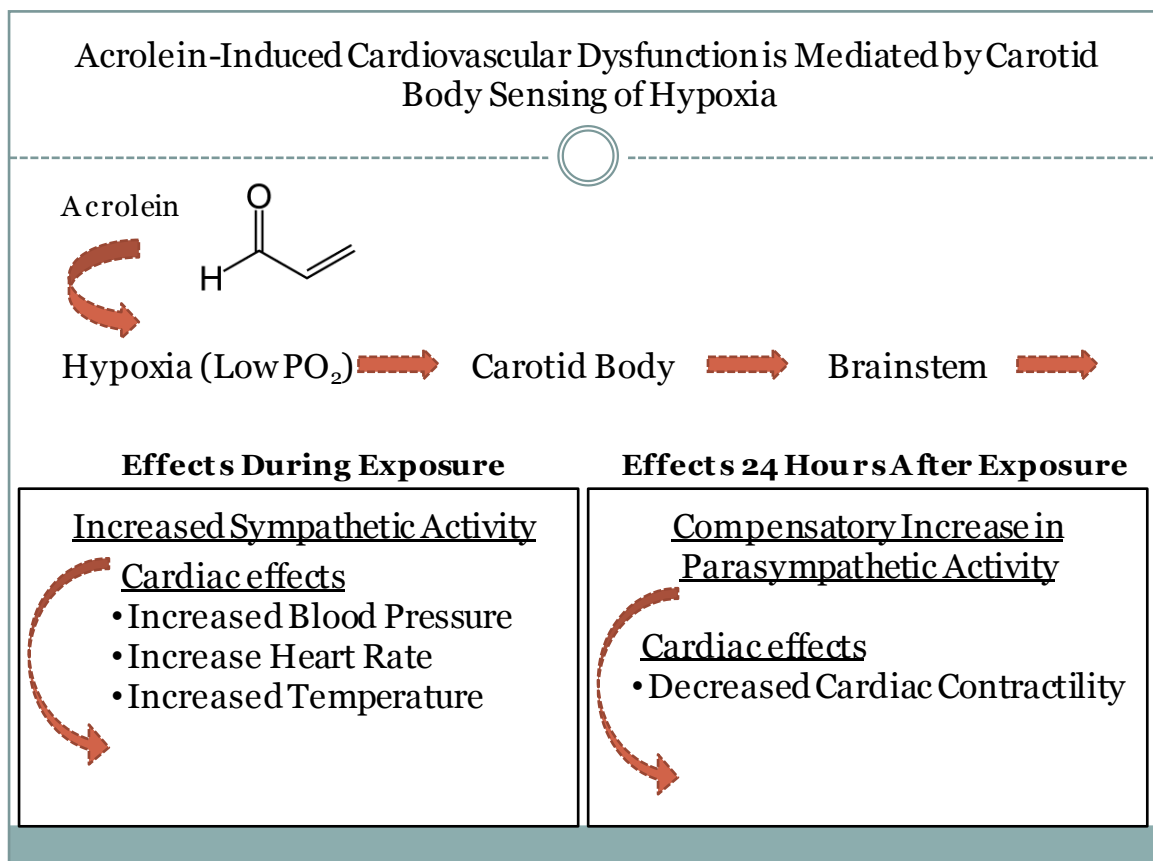


Figure 4.6. Acrolein-induced cardiovascular dysfunction is mediated by carotid body sensing of hypoxia. We propose that adverse cardiovascular events following acrolein exposure are likely due to the carotid body response to hypoxia leading to increased sympathetic tone and downstream cardiac effects.

Chapter 5

Conclusions and Implications

This dissertation research project sought to uncover the potential mechanisms responsible for the adverse cardiovascular events associated with air pollution exposure. This goal is predicated on a wealth of epidemiological studies showing that exposure to air pollution causes increased cardiovascular morbidity and mortality, especially in individuals with underlying cardiopulmonary diseases. Because epidemiological and experimental data exists that suggest that hypoxia may play a role in the adverse cardiovascular effects of air pollution, we tested the hypothesis that hypoxia mediates in part the cardiovascular effects of air pollution. Spontaneously Hypertensive (SH) rats were used to model humans with hypertension, a subgroup with acute sensitivity to the effects of air pollution exposure. A number of studies were carried out in the SH rat to determine 1) if exposure to whole or filtered DE and the toxic DE component acrolein cause adverse cardiovascular responses in hypertensive rats compared to normotensive controls, 2) if a hypoxia stress test reveals altered sensitivity in the hypertensive rat following acrolein exposure, 3) if acrolein exposure itself causes decreases in blood oxygen levels, and 4) if the adverse cardiovascular effects of acrolein are mediated by chemoreflex signaling in the carotid body.

Divergent Responses to Whole and Filtered Diesel Exhaust Inhalation in Spontaneously Hypertensive Rats.

The initial set of experiments was a characterization study that examined the effects of two concentrations of whole and filtered DE exposure in hypertensive (SH) and normal (WKY) rats. Exposure to fDE, but not wDE, caused immediate electrocardiographic alterations in cardiac repolarization (ST depression) and atrioventricular conduction block (PR prolongation) as well as bradycardia in SH rats. Exposure to wDE, but not fDE, caused post-exposure ST depression and increased sensitivity to the pulmonary C fiber agonist capsaicin in SH rats. The divergent responses of fDE and wDE highlight the real challenge of studying complex pollutants such as DE. It is apparent from this study that the gaseous and particulate components of DE may be functioning through separate mechanisms and therefore eliciting different responses. This is of great importance in epidemiological studies because human exposures are rarely confined to a single pollutant exposure, and this study demonstrates that multiple pollutants have the ability to interact and cause multiple responses. Most of the responses in this study were seen with exposure to the gaseous components of DE. It is possible that gases trigger immediate pulmonary effects through nasal irritation. Gases will also quickly react with pulmonary receptors and elicit cardiovascular responses such as bradycardia. Another possibility is that in wDE exposure, particles and gases interact and alter the chemistry of the pollutant itself, leading to altered responses compared to separate components alone. This demonstrates the need to model multipollutant environments to understand interactions of constituents.

Notably, the responses to fDE occurred with the low concentration ($150\mu\text{g}/\text{m}^3$), showing the importance of modeling lower concentrations in experimental designs. Although

seemingly counterintuitive, lower concentrations of pollutants may cause more effects than higher concentrations because lower doses of pollutants may activate receptors while higher doses can overload the sensory network and blunt downstream responses. In a study that examined causes for emergency room visits for elevated blood pressure, ambient levels of NO₂, SO₂, PM_{2.5}, and PM₁₀ all had significant associations with increased hypertension-related hospitalizations (Szyszkowicz *et al.*, 2012). Researchers are becoming more and more interested in environmentally relevant concentrations, and the most recent American Heart Association statement on particulate matter air pollution and cardiovascular disease includes recommendations that experimental studies better “describe the physiological relevance in humans and the fundamental details of the mechanisms underlying the intermediate general mediating pathways” (Brook *et al.*, 2010). Future studies should take into account actual exposure concentrations of human populations and extrapolate those concentrations to the animal model.

One of the important findings of this study was that the gaseous components of DE caused significant ST depression, a finding associated with myocardial ischemia. Although myocardial ischemia was not directly measured, the potential of reduced oxygen availability was of great interest and influenced the direction of subsequent studies. It turns out that several studies have found decreases in oxygen saturation in humans following exposure to PM and CAPs (DeMeo *et al.*, 2004; Gong *et al.*, 2004; Gong *et al.*, 2005), which spurred our interest in examining arterial blood oxygen levels in the final study. These findings were in the development of the hypothesis that hypoxia is a mechanism by which the adverse cardiovascular responses following air pollution exposure take place. Another major conclusion from this study that affected the direction of the research was the finding that SH

rats were more sensitive to the effects of DE compared to WKY rats. Multiple epidemiological studies have demonstrated that adverse events following PM exposure are heightened in susceptible populations such as the elderly (Pope et al., 2002) and individuals with diabetes (Zanobetti and Schawart, 2001), preexisting heart disease (Katsouyanni *et al.*, 2001) or heart failure (Goldberg *et al.*, 2001). Clinically, hypertension can be a major risk factor for adverse responses to air pollution. There may be several reasons for this including inability to alter heart rate variability to respond to stressors as well as increased risk of arrhythmia with hypertension that could predispose to more severe responses to air pollutants. Hypertension is associated with several remodeling events that can lead to heightened susceptibility including increased protein synthesis such as myosin and collagen, mutations in genes encoding ion channels that lead to increased action potential duration, altered calcium homeostasis leading to changes in cellular energetics, and decreased sensitivity of β -adrenergic receptors (Swynghedauw, 1999). These changes may explain why hypertension predisposes to adverse responses to air pollution seen clinically. Our studies used the SH rat to model hypertension. The SH rat is an excellent model for hypertension, and has been shown to develop increased vascular resistance, high systemic blood pressure, and increased activation of the rennin-angiotensin system (Kodavanti *et al.*, 2000). Clinically, there are several different causes of hypertension including aging, obesity, and insulin resistance. This is important to remember when extrapolated results from the SH rats. In addition, SH rats have larger carotid bodies (Habeck *et al.*, 1985) and are more sensitive to the effects of hypoxia as evidenced by increased carotid sinus nerve activity and intracellular Ca^{2+} changes (Weil *et al.*, 1998). Despite hypertension originating in the kidney of the SH rat, it can still be used as a model for humans because increased blood pressure leads to

similar phenotypes seen in diverse human populations such as high blood pressure, evidence of cardiac remodeling, and vascular dysfunction.

Modern diesel engines have much stricter emissions criteria, creating much cleaner exhaust emissions and limiting some of the more toxic air pollutants created by older diesel engines (EPA Diesel, <http://www.epa.gov/otaq/marine.htm>). The diesel engine used in this study was an older Yanmar L70 V diesel generator that did not have the more stringent emission standards. Despite more efficient and cleaner engines in our modern diesel vehicles, this study is still extremely relevant because diesel engines have an extremely long life span, so many older, less efficient engines are on the road today and will be for decades to come. In addition, the use of the newer diesel engines has not been adopted in the majority of the developing world where air pollution is a major public health concern and particulate matter levels are significantly higher than those in the United States. In addition, our studies found that the gaseous components of DE causes most of the responses, so even fuel that greatly reduces the sulfur content and particulate matter levels could still create emissions that contain toxic gases. Because most of the responses observed in this study occurred with the gaseous components of DE in hypertensive rats, we decided to focus on the effects of acrolein, a key toxic gas found in DE, for future studies.

Acrolein Exposure Causes Significant Increases in Heart Rate and Blood Pressure in Hypertensive Rats.

DE is a chemically complex source of ambient PM and thus defining modes of action is challenging. In addition to PM, DE also consists of a mixture of gases including nitrogen oxides (NO_x), sulfur oxides (SO_x), carbon monoxide (CO), and volatile organics including

aldehydes, benzene, and polycyclic aromatic hydrocarbons. The previous study with DE revealed the importance of the gaseous components in driving the majority of cardiovascular responses during exposure. Because of the complexity of DE and the importance of the gaseous components in driving the cardiovascular responses, we decided to focus on a toxic gaseous component of DE for future studies to explore the mechanisms controlling cardiovascular responses to air pollution exposure. Acrolein is an α,β -unsaturated aldehyde that is formed during the combustion of organic materials and is a toxic component of cigarette smoke and internal combustion engine exhaust (Baeuchamp *et al.*, 1985). Acrolein exposure in SH rats increased heart rate, blood pressure, breathing frequency, minute volume, and the heart rate variability parameter LF, suggesting a potential role for increased sympathetic tone. Increased sympathetic tone is associated with an elevated risk of heart disease (Pope *et al.*, 2001) and may have driven the adverse cardiovascular effects of acrolein. Green and Egle (1983) found that intravenous injection of acrolein in rats causes significant increases in blood pressure that are reversed with guanethidine, a sympatholytic drug. The acrolein concentration used in the study was 3ppm. In ambient air, 0.04 - 0.08 ppm acrolein have been measured (Costa and Amdur, 1996), but cigarette smoke contains up to 90 ppm acrolein (Esterbauer *et al.*, 1991), and acrolein levels in sidestream tobacco smoke are as high as 10 ppm (Esterbauer *et al.*, 1991). Acrolein is a highly reactive gas that will react with any nucleophile it contacts in the airway. Acrolein may react with cysteine residues of TRP channels that activate pulmonary C fibers (Takahashi and Mori, 2011). Pulmonary C fiber activation in the lung can lead to reflex cardiac responses, characterized by apnea and bradycardia (Widdicombe and Lee, 2001). It is clear from the data, however, that acrolein exposure also causes hypoxia, which through the carotid body led to reflex increases in heart

rate and blood pressure among other changes. It is likely that both responses are occurring with the irritant response initiated first as evidenced by the immediate ventilatory responses with exposure. Signals from the pulmonary receptors, baroreceptors, and chemoreceptors will all be integrated in the brain stem, and it is likely that interpreting multiple signals leads to diverse responses in autonomic tone to balance conflicting input. The hypertensive rat was clearly more susceptible to the effects of acrolein, a finding that supports our previous findings with DE as well as epidemiological data. Although acrolein exposure alone suggested enhanced sensitivity in hypertensive rats, we decided to conduct a stress test to further delineate the enhanced susceptibility in rats with underlying hypertension. A stress test is a common way to reveal enhanced sensitivity or the potential for adverse responses because increased cardiovascular effort causes reduced oxygen availability to tissues and may provoke autonomic imbalance (Goldberger *et al.*, 2006). Numerous studies have shown that stress tests trigger many adverse cardiovascular effects including increased cardiac arrhythmias and deleterious changes in heart rate (HR), heart rate variability (HRV), an indirect indicator of cardiac autonomic tone, and ECG parameters (Watanabe *et al.*, 2001). Multiple studies have shown that hypertensive individuals have abnormal responses to high altitude with low atmospheric oxygen. In addition, exposures to the air pollutants tobacco smoke (Adgent, 2006), sulfur dioxide and nitrogen dioxide (Hoppenbrouwers *et al.*, 1981) have been linked to abnormal cardiopulmonary sensitivity responses to hypoxia. These findings suggest that exposure to hypoxia may be a useful stress test to unmask the latent effects of air pollution.

Hypoxia Stress Test Reveals Exaggerated Cardiovascular Effects in Hypertensive Rats after Exposure to the Air Pollutant Acrolein

The studies with DE and acrolein revealed heightened sensitivity in SH rats, but we wanted to further determine the importance of underlying susceptibility in adverse cardiovascular events 24 hours after acrolein exposure. Epidemiological studies indicate that effects of air pollution exposure are not always immediate, usually manifesting some time after exposure. For example, Peters *et al.* (2004) showed that humans exposed to traffic were more susceptible to myocardial infarction up to 24 hours after exposure. In addition, patients with implanted cardioverter defibrillators had increased incidence of life-threatening arrhythmias up to 2 days after air pollution exposure (Peters *et al.*, 2000). These findings suggest that techniques that help manifest latent responses may be useful in uncovering subtle subclinical effects of air pollution exposure. This approach is predicated on the idea that air pollution primes the cardiovascular system to exaggerated responses to subsequent nonspecific stressors and has been put to practice in the form of exercise stress testing in humans (Gold *et al.*, 2005) and dobutamine and isoproterenol pharmacologic challenge in rats (Hazari *et al.*, 2012; Andre *et al.*, 2011). A novel stress test was developed that set out to mimic the heightened sensitivity of individuals with hypertension to sub-atmospheric oxygen as happens at high altitude. In order to determine latent responses of acrolein exposure, we developed an acute hypoxia stress test that involved short-term exposure to a 10% oxygen atmosphere. The hypoxia stress test after acrolein exposure revealed increased diastolic blood pressure only in hypertensive rats and increased minute volume and expiratory time only in normotensive rats. The hypoxia stress test further demonstrated the enhanced susceptibility of the hypertensive rat and created an additional stress test to determine enhanced sensitivity

to air pollutants. Importantly, hypoxia stress test caused ventilatory responses in WKY rats that were absent in the SH rat. Researchers often assume that enhanced susceptibility means a greater response, but it can also mean the lack of a response when one is expected or required to compensate for an environmental insult. This is critical to remember when defining susceptibility and looking for risk factors in the clinic. Future studies should incorporate additional pollutants to further define the usefulness of this stress test. Given that acrolein induced blood pressure and heart rate changes are preceded by apnea in hypertensive rats (Hazari *et al.*, 2008), we explored the possibility that acrolein exposure causes drops in blood oxygen levels.

Acrolein Exposure Causes Significant Decreases in pO_2 and Significant Increases in pCO_2 in Hypertensive Rats.

Acrolein exposure caused significant decreases in arterial blood pO_2 and significant increases in arterial blood pCO_2 in hypertensive rats. While we did not see significant reductions in SaO_2 as found in humans exposed to air pollution, this is likely due to the fact that SaO_2 is well buffered from rapid decreases due to the sigmoidal nature of the oxy-hemoglobin dissociation curve (Urbano and Mohsenin, 2006). Additionally, the epidemiological studies linking reduction in SaO_2 and pollutant exposure were based on pulse oximetry recordings (Gong *et al.*, 2004; Gong *et al.*, 2005). These measurements are not as sensitive as direct analysis of arterial blood, as used in this study. This is important to note as reductions in pO_2 may be a more sensitive indicator of a pollutant's effect on blood oxygen levels because a large drop in pO_2 is required to alter SaO_2 . Normal pO_2 levels in humans and rats range from 80-100 mmHg, and a drop in partial pressure below 60 mmHg is

required for hypoxemia. Although we did not see a decrease to this extent, small decreases in pO_2 as well as small increases in pCO_2 are able to activate the carotid body. Acrolein exposure also caused an increase in pCO_2 and increases in pCO_2 as small as 5 mmHg have been shown to activate the body's response to hypoxia (Whipp, 1994). Importantly, hypoxia is associated with increased sympathetic tone and increased blood pressure, which were both observed in our exposure studies with acrolein. In addition to these changes, SH rats also had significant decreases in K^+ during acrolein exposure. The reason for this is unclear, but it may be related to increased aldosterone levels in the SH rat from acrolein-induced increases in blood pressure. Changes in blood gas parameters only occurred in SH rats. Future studies should treat SH rats with antihypertensive drugs and determine if the blood gas levels remain unchanged with acrolein exposure. This would allow us to determine if underlying hypertension is responsible for the reduction in pO_2 . This could be extremely important in clinical studies looking at the effects of air pollutants on oxygen saturation because susceptible populations could have a more severe decrease in SaO_2 and be potentially more susceptible to harmful effects. The fact that acrolein exposure resulted in decreased pO_2 , increased pCO_2 , and significant increases in blood pressure and heart rate suggests that the carotid body may be mediating some of the cardiovascular responses to acrolein, a possibility that was examined in the final study.

Pharmacological Inhibition of the Carotid Body Attenuates Blood Pressure Increases during Acrolein Exposure.

In order to determine if the cardiovascular effects of acrolein exposure are mediated by the chemoreflex response to hypoxia, we pharmacologically inhibited the carotid body

prior to acrolein exposure using the CSE-inhibitor PAG . Control rats exposed to acrolein had significant increases in MAP, systolic, and diastolic blood pressure, consistent with our previous study with acrolein (Perez *et al.*, 2013). Pretreatment with the CSE-inhibitor PAG blocked acrolein-induced blood pressure increases, suggesting that the carotid body is at least in part mediating the cardiovascular response to acrolein. Acrolein also diminished cardiac contractility one day after exposure; this response was also blocked by pre-treatment with PAG. The prevention of the acrolein-induced decrease in contractility with PAG pretreatment suggests that the impaired contractility response one day after exposure with acrolein was a compensatory mechanism characterized by rebound increases in parasympathetic tone mediated by carotid body chemoreceptor signaling. Early autonomic responses triggered by the carotid were likely characterized by increased sympathetic tone during exposure, which were followed by rebound increases in parasympathetic tone after exposure. This explanation is strengthened by the fact that bilateral vagotomy reversed decreases in contractility 24 hours after exposure, suggesting parasympathetic mediation. While these findings combined with the blood oxygenation data strongly suggest a role for the carotid body, mediation by the carotid body may be further demonstrated by measuring carotid body nerve impulse formation during exposure to acrolein to confirm increased carotid body activity. Another option is to look at activation of the sympathetic trunk. Carotid body signaling leads to increased sympathetic activation, which increases blood pressure, ventilation, heart rate, and arrhythmogenicity (Lopez-Barneo *et al.*, 2008). Measurement of sympathetic nerve activity during or immediately following acrolein exposure would further strengthen the hypothesis that the carotid body is mediating the cardiovascular response. Acrolein served as an excellent positive control pollutant in this study, but it may be useful to repeat the

experiments with a less irritating air pollutant to determine if the carotid body is still involved. Because acrolein reacts so quickly with the lung and causes a significant irritant response, exposure to a less irritating air pollutant may determine if carotid body activation is related to the irritant response or separate mechanisms. We are currently analyzing ECG, HRV, and arrhythmogenesis, and these results will give more insight into the potential importance of the carotid body in the response to acrolein exposure and may provide additional evidence of autonomic modulation.

We also looked at the effects of dobutamine, a sympathomimetic, on contractility one day after acrolein exposure. Acrolein exposed rats showed enhanced sensitivity to dobutamine one day after exposure. This response may be due to increased vascular resistance, as has been found with exposure to acrolein, and may not be the result of exaggerated sympathetic tone. Increases in contractility during dobutamine infusion were eliminated with bilateral vagotomy. Although the mechanism causing the responses one day after exposure are difficult to decipher, the body has many ways to respond to air pollutants, and it is likely that more than one mechanism is activated upon inhalation of different pollutants. The type, size, shape, and reactivity of the pollutant as well as the duration of exposure and underlying susceptibility of the individual or animal will determine the differential activation of pulmonary reflex responses, baroreceptor reflex responses, chemoreceptor reflex responses and other physiological responses. These responses, particularly in a multipollutant setting as evident from our findings with whole and filtered DE can often times lead to opposing effects.

Summary and Broad Perspective of Findings.

There are several postulated mechanisms of action explaining the cardiovascular effects associated with exposure to air pollution including irritant or sensory receptor activation leading to changes in autonomic balance, activation of systemic inflammatory pathways that impact vascular function, and direct effects of translocated air pollution components (Brook *et al.*, 2010). Based on our findings, air pollutant-induced hypoxia is a potential mechanism controlling adverse cardiac responses. This study provides evidence that exposure to air pollution, especially irritants like acrolein, may cause decreases in blood oxygen levels that activate the carotid body and lead to increased blood pressure and heart rate and impaired contractility. This is of potentially great interest to the field of air pollution epidemiology because the potential repercussions of decreased oxygen availability in cardiac morbidity and mortality following exposure to air pollution are underexplored.

This research also helps fill significant data gaps in our current understanding of the effects of air pollution on cardiovascular health. The American Heart Association (AHA) scientific statement on PM air pollution and cardiovascular disease states that mechanistic studies should “investigate the interaction between preexisting traditional cardiovascular risk factors and PM exposure, as well as the potential of air pollutants to exacerbate or worsen these risk factors” (Brook *et al.*, 2010). All of the studies focus on a rat model of hypertension, a known susceptibility factor for humans exposed to air pollution (Dong *et al.*, 2013). We found heightened sensitivity to DE and acrolein in hypertensive rats compared to normotensive controls. The hypertensive phenotype was associated with increased electrocardiographic abnormalities, increased sensitivity to hypoxia stress test, and significant changes to blood pressure and heart rate with air pollutant exposure. Importantly,

decreased pO₂ and carotid body activation were only observed in the hypertensive rat, suggesting that hypertensive individuals may be at risk through previously understudied mechanisms. This research adds a wealth of new data that better characterizes hypertension as a risk factor for cardiac dysfunction in air pollution exposures. These findings are consistent with epidemiological data and suggest further exploration of the interactions between air pollutants and the physiological and biological sequelae in hypertension. In addition, the AHA recommends that exposure studies “expand our knowledge related to the “responsible” PM pollution constituents (eg, metals, organic compounds, semiquinones, endotoxin, and VOC and SVOC compounds), size fractions (eg, UFPs), sources (eg, traffic, power generation, and biomass combustion), and mixtures of pollutants” (Brook *et al.*, 2010). Our studies with DE examined the separate effects of whole (particles + gases) and filtered (gases alone) components and found that greater toxicity (PR prolongation and ST depression) was associated with the gaseous components of DE. This study demonstrated for the first time that whole DE and particulate-free DE from the same diesel engine source trigger divergent electrocardiographic responses. Subsequent studies revealed enhanced sensitivity to the pulmonary irritant acrolein and suggest that the toxic gases found in DE may be responsible for some of the cardiac responses following exposure.

All of the epidemiological studies that examine changes in oxygen saturation following air pollution exposure relied on pulse oximetry measurements (Gong *et al.*, 2004; Gong *et al.*, 2005), a device placed on the finger that measures arterial blood oxygen saturation using light absorbance. This measurement is not as sensitive as direct analysis of arterial blood and demonstrates the increased sensitivity of the system used in this study. This presents a great strength of our mechanistic studies, and shows that even with small

decreases in SaO₂ in humans during air pollution exposure, the potential reductions in pO₂ may be significantly greater. Therefore, little to no changes in SaO₂ may underestimate the cardiovascular risk associated with hypoxic responses attributed to air pollution exposure. In addition, we found significant cardiac effects during exposure to DE at a relatively low concentration (150 µg/m³). This finding is significant because responses may not follow conventional dose-response relationships, thus complicating standard setting. In addition, most of the adverse cardiac effects we found were in the filtered DE, suggesting that gaseous components of DE may be driving some of the responses. There are currently no NAAQS standards for most of the gaseous components of DE. This presents a potential gap in current human risk factor settings because diesel components such as acrolein are known to cause adverse effects, even at low doses. Furthermore, whole DE with its full complement of particulate and gaseous constituents elicited fewer effects than DE gases alone. This suggests the possibility that constituents interact and/or elicit divergent and potentially opposing mechanisms of toxicity, which may complicate the hazard identification and risk assessment of multipollutant air sheds. This research provides important information for risk assessment and fills data gaps in our knowledge of pollutant effects on the heart, but it also raises additional questions and provides future study directions in animal models and humans.

Multiple questions still exist regarding the role of hypoxia in air pollutant-induced cardiovascular dysfunction. Our studies utilized pharmacologic inhibition of the carotid body with PAG, but there are additional methods that may more reliably inhibit carotid body signaling. Peng *et al.* (2010) used the CSE knockout mouse which has a genetic deletion of the CSE enzyme and therefore has a severely impaired carotid body response to hypoxia. Although PAG has been shown to inhibit the carotid body in rats (Peng *et al.*, 2010), we did

not directly measure carotid body signaling, so similar findings to our inhibition studies in a knockout model would provide additional evidence for carotid-body mediation of the cardiovascular effects of acrolein exposure. In addition, the role of the vasculature was not a focus of this research, but vasoconstriction following acrolein exposure could be a potential driver of the sustained decreases in oxygen we found during exposure. Hypoxia is known to cause pulmonary vasoconstriction (Voelkel *et al.*, 2013), and studies have found that acrolein causes increased blood pressure and vasoconstriction as well as depletion of antioxidants (Yousefipour *et al.*, 2005). Measurements of vascular tone could provide potential explanations for sustained decreases in oxygen and large increases in blood pressure observed in these studies. Also, the role of pulmonary receptors in driving the irritant response to acrolein is an important avenue to consider. Acrolein elicits a host of responses in pulmonary cells including epithelial damage, activation of stress kinases, induction of mucus secretion, and altered production of inflammatory mediators (Moretto *et al.*, 2012). Pharmacological inhibition or knock out models of specific pulmonary receptors such as TRPV receptors may reveal that the downstream cardiac responses to acrolein are mediated by a primary response in the lung. Finally, there are several epidemiological studies linking exposure to air pollutants with decreased oxygen saturation (Gong *et al.*, 2004; Gong *et al.*, 2005), but all of these studies used pulse oximetry to measure SaO₂. Pulse oximetry is not as sensitive as direct analysis of arterial blood and small changes in SaO₂ may be coupled with large decreases in pO₂ due to the sigmoidal shape of the oxy-hemoglobin dissociation curve. Ideally, serial blood draws in humans exposed to air pollution would provide more robust measurements of changes in blood gases during exposure. This may reveal even more significant decreases in oxygen levels during exposure and would add tremendous strength to

the hypothesis that air pollutant-induced hypoxia is driving the cardiac responses during exposure.

Taken together, the findings of these studies further characterize the effects of DE and acrolein exposure on cardiac health, suggest divergent responses with gaseous constituents of DE relative to whole DE, demonstrate that underlying disease confers greater susceptibility to adverse cardiac responses following air pollution exposure, and provide evidence that the adverse cardiovascular effects of acrolein exposure are due, at least in part, to carotid body reflex signaling leading to increased sympathetic tone followed by rebound increases in parasympathetic tone. The findings described in this research dissertation provide biological plausibility to the epidemiological findings that link air pollution to adverse cardiovascular effects, which may reduce uncertainty in standard setting and facilitate risk assessment.

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