THE INFLUENCE OF AUTONOMIC IMBALANCE ON DIESEL EXHAUST-INDUCED CARDIAC DYSFUNCTION IN HEART FAILURE-PRONE RATS

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ABSTRACT

ALEX P. CARLL: The Influence of Autonomic Imbalance on Diesel Exhaust-Induced Cardiac Dysfunction in Heart Failure-Prone Rats
(Under the direction of Drs. Aimen K. Farraj & Daniel L. Costa)

Short-term exposure to vehicular emissions is strongly associated with adverse cardiac events. Diesel exhaust (DE) is a ubiquitous air pollutant hypothesized to provoke adverse cardiac events partly through defective co-ordination of the sympathetic and parasympathetic branches of the autonomic nervous system. To investigate this putative mechanism, cardiophysiologic responses to a single DE inhalation exposure (500 $\mu g/m^3$, 4 h, whole-body) were examined in heart failure-prone rats and age-related susceptibility or autonomic challenges were incorporated to reveal latent effects. Challenges included sympathetic stimulation (dobutamine) with and without parasympathetic ablation (vagotomy) and, separately, treadmill exercise and pretreatment with a sympathetic or parasympathetic inhibitor. Measures of cardiac function by left ventricular (LV) pressure and echocardiography, autonomic balance by heart rate (HR) and HR variability (HRV), electrocardiogram, and aortic pressure were performed. DE increased cardiac output, bradyarrhythmias, and parasympathetic tone while altering ventricular repolarization in aged heart failure-prone rats during or shortly after exposure. Exercise also revealed a DE-induced increase in parasympathetic tone in young adult rats shortly after exposure. At 1 day post-exposure, dobutamine and treadmill challenges indicated that DE increased sympathetic influence, but pre-treatment with autonomic inhibitors prevented this. Only sympathetic inhibition prevented a DE-induced decline in contractility and systolic blood

pressure at exercise 1 day after exposure. Vagotomy revealed that DE caused systolic and diastolic dysfunction and altered diastolic and chronotropic responses to dobutamine through impaired parasympathetic regulation. Thus, altered autonomic regulation of the heart, characterized by an early parasympathetic dominance and a delayed sympathetic dominance, mediates adverse cardiac effects of air pollution exposure. This research elucidates a major physiologic mechanism driving the adverse health effects of air pollutant exposure. Consequently, these findings will inform health risk assessments, medical therapies, and environmental controls for air pollution.

PREFACE

The first and second manuscripts of this dissertation (Chapters 2 & 3, respectively) are pre-copy-editing, author-produced versions of articles accepted for publication in *Toxicological Sciences* following peer review. The definitive publisher-authenticated version of the manuscript in Chapter 2 is available online at http://toxsci.oxfordjournals.org/content/128/2/490.long and is cited as follows:

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The definitive publisher-authenticated version of the manuscript in Chapter 3 is available online at http://toxsci.oxfordjournals.org/content/early/2012/10/09/toxsci.kfs295.long and is cited as follows:

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The third manuscript (Chapter 4) will be submitted to *Environmental Health Perspectives* in November 2012 following revisions.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE, angiotensin-converting enzyme

ANP, atrial natriuretic peptide

ANS, autonomic nervous system

AV, atrioventricular

βAR, β-adrenergic receptor

βMHC, β-myosin heavy chain

BNP, B-type natriuretic peptide

BP, blood pressure

BPM, beats per minute

CAA, Clean Air Act

CAPs, concentrated ambient particles

CO, carbon monoxide

COPD, chronic obstructive pulmonary disease

CRP, C-reactive protein

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

EP, epinephrine

EPA, Environmental Protection Agency

ESV, end systolic volume

ET-1, endothelin 1

fDE, filtered diesel exhaust

FS, fractional shortening

HDL, high density lipoprotein

HF, high frequency

HR, heart rate

HRV, heart rate variability

IL-6, interleukin 6

IT, intra-tracheal instillation

LF, low frequency

MAP, mean arterial pressure

MI, myocardial infarction

NAAQS, National Ambient Air Quality Standards

NE, norepinephrine

nNOS, neuronal nitric oxide synthase

NO, nitric oxide

NO_2, nitrogen dioxide

NOS, nitric oxide synthase

O_2•-, superoxide

OH•, hydroxyl radical

PEP, pre-ejection period

PM, particulate matter

PM_{10}, particulate matter ≤ 10 µm in diameter

$PM_{10-2.5}$, “coarse particulate matter”, between 2.5 and 10 µm in diameter

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

pNN15, percent of adjacent normal RR intervals differing by ≥15 milliseconds

PVN, paraventricular nucleus

QTc, heart rate-corrected QT interval

RAS, renin-angiotensin system

ROFA, residual oil fly ash

ROS, reactive oxygen species

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

SDNN, standard deviation of the RR interval

SH, Spontaneously Hypertensive

SHHF, Spontaneously Hypertensive Heart Failure

SO_2, sulfur dioxide

SOD, superoxide dismutase

TNF-α, tumor necrosis factor alpha

TRPA1, transient receptor potential ankyrin 1

TSP, total suspended particulate

UFP, ultrafine particulate matter

wDE, whole diesel exhaust

WKY, Wistar Kyoto

CHAPTER 1

INTRODUCTION

Background

The U.S. Congress passed the Clean Air Act (CAA) in 1963, amended it in 1970, and re-amended it most recently in 1990. The CAA requires that the U.S. Environmental Protection Agency (EPA) set and enforce air quality and emissions regulations in order to protect the health and welfare of the public, including sensitive populations such as asthmatics, children, and the elderly. Based on evidence from epidemiological, clinical, and toxicological studies, the EPA sets the National Ambient Air Quality Standards (NAAQS) to limit six criteria air pollutants—nitrogen dioxide, sulfur oxides, carbon monoxide, lead, ozone, and particulate matter (PM). These standards are adjusted by the EPA Administrator according to an "adequate margin of safety" (Section 109) based on input from scientific advisory committees and the agency's internal assessments of the current body of scientific research on health effects of air pollutants. As well, the Administrator is responsible for maintaining a national research and development program for prevention and control of air pollution that, per the CAA, "conduct[s], and promote[s], the coordination and acceleration of, research, investigations, experiments, demonstrations, surveys, and studies relating to the causes, effects (including health and welfare effects), extent, prevention, and control of air pollution" (United States, 1970). The report from the Senate regarding the 1970 amendment noted that the purpose of air quality standards is to guarantee "an absence of adverse effects on the health of a statistically related sample of persons

in sensitive groups", including "bronchial asthmatics and emphysematics who in the normal course of daily activity are exposed to the ambient environment" (Coglianese & Marchant, 2004). Thus, the federal courts have interpreted Section 109 as a mandate that the NAAQS "be set at a level at which there is 'an absence of adverse effect' on . . . sensitive individuals" (Coglianese & Marchant, 2004). The determination of this threshold is contingent upon scientific understanding of the adverse health effects of air pollutant exposure in humans. While epidemiologic and observational laboratory studies assist in this understanding, the elucidation of toxic mechanisms enhances scientific knowledge about the biological cause of adverse effects. Such an enhancement may inform observation and epidemiologic studies that guide regulatory decisions, including emissions standards and control technologies, while it also may inform efforts to mitigate the adverse effects of exposure.

Particulate Matter Health Effects – Past and Present

Descriptions of the detrimental health effects of urban air pollutants originate from the 11th century with accounts of Maimonides—Jewish philosopher, theologian, and physician (Bloch, 2001). Nevertheless, detailed historic information on air pollution is limited mostly to the 20th century. The most notable air pollution events occurred as "killer fogs" in the Meuse Valley, Belgium (1930); Donora, Pennsylvania (1948); and London (1952 & 1956). While the Meuse Valley and Donora experienced 60 and 20 smog-induced fatalities respectively, researchers have attributed 12,000 deaths in 1952 and 1,000 in 1956 to unusually high levels of London smog (Hunt *et al.*, 2003). Over 5 days in 1952, London's daily concentrations of total suspended particulate (TSP) peaked at about 7.0 mg per cubic meter of air, with maximum concentrations of smoke at 4.46 mg/m^3 and sulfur dioxide at 1.34ppm (Whittaker *et al.*, 2004).

Meanwhile, 98% of the total PM collected during a similar episode in London (1955) were respirable fine particles (< 2.5μm in diameter; $PM_{2.5}$), and 89% were less than 1μm (Whittaker, et al., 2004).

Today, PM occurs at substantially lower ambient concentrations but continues to adversely affect public health. Numerous epidemiological studies have linked coarse and fine PM with increased mortality and morbidity (Biggeri *et al.*, 2004; Schwartz, 1996; Schwartz & Marcus, 1990; United States, 2004; Zanobetti *et al.*, 2002; Zanobetti *et al.*, 2003). Although sulfur dioxide (SO_2) exposure was originally believed to be the cause of the adverse health responses seen in London, more recent analyses have determined PM was the dominant culprit (Schwartz, 1996; Schwartz & Marcus, 1990). Fittingly, a positive correlation between mortality and PM_{10} concentration was observed in the Utah Valley in winter 1985-86 (WHO, 2000). Increased mortality and morbidity occur with both acute and chronic exposures to particulate matter. The excess deaths observed in London in 1952 occurred almost entirely from cardiopulmonary complications—especially among those with preexisting cardiopulmonary diseases (Whittaker, et al., 2004). While some skeptics initially argued that increased deaths during high smog events resulted from displaced short-term deaths, several studies have since disproved these arguments (Biggeri, et al., 2004; Zanobetti, et al., 2002; Zanobetti, et al., 2003).

Among cardiopulmonary diseases, respiratory illnesses such as bronchitis, chronic obstructive pulmonary disease (COPD), and asthma, as well as cardiovascular diseases such as atherosclerosis, heart failure, ischemic heart disease, coronary artery disease, hypertension, and diabetes are particularly relevant to PM-induced disease exacerbation and death. A plethora of recent epidemiological studies report PM-associated increases in cardiopulmonary symptoms, diseases and lung cancer. EPA's *2004 Criteria Document for Particulate Matter* cites over 100

journal articles published between 1995 and 2004 that revealed positive associations between short-term PM exposure and cardiopulmonary and lung cancer mortality (United States, 2004). Peters et al. (2001) observed a 69% increase in Boston-area heart attacks per 20 $\mu g/m^3$-increase in 24-hour $PM_{2.5}$ on the preceding day. In 2006, 23 U.S. cities (accounting for 46 million people) had peak 24-hour $PM_{2.5}$ levels of at least 40 $\mu g/m^3$ (United States, 2006). Thus, acute fluctuations in fine particulate matter likely contribute to major surges in the national incidence of heart attacks.

The adverse cardiovascular effects of particulate matter have recently become a topic of mounting interest in the scientific community. The epidemiological field has reached relative consensus that PM exposure damages the cardiovascular system and exacerbates pre-existing cardiovascular diseases (Brook *et al.*, 2010; United States, 2004). Many investigators have demonstrated evidence of cardiac dysfunction in humans following elevated ambient PM. As previously noted, Peters *et al.* (2001) reported that a 20 $\mu g/m^3$ increase in the 24-hour concentration of $PM_{2.5}$ corresponded with a 69% increase in risk of myocardial infarction (MI). Others have observed stronger effects of PM on mortality and morbidity in humans with pre-existing cardiovascular disease. Zanobetti and Schwartz recently (2007) observed among heart attack survivors that a 10 $\mu g/m^3$ increase in annual PM_{10} levels was linked with a 43% increased occurrence of subsequent heart attacks and a 34% increased mortality over three years. By comparing pollution records with data from implanted defibrillators, Dockery and colleagues (2005) found positive correlations between PM levels and potentially fatal ventricular tachyarrhythmias. Henneberger *et al.* (2005) revealed an association between elevated PM and impairments in ventricular repolarization by examining electrocardiograms (ECGs) of patients with coronary artery disease.

Particulate Matter Concentrations – Evidence That the Threat Persists

In light of the aforementioned studies, a review of present-day PM concentrations reveals that the United States and elsewhere still frequently reach PM concentrations that threaten public health. One of the worst modern PM episodes within the U.S. occurred in Utah Valley, Utah, where 24-hour PM_{10} concentrations peaked at 365 $\mu g/m^3$ from 1985-1989 (WHO, 2000). Over two months from December 1985 to January 1986, PM_{10} levels in Utah Valley averaged 120 $\mu g/m^3$ and, for 13 days, exceeded 300 $\mu g/m^3$ (United States, 2004). The highest annual PM_{10} levels of 2005 within U.S. cities were recorded in Phoenix (74 $\mu g/m^3$) and St. Louis (57 $\mu g/m^3$) (United States, 2005). In 2006, Bakersfield, CA; Birmingham, AL; and Riverside-San Bernadino, CA, had the highest peak 24-hour PM_{10} levels, reaching 192, 169, and 155 $\mu g/m^3$, respectively (United States, 2006). Meanwhile, in developing nations, peak PM concentrations may approach levels comparable to those of London in 1952. For instance, maximum 24-hour TSP and PM_{10} for Gujranwala, Pakistan was recently recorded at 5.19 mg/m^3 and 1.1 mg/m^3, respectively (Pak-EPA and JICA, 2003). Among major cities surveyed by the World Health Organization (WHO), those most burdened by PM_{10} included Cairo, Beijing, Delhi, Calcutta, and Taiyuan, China, with annual levels at 169, 161, 150, 128, and 125 $\mu g/m^3$ respectively (WHO, 2007). Data on ambient $PM_{2.5}$ is more limited than that of PM_{10}, but is also of major concern. In Beijing, in defiance of the Chinese government and in contradiction of publicly available government data, the U.S. embassy 'tweets' hourly $PM_{2.5}$ concentrations on-line, which recently peaked at 248 $\mu g/m^3$ (twitter.com/#!/beijingair, 3 June 2012). The U.S. cities with the three highest peak 24-hour $PM_{2.5}$ levels include Bakersfield, CA (64 $\mu g/m^3$); Chico-Paradise, CA (59 $\mu g/m^3$); and Pittsburgh, PA (58 $\mu g/m^3$) (United States, 2006). Meanwhile, UFPs reach peak

environmental concentrations usually in settings of traffic settings, including on a busy Los Angeles highway (# concentration: 1.2×10^5 / m^3), behind a cement truck in France (1.47×10^6 / m^3) or in a traffic tunnel (1.1×10^6 / m^3) (Gouriou *et al.*, 2004; Zhu *et al.*, 2007).

Particulate Matter Toxicity and Classifications

In addition to concentration, the toxicity of an exposure to particulate matter depends upon the size and chemical composition of the particles. As size is among the most distinguishable determinants of PM toxicity in an air-shed, the EPA classifies, monitors, and regulates two different size classes of PM: particles 10 micrometers in diameter or less (PM_{10}) and those less than 2.5 μm ($PM_{2.5}$) (United States, 2007). PM less than 10 μm but greater than 2.5 μm ($PM_{10-2.5}$) are called "inhalable coarse particles" because they can enter the airways and lungs. In contrast, $PM_{2.5}$ are "respirable fine particles" because they can enter deeper into the lungs where gas exchange occurs. Although sources of particulate matter vary depending on time and location, the majority of fine particles originate from fossil fuel combustion while most coarse particles come from dust, sea salt, pollen, mold, fungal spores, and mechanical fragmentation of solids from grinding, crushing and abrasion (United States, 2004, 2007). Fine particles in the eastern and central U.S. consist mostly of organic compounds and sulfate, while in the western U.S. they consist of nitrate in addition to organics and sulfates. In addition to PM_{10} and $PM_{2.5}$, there are also ultrafine particles (UFPs; diameter < 0.1 μm), which are neither regulated nor consistently monitored by the U.S. EPA. Relative to $PM_{2.5,}$ UFPs have a substantially greater surface area per given mass concentration; for example, 16,000 UFPs with diameters of 0.1 μm are required to achieve the same mass as a single 2.5-μm particle—leading

to a total surface area among these UFPs that is roughly 25-times greater than the fine particle when controlling for mass.

Myriad studies have revealed increased PM toxicity with decreasing particle size and increasing transition metal content. Exceptions to the metal-toxicity association in PM include diesel exhaust particles (DEPs), which have relatively low metal content but remain harmful possibly due to organic material. Meanwhile, size determines the depth to which particles can penetrate the respiratory tract. Inhaled coarse particles usually enter the conducting airways where they may deposit by impaction. In contrast, inhaled fine particles can deposit in the lung parenchyma—which include the respiratory bronchioles, alveolar ducts, and alveolar sacs—where they may exert greater toxicity due to weaker defenses (i.e., clearance mechanisms and blood barriers) and greater biochemical responsiveness. Given their smaller size, UFPs have an even greater capacity to be respired within the lower airways and alveoli, to penetrate the lung lining tissue and cell barriers, and to translocate to extra-pulmonary organs including the heart and brain (Oberdorster *et al.*, 2002; Peters *et al.*, 2006). Other physical and chemical factors may determine particle toxicity including the solubility of metals and acidity, which is often affected by sulfate content and surface charge (United States, 2004).

Other Criteria Pollutants: Concentrations and Cardiovascular Effects

Because PM levels frequently correspond with those of many other co-pollutants, it is often not possible for epidemiologists to assert a causal role for any single pollutant in an adverse health outcome. Other common air pollutants of public health concern that are immediately derived from vehicular emissions include CO, NO_2, and SO_2. A recent study (Bhaskaran *et al.*, 2011) found the highest ambient concentrations of pollutants among 15 metropolitan areas in the

United Kingdom reached an hourly upper quartile (75th percentile) of 0.65 ppm for CO, 32 ppb for NO_2, and 6 ppb for SO_2. These values compared closely to those of ten Italian cities (Chiusolo *et al.*, 2011) as well as Erfurt, Germany, the latter of which had comparable daily ambient concentrations and reported a peak CO level at 1.56 ppm (Berger *et al.*, 2006). An additional study conducted over 126 U.S. counties observed similar 24-hour levels, with only a notable difference in CO concentrations (peak hourly observation = 9.7 ppm; peak daily = 2.5 ppm) (Bell *et al.*, 2009). Yet, levels of these pollutants may reach much higher levels indoors, within traffic tunnels, or in occupational settings. For instance, while hourly NO_2 concentrations are unlikely to surpass 0.2 ppm outdoors, they can range from 0.4 to 1.5 ppm indoors during gas cooking (Hesterberg *et al.*, 2009). In traffic tunnels, NO levels (which are generally at higher levels than NO_2 in vehicular emissions) can reach 1.5-2.2 ppm , while CO has been shown to reach levels as high as 19-22 ppm (Gertler *et al.*, 2002).

Significant associations have been demonstrated between these pollutants and adverse health outcomes. Fluctuations in NO_2 and CO have both been shown to correspond with increased hospitalizations for cardiovascular disease across the U.S. (Bell, et al., 2009; Mann *et al.*, 2002), while cardiac mortality has been shown to increase with increasing NO_2 throughout Italy (Chiusolo, et al., 2011). In a study spanning 15 cities within the United Kingdom, Bhaskaran et al. (2011) demonstrated an association between exposure to NO_2 and onset of myocardial infarction 1-6 hours later. It is plausible that several of these pollutants exert toxicity in concert. Conversely, one pollutant may be the primary culprit, but it cannot be isolated from its co-pollutants in an epidemiologic setting. For example, Berger and colleagues (2006) found that three different size classes of PM (UFP, $PM_{2.5}$, and accumulation mode [1.0 -

0.1 μm]) as well as NO_2 concentrations correlated with episodes of ventricular tachycardia in men with coronary artery disease in Erfurt, Germany.

Diesel Exhaust Cardiovascular Effects

Diesel exhaust (DE) is a ubiquitous source of urban PM, NO_2, and CO, among other pollutants. Consequently, DE is strongly believed to contribute to adverse cardiovascular effects. Ischemic heart disease hospitalizations in eight European cities have been attributed to DE exposure (Le Tertre *et al.*, 2002). In addition, Mills et al. (2007) found that DE exposure exacerbated exercise-induced electrocardiographic ST depression in human subjects with known coronary artery disease. Several mechanisms underlying the acute cardiovascular toxicity of DE exposure have been implicated, including electrophysiological dysfunction, autonomic imbalance, vascular dysfunction, coagulation, and low-level systemic inflammation (Anselme *et al.*, 2007; Brook, 2008; Campen *et al.*, 2005; Lucking *et al.*, 2011; Mills, et al., 2007; Peretz *et al.*, 2008b).

Although many components of DE are suspected to play a role in DE-induced cardiovascular (CV) dysfunction, recent investigations using relatively healthy individuals have implicated particles as the predominant mediators (Lucking, et al., 2011; Mills *et al.*, 2011b). Studies have demonstrated pathophysiologic effects on the CV system following acute exposure to either particle-containing whole diesel exhaust (wDE) (Anselme, et al., 2007; Miller *et al.*, 2009; Mills, et al., 2007) or DE particles alone (Huang *et al.*, 2010). Likewise, removal of particles by modern DE filters can prevent DE-induced thrombosis and vasoconstriction in healthy humans (Lucking, et al., 2011; Mills, et al., 2011b). Other studies suggest that the gaseous components of DE contribute to the pathophysiologic effects documented in

epidemiologic studies. Several reports have shown that particle-free DE exposure promotes acute physiologic alterations that can trigger cardiac dysfunction and injury—including increased blood pressure, vascular plaque formation, cardiac arrhythmia, and enhanced responsiveness to a vasoconstrictor (Campen, et al., 2005; Mills, et al., 2011b). However, neither the dominant constituents nor the primary mechanisms behind DE-induced cardiac toxicity are resolved.

Mechanisms of Air Pollutant Cardiovascular Toxicity

Observations of increased detrimental cardiovascular events such as cardiac arrhythmia, endothelial lipid peroxidation, ischemic myocardial lesions, MI, decompensation of heart failure, and changes in heart rate variability have guided many hypotheses about mechanistic pathways of air pollutant-induced cardiovascular insult—all of which concern neuroregulatory, vascular, cardiac, and/or pulmonary effects. Among the hypothesized mechanisms, the most substantiated include: (i) dysfunction of the autonomic nervous system (ANS) resulting from lung receptor reflexes and/or pulmonary inflammation; (ii) cardiac dysfunction following heart tissue responses to inadequate blood supply (ischemia) and alterations of ion channels in heart cells (cardiomyocytes); and (iii) inflammatory responses (systemic and pulmonary) that lead to vascular changes including pro-coagulant alterations of blood, endothelial malfunction, and structural deterioration of the endothelium. (Schulz *et al.*, 2005; Zareba, 2001). Although the scientific community lacks precise evidence for mechanisms involving the exceptional vulnerability of heart failure patients to PM, the pathological features of heart failure may confer a hypersensitivity that predisposes the myocardium to exaggerated responses to PM inhalation.

Pathophysiologic Relevance of the Autonomic Nervous System

As the primary mediator of cardiovascular function, the autonomic nervous system is divided into the parasympathetic and sympathetic branches. These branches have generally opposing influences and are coordinated by the cardiovascular control center within the medulla according to external and internal stimuli. The parasympathetic branch governs basal control of cardiovascular physiology ("rest and digest" homeostatic mechanisms), whereas the sympathetic branch enables increased cardiac output to support muscle movement, alertness, and quick physical action ("fight or flight" responses).

Autonomic control of heart function is frequently assessed by measuring heart rate variability (HRV)—the variation in time between successive heart beats. Significant declines or increases in HRV parameters may indicate autonomic dysfunction. Decreased HRV has been associated with a risk of cardiovascular events such as myocardial infarct (MI), cardiac arrhythmias, and sudden cardiac death, as well as progression of heart failure and atherosclerosis (Ponikowski *et al.*, 1996; Singh *et al.*, 2003). PM exposure has been associated with significant decreases in HRV parameters among the elderly that intensify with preexisting arrhythmia, coronary heart disease, and hypertension (Devlin *et al.*, 2003; Liao *et al.*, 1999); however, air pollution's effects on autonomic function can vary with specific cardiopulmonary diseases. Wheeler *et al.* (2006) observed that increases in ambient $PM_{2.5}$ significantly increased HRV parameters in subjects with COPD but decreased HRV parameters in individuals with prior MI.

Cardiovascular Susceptibility to Air Pollutant Exposure

Among those with cardiovascular disease, heart failure patients are particularly susceptible to the effects of PM. Elevated fine ambient PM levels have been associated more

strongly with heart failure hospitalizations than hospitalizations for other cardiopulmonary diseases—including cerebrovascular disease, peripheral vascular disease, ischemic heart disease, COPD, heart rhythm problems, and respiratory tract infection (Dominici *et al.*, 2006). Limited yet compelling evidence suggests that chronic particulate exposure promotes the development of heart failure. Among heart attack survivors, a 10 $\mu g/m^3$ increase in annual PM_{10} levels corresponded with a 40% increased incidence of development of heart failure over three years (Zanobetti & Schwartz, 2007). Lastly, epidemiological studies have linked acute particulate exposure with acute exacerbation of heart failure. Schwartz and Morris (1995) observed that a 32 $\mu g/m^3$ increase in daily PM_{10} was followed by a 3.2% increase in daily heart failure hospital admissions among people over 64 years old.

Potential Biochemical Links between Heart Failure and Air Pollutant Toxicity

The striking similarity in biochemical effects between heart failure and air pollutant exposure add further plausibility to heart failure conferring particular susceptibility to air pollutants. Heart failure has been associated with elevations in circulating inflammatory markers (such as fibrinogen and C-reactive protein), endothelin 1 (ET-1), and the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) (Seta *et al.*, 1996). Similarly, short- and long-term air pollutant exposures have been shown to increase these markers (Brook *et al.*, 2003; Calderon-Garciduenas *et al.*, 2007; Lund *et al.*, 2009; Peretz, et al., 2008b; Ruckerl *et al.*, 2007; van Eeden *et al.*, 2001). Researchers demonstrated a strong association between exposure to $PM_{2.5}$ and ET-1, as well as pulmonary arterial pressure, in children in Mexico City (Calderon-Garciduenas, et al., 2007). Likewise, others detected an association between PM exposure and circulating IL-6 and fibrinogen in survivors of MI

(Ruckerl, et al., 2007). Heart failure and air pollutant exposure also both appear to increase natriuretic peptides, which are released upon myocardial stretch. Atrial and B-type natriuretic peptides (ANP and BNP) correlate closely with severity of cardiac dysfunction and heart failure (Clerico *et al.*, 2006; de Denus *et al.*, 2004), and have been demonstrated to increase upon short-term PM inhalation exposure (Tankersley *et al.*, 2008).

ET-1, TNF-α, IL-6, ANP, and BNP bear several effects on cardiovascular physiology. ET-1 is a powerful vasoconstrictor that temporarily increases the contractile force of cardiomyocytes and expedites myocyte protein synthesis (Seta, et al., 1996). TNF-α is a vasodepressor (decreases blood pressure) that promotes pulmonary edema and left ventricular dysfunction at high levels, is closely associated with myocyte hypertrophy, and is believed to trigger cardiomyocyte apoptosis in conjunction with ANP (Francis, 2001; Kang, 2006). TNF-α may also induce IL-6, which is another vasodepressor that promotes myocardial dysfunction as well as myocyte atrophy (Seta, et al., 1996). Meanwhile, ANP and BNP compensate for myocardial stretch by decreasing blood volume and blood pressure through induction of natriuresis (the excretion of sodium in the urine by the kidneys) and peripheral vasodilation, thereby reducing the symptoms and progression of heart failure (Stoupakis & Klapholz, 2003).

Heart failure and air pollutant exposure have both been shown to promote oxidative stress through formation of reactive oxygen species (ROS) and sympathetic activation—each of which can compromise cardiac health. In addition to sympathetic excitation involving marked increases in circulating catecholamines (epinephrine [EP], norepinephrine [NE], and dopamine) heart failure is typically accompanied by increased production of ROS (especially superoxide [O_2•-] and hydroxyl radical [OH•]) leading to lipid-peroxidation (Dhalla *et al.*, 2000; Diwan & Dorn, 2006; Peng *et al.*, 2003). Catecholamines themselves can promote oxidative stress, as they

auto-oxidize to generate highly reactive OH•, O_2•-, aminochromes, and o-semiquinones, thereby promoting lipid peroxidation and cellular necrosis (Dhalla, et al., 2000; Remiao *et al.*, 2001; Remiao *et al.*, 2002; Singal, 1983; Singal *et al.*, 1982). An extensive body of research has demonstrated that PM exposure also increases ROS and oxidative stress (Brook, et al., 2010), while there is suggestive (yet inconclusive) evidence for autonomic effects of air pollutant exposure (Brook, et al., 2010; Mills *et al.*, 2011a). Only a limited few studies have demonstrated air pollutant effects on catecholamines. For instance, Orgacka *et al.* (1983) observed elevated urine NE among Polish children exposed to moderately high levels of "falling dust"; meanwhile, the highest exposures corresponded with decreases in NE, EP, and dopamine, potentially stemming from an inverted U-shaped PM dose-response or from differences in air pollutant components between groups. Another group noted strong associations between sympathoexcitation and oxidative stress after PM exposure in rats (Rhoden *et al.*, 2005), revealing by pharmacologic inhibition that PM-induced autonomic stimulation promotes oxidative stress. Others have demonstrated that exposure to concentrated ambient particulates (CAPs) increases NE within the paraventricular nucleus (PVN) of the murine hypothalamus (Sirivelu *et al.*, 2006). The PVN has subpopulations of neurons that relate directly to neuroendocrine and autonomic effector mechanisms, making it important in the regulation of visceral responses within both the central and peripheral nervous systems (Swanson & Sawchenko, 1980). Interestingly, the PVN not only has increased NE during heart failure, but also activates multiple sympathetic nerves and increases serum NE when it is stimulated (Patel, 2000). Sympathetic activation from elevations in NE can lead to arrhythmia, MI, and sudden cardiac death, while also exacerbating heart failure (Patel, 2000). Ultimately, the biochemical

influences air pollutant exposure may lead to pronounced autonomic and cardiac dysfunction in those predisposed to heart failure.

In part, disruption of vascular homeostasis (involving neurohormonal responses and altered NO homeostasis) mediates heart failure progression and underlies air pollutant toxicity. Patel and colleagues (2001) observed significant depressions in neuronal nitric oxide synthase (nNOS) within the PVN of rats with experimentally induced heart failure. Others have noted decreased NO production or depressed NOS activity in multiple vascular tissues during heart failure (Schultz & Sun, 2000). Similarly, multiple studies have demonstrated that air pollutant exposure can disrupt NO homeostasis through uncoupling of NOS (Campen, 2009; Cherng *et al.*, 2011; Knuckles *et al.*, 2008; Tankersley *et al.*, 2008), and also the conversion of inhaled nitrogen oxides into bio-active nitric oxide (Knuckles *et al.*, 2011). With diesel exhaust exposure, such effects have been shown to promote venoconstriction (Knuckles, et al., 2008). Since NO is a major mediator of vasodilation, disrupted NO synthesis has major implications for vascular function. Additionally, altered NO homeostasis can effect autonomic function, as NO has been demonstrated to inhibit sympathetic influence over the heart by inhibiting cardiac NE release (Schwarz *et al.*, 1995) and promoting catecholamine oxidation (Klatt *et al.*, 2000). Accordingly, decreases in NO correspond with increased sympathetic stimulation in heart failure (Patel, et al., 2001). This impairment in NO homeostasis, as well as other neurohormonal effects, causes vasoconstriction in heart failure patients (Ferro & Webb, 1996). Likewise, PM exposure elicits vasoconstriction (Brook *et al.*, 2002), a response likely due to NO depletion (via superoxide generation) (O'Neill *et al.*, 2005; Rajagopalan *et al.*, 2005) and direct nerve stimulation by neurotransmitters. Therefore, air pollutant exposure and heart failure may concomitantly promote lipid peroxidation, sympathoexcitation, and vasoconstriction by depleting

NO, promoting catecholamine release, and generating ROS. Ultimately, there is substantial evidence that air pollutant exposure may bear an additive effect on multiple pathologic traits of heart failure. Heart failure may thus confer heightened sensitivity to the physiologic effects of air pollutant exposure.

Incorporation of Animal Models of Cardiac Disease into Air Pollution Studies

Very few animal studies support the epidemiological findings that people with preexisting heart failure are exceptionally sensitive to the adverse effects of PM. Research incorporating animal models of disease may further elucidate the extent to which inflammation, autonomic alterations, and/or cardiomyocyte degradation contribute to PM-induced cardiac dysfunction. Results of previous animal disease model studies have mostly complemented epidemiological observations, but have failed to elicit responses at comparable PM concentrations. Kodavanti *et al*. observed histological evidence of myocardial injury—including chronic-active inflammation, necrosis, and fibrosis—in the hearts of Wistar Kyoto (WKY) rats following long term inhalation of particles < 2.5μm diameter (10 mg/m^3, 6 hours/day, 1 day/week, for 16 weeks) (Kodavanti *et al.*, 2003), indicating that PM could exacerbate heart failure by directly damaging heart tissue. Wellenius and colleagues induced MI in Sprague-Dawley rats and observed that inhalation of residual oil fly ash (ROFA; < 2.5μm, 3 mg/m^3 for 1 hour) 15 hours later increased arrhythmia and decreased HRV during exposure (Wellenius *et al.*, 2002). Subsequently, the investigators found that a similar regimen using concentrated ambient particulates (CAPs; < 2.5μm, 350$\mu g/m^3$ for 1 hour) instead of ROFA also increased arrhythmia in MI rats during exposure (Wellenius *et al.*, 2004). Very few studies have reported on the effects of PM exposure in animals with heart failure. Gordon and coworkers saw no adverse cardiac or

pulmonary effects in a hamster model of genetic cardiomyopathy exposed to CAPs; however, at no point in the publication did the authors provide evidence of preexisting cardiac insufficiency in the test animals (Gordon *et al.*, 2000). Muggenburg *et al.* exposed 7 old dogs with various cardiac abnormalities to aerosols of lone transition metal oxides or sulfates by oral inhalation (manganese, nickel, iron, vanadium, and copper oxides or vanadyl and nickel sulfates; 0.7-2.9μm, 0.05-0.1 mg/m^3, 3 hours/day for 3 days) (Muggenburg *et al.*, 2003). Two of these dogs had pre-existing heart failure with depressed baseline HRV. HRV decreased in one of the heart failure dogs following VSO_4 exposure. In contrast, the other dog with heart failure responded to separate MnO_2 and $NiSO_4$ exposures with increased HRV and did not show a response to VSO_4.

Anselme *et al.* exposed rats with surgically induced post-myocardial infarction heart failure to diesel exhaust (DE) containing a mixture of 500 $\mu g/m^3$ ultra-fine PM (0.085 μm diameter), hydrocarbons, nitrogen dioxide, and carbon monoxide (Anselme *et al.*, 2007). Exposure immediately decreased HRV in normal and heart failure rats, but increased arrhythmias (ventricular premature beats) in heart failure rats only. In this instance, exposure to filtered DE (with PM removed by filtration) may have better elucidated PM's role in the observed adverse effects. In a related study, Morin observed that the removal of PM from DE by filtration did not alter the toxic profile of lung cultures exposed to diesel emissions (Morin, 2006). Likewise, Campen and colleagues (2005) observed similar effects of whole- and filtered DE on ECG measures of ventricular repolarization and heart rate in a mouse model of atherosclerosis. Regardless of filtration, the findings of Anselme (2007) and Campen (2005) indicate that the cardiophysiologic effects of air pollutants are more readily observed in rodent models of cardiovascular disease.

The SHHF rat: A Model of Cardiovascular Susceptibility and Age-dependent Heart Failure

The SHHF/*Mcc-fa*^{cp} strain originates from the seventh back-cross of the normal Spontaneously Hypertensive rat (SHR) with "Koletsky obese" rats (inbred from the hypertensive offspring of a Sprague Dawley/SHR cross) (Koletsky, 1975; McCune *et al.*, 1990). SHHF rats possess characteristics similar to the SHR, except 100% eventually acquire dilated cardiomyopathy and heart failure preceded by Type II diabetes mellitus and consequent diabetic nephropathy accentuated in obese and male rats (McCune *et al.*, 1990; Muders & Elsner, 2000). The SHHF's additional pathology is attributed to a nonsense mutation, *fa*, which encodes a premature stop codon in the leptin receptor (Muders & Elsner, 2000; Roncalli *et al.*, 2007). "Lean" and "obese" SHHF rats differ in disease severity and progression primarily by their responsiveness to leptin—a hormone released upon eating that inhibits appetite, provokes a sense of satiation, stimulates the sympathetic nervous system, and increases energy expenditure in a receptor-dependent manner (Mark *et al.*, 2003). The autosomal recessive corpulence trait (cp) manifests as obesity in rats homozygous for the *fa* mutation (fa^{cp} / fa^{cp}), while homozygous wild-type (+ / +) or heterozygous (+ / fa^{cp}) SHHFs are lean (Jackson *et al.*, 2001; Radin *et al.*, 2003; Roncalli, et al., 2007). Among lean males, heterozygotes develop congestive heart failure and die sooner than the homozygous wild-types (McCune *et al.*, 1995; Radin, et al., 2003). Heterozygosity confers mild hyperleptinemia and insulin resistance, with marked effects in homozygous mutant (fa^{cp} / fa^{cp}) rats (Emter *et al.*, 2005; Radin, et al., 2003). Notably, leptin administration has been shown to induce eccentric dilatation of the left ventricle(Abe *et al.*, 2007), while leptin receptor polymorphisms and circulating leptin associate with human heart failure (Bienertova-Vasku *et al.*, 2009). In contrast to 10-12 month old SHRs with concentric hypertrophy, age-matched lean male SHHF rats develop eccentric hypertrophy and lack

ventricular wall thickening (Haas *et al.*, 1995). Although leptin stimulates sympathetic nerve activity and may increase arterial pressure, leptin-induced sympathetic excitation is absent in the obese phenotype of another rat strain (Zucker) homozygous for the same mutated leptin receptor gene (Mark, et al., 2003). Thus, heart failure in the SHHF is not entirely a result of hypertension-induced increases in afterload and may result partly from preload-driven volume overload. Un-anesthetized, unrestrained lean male SHHF rats have hypertension exceeding the SHR (24-hour MAP: 161 mmHg at 10 weeks and 145 mmHg at 15 weeks) (Carll, 2010; Carll *et al.*, 2010), while several studies suggest that un-anesthetized, un-restrained obese male SHHFs have less severe hypertension (MAP: 119 mmHg at 18-26 weeks, 133 mmHg at 40 weeks, and 127 mmHg at 54 weeks) (Poornima *et al.*, 2008; Schlenker *et al.*, 2004). Some studies have reported higher systolic pressure in the obese relative to the lean; however, pressure measurements in these studies use anesthesia or restraint (e.g. tail-cuff), which may differentially affect the two phenotypes (McCune, et al., 1995; Radin, et al., 2003; Roncalli, et al., 2007).

SHHFs express LV hypertrophy at 3 months regardless of gender or obesity. Decompensated heart failure with gross symptoms occurs at 10-13 months in obese males(McCune, et al., 1995; Peterson *et al.*, 2001; Schlenker, et al., 2004), 15 months in obese females(Hohl *et al.*, 1993), 18 months in lean males(Anderson *et al.*, 1999; Heyen *et al.*, 2002; Janssen *et al.*, 2003; Reffelmann & Kloner, 2003; Tamura *et al.*, 1999), and 24 months in lean females.(Gerdes *et al.*, 1996; McCune, et al., 1995; Onodera *et al.*, 1998; Tamura, et al., 1999) The overt signs of decompensated heart failure found in the SHHF often include subcutaneous edema, tachypnea and shallow rapid breathing, cold tails, cyanosis, lethargy, piloerection, pulmonary edema, pleural effusion, ascites, cardiomegaly, left and right atrial dilatation, and hepatomegaly.(McCune, et al., 1995) Death typically occurs at 18 months in obese females(Park

et al., 1997) and 19 months in lean males(Emter, et al., 2005; Heyen, et al., 2002) as heart failure severity increases with decompensation. Although heart failure onset is more rapid in obese SHHFs, lean males compare well to the hypertrophic qualities of obese SHHF's and also compare closely to human heart failure pathogenesis.(Roncalli, et al., 2007) In 18-20-month old lean male SHHFs, a reduction in αMHC and β-adrenergic receptor (βAR) density as well as increases in ventilatory rate (>200 breaths/min), βMHC, circulating TNF-α, IL-6, natriuretic peptides, and leptin suggests a profound comparability between heart failure pathogenesis in SHHF rats and humans.(Anderson, et al., 1999; Emter, et al., 2005; Ferrara *et al.*, 1996; Heyen, et al., 2002) Furthermore, exceptional homology has been demonstrated between lean and obese male SHHFs in increases in neurohormonal, apoptotic, fibrotic, inflammatory, metabolic, hypertrophic, and structural gene expression at 10 months relative to 4 months of age.(Roncalli, et al., 2007) Thus, the pathogenesis of heart failure in lean and obese males is strikingly similar with exception to rate of progression.

Among SHHFs, the lean male has been the most thoroughly studied for cardiac dysfunction. Yet, timing of systolic and diastolic dysfunction has been inconsistent between several of these studies (Anderson, et al., 1999; Carll *et al.*, 2011b; Heyen, et al., 2002; McCune, et al., 1995). The variability in observations may stem from different anesthetics used during physiologic measurements; however, similar variability has not been observed in studies using other models and different anesthetics. Female SHHFs differ from males in timing to progression and gross signs of heart failure. A few studies have noted the absence of several common heart failure traits in 24-month old lean female SHHFs (Onodera, et al., 1998; Tamura, et al., 1999) despite marked declines in LV systolic and diastolic performance and significant

cardiomegaly (Gerdes, et al., 1996; Tamura, et al., 1999).

Project Summary

The brain regulates cardiovascular function through the opposing influences of the sympathetic ("fight or flight") and parasympathetic ("rest and digest") branches of the autonomic nervous system. Epidemiological data indicate that a defective co-ordination of these two branches, known as autonomic imbalance, is a mechanism mediating the adverse cardiac effects of air pollution. In the presented research, rats with preexisting cardiovascular disease were used to test the hypothesis that DE inhalation causes cardiac dysfunction through imbalance of the autonomic nervous system. To this end, heart failure-prone rats were exposed one time for 4 hours to DE (500$\mu g/m^3$) and examined for changes in cardiovascular physiology through multiple endpoints: heart rate variability, electrocardiogram, blood pressure, arrhythmia, and left ventricular function and dimension. Most of the studies performed herein involved young adult SHHF rats (2-2.5 months old). To initially characterize the effects of DE on rats with even greater cardiovascular susceptibility, aged SHHF rats (16 months old, pre- heart failure) were also used. Physiologic stress tests (treadmill challenge or infusion of a sympathetic agonist) were also applied to unmask latent effects of DE on autonomic balance and cardiovascular function in the young adult SHHF. Additionally, sympathetic and parasympathetic inhibitions were performed in young adult SHHF rats either pharmacologically (atenolol or atropine), or surgically (vagotomy), to determine the contribution of each autonomic branch to DE-induced cardiovascular dysfunction.

Human studies have repeatedly demonstrated associations between exposure to fossil fuel combustion-derived air pollutants and adverse cardiac events—especially in individuals with

preexisting heart disease. Although still unclear, multiple mechanisms of toxicity have been postulated including direct effects of components on the myocardium, systemic/vascular inflammation, and autonomic imbalance. Preliminary work on rats in our laboratory has demonstrated that air pollutant exposure triggers irritant responses that are characterized by immediate decreases in heart rate and/or blood pressure suggesting a role for the autonomic nervous system. The goal of this research is to determine if autonomic imbalance from acute inhalation of diesel engine exhaust (DE—a major source of fine urban particulate matter, $PM_{2.5}$) provokes cardiac dysfunction in rats with underlying cardiovascular pathology. The lean male Spontaneously Hypertensive Heart Failure (SHHF) rat progresses from cardiac hypertrophy at 2 months to overt decompensated heart failure at approximately 18 months. Young adult (2-3 months) and aged adult (16 months) SHHFs were used as models of early and late compensated hypertrophy (before any sign of overt decompensated heart failure), respectively. In the following studies, lean male SHHF rats received a single inhalation exposure to either DE or filtered air. Radiotelemetry was used to analyze for effects on the electrocardiogram (ECG), heart rate variability (HRV), heart rate, core body temperature, and—in some instances—blood pressure. Left ventricular catheterization and echocardiography were also performed in some instances to generate measures of cardiac function and dimensions.

The studies conducted toward Specific Aim 1 sought to characterize the cardiovascular effects of DE in lean SHHFs through the use of telemetry and echocardiography. The experiments performed for Specific Aim 2 sought to unveil latent effects of DE exposure on autonomic balance through the use of physiologic stress tests, including treadmill exercise and administration of a sympathomimetic drug (dobutamine). For Specific Aim 3, the role of each

branch of the autonomic nervous system in DE-induced cardiac dysfunction was investigated through pharmacologic or surgical inhibition and subsequent stress test.

DE is a major source of common air pollutants to which the majority of humans are exposed on a daily basis. Such acute exposures increase the short-term likelihood of hospitalization and death due to cardiac complications. The link between acute air pollutant exposure and adverse cardiac events remains poorly understood, but it may be driven by alterations in the autonomic nervous system. Such an elucidation of mechanism may enhance biological plausibility of a causal relationship, thereby reducing uncertainty in standard setting and in the linkage between exposures and health outcomes.

Specific Aims

Hypothesis: Diesel exhaust exposure promotes cardiac dysfunction in heart failure-prone rats through autonomic imbalance

Specific Aim 1: Characterize the cardiophysiologic effects of diesel exhaust on a heart failure-prone rat using ECG & blood pressure telemetry to unmask potential autonomic effects.

Specific Aim 2: Determine if diesel exhaust exposure modifies cardiac responses to physiologic stress tests, including treadmill exercise and administration of a sympathomimetic drug (dobutamine).

Specific Aim 3: Elucidate the role of the autonomic nervous system in DE-induced cardiac effects by inhibiting the parasympathetic (atropine or vagotomy) or sympathetic (atenolol) branches and incorporating stress tests.

Aim 1: The goal of Aim 1 was to determine the effects of a single acute inhalation of DE (4 hours) on cardiac function in lean SHHF rats. Prior to these studies, the electrophysiologic, hemodynamic, and pulmonary effects of acute DE inhalation had not been examined in the heart-failure prone SHHF rat, and very few studies had examined such effects in animals genetically predisposed to heart failure. The studies addressing Aim 1 were performed to provide descriptive evidence of air pollutant-induced autonomic imbalance both early (2-2.5 months of age) and late (16 months of age) in the SHHF's progression through compensated hypertrophy in order to guide subsequent mechanistic studies.

Aim 2: In the studies toward Aim 1, we found that the aged (16 month-old) SHHF rat provided a useful model with which to demonstrate autonomic effects of DE exposure, while the *resting* young adult SHHF rat offered little insight into the role of autonomic imbalance on DE-induced cardiac dysfunction. Yet, since the aged SHHF rat was difficult to reliably obtain, a pilot study was performed in young adult SHHFs intratracheally instilled with diesel exhaust particles and challenged by treadmill exercise in order to reveal effects of DE exposure unapparent in resting rats. The indications of autonomic imbalance in DE particle-exposed rats (Appendix, Figures 2 & 3) justified the studies performed toward Specific Aim 2, which were conducted on the young adult SHHF rat with the goal of providing reproducible methods for demonstrating cardiophysiologic and autonomic effects of DE. As such, physiologic stress

tests—including treadmill exercise and administration of a sympathomimetic drug (dobutamine)—were conducted to determine if DE exposure modifies cardiac responses consistent with autonomic dysfunction. Additionally, left ventricular pressure was measured in the young SHHF rat before and after dobutamine infusion to determine if more direct assays of cardiac function could demonstrate effects of DE unapparent in our previous characterization studies.

Aim 3: The studies toward Aims 1 and 2 demonstrated effects of DE that implicated dysregulation of cardiac function through the autonomic nervous system. The goal of Aim 3 was to determine more conclusively if autonomic imbalance mediates pollutant-induced cardiophysiologic effects. Young adult SHHFs were injected with a parasympathetic inhibitor or a sympathetic inhibitor shortly before exposure to DE and subjected to treadmill exercise challenge after DE exposure. Additionally, a subgroup of DE- or air-exposed rats received left ventricular pressure measurements and dobutamine infusion before and after surgical ablation of the vagus nerve—the primary route of parasympathetic regulation of the heart.

CHAPTER 2

WHOLE AND PARTICLE-FREE DIESEL EXHAUSTS DIFFERENTIALLY AFFECT CARDIAC ELECTROPHYSIOLOGY, BLOOD PRESSURE, AND AUTONOMIC BALANCE IN HEART FAILURE-PRONE RATS

Overview

Epidemiologic studies strongly link short-term exposures to vehicular traffic and particulate matter (PM) air pollution with adverse cardiovascular events, especially in those with preexisting cardiovascular disease. Diesel engine exhaust (DE) is a key contributor to urban ambient PM and gaseous pollutants. To determine the role of gaseous and particulate components in DE cardiotoxicity, we examined the effects of one 4-hour inhalation of whole DE (wDE; target PM concentration: 500 $\mu g/m^3$) or particle-free filtered DE (fDE) on cardiovascular physiology and a range of markers of cardiopulmonary injury in hypertensive heart failure-prone rats. Arterial blood pressure (BP), electrocardiography (ECG), and heart rate variability (HRV, an index of autonomic balance) were monitored. Both fDE and wDE decreased BP and prolonged PR interval during exposure, with more effects from fDE, which additionally increased HRV triangular index and decreased T-wave amplitude. fDE increased QTc interval immediately after exposure, increased atrioventricular (AV) block Mobitz II arrhythmias shortly thereafter, and increased serum high-density lipoprotein 1 day later. wDE increased BP and decreased HRV root mean square of successive differences (RMSSD) immediately post-exposure. fDE and wDE decreased heart rate during the 4th hour of post-exposure. Thus, DE gases slowed AV conduction and ventricular repolarization, decreased BP, increased HRV, and

subsequently provoked arrhythmias, collectively suggesting parasympathetic activation; conversely, brief BP and HRV changes after exposure to particle-containing DE indicated a transient sympathetic excitation. Our findings suggest that whole and particle-free DE differentially alter cardiovascular and autonomic physiology and may potentially increase risk through divergent pathways.

Introduction

Exposure to vehicular traffic air pollution poses a significant threat to public health, especially in individuals with pre-existing cardiovascular disease (Brook, 2008). Diesel engine exhaust (DE) is a major source of urban fine and ultrafine particulate matter, as well as volatile organics, carbonyls, and gases such as sulfur dioxide (SO_2), nitrogen oxides (NO and NO_2), and carbon monoxide (Krivoshto, et al., 2008; Peretz, et al., 2008a). Moreover, DE is an important contributor to vehicular emissions attendant to biochemical and physiological responses and adverse clinical outcomes near roadways. For instance, in a study spanning15 cities within the United Kingdom, Bhaskaran *et al.* (2011) demonstrated an association between exposure to NO_2 and onset of myocardial infarction 1-6 hours later. Further, ischemic heart disease hospitalizations in eight European cities have been attributed to DE exposure (Le Tertre, et al., 2002). In addition, Mills *et al.* (2007) found that DE exposure exacerbated exercise-induced electrocardiographic ST depression in human subjects with known coronary artery disease. Several mechanisms underlying the acute cardiovascular toxicity of DE exposure have been implicated, including electrophysiological dysfunction, autonomic imbalance, vascular dysfunction, coagulation, and low-level systemic inflammation (Anselme, et al., 2007; Brook, 2008; Campen *et al.*, 2005; Lucking, et al., 2011; Mills, et al., 2007; Peretz, et al., 2008a).

Although many components of DE are suspected to play a role in DE-induced cardiovascular (CV) dysfunction, recent investigations using relatively healthy individuals have implicated particles as the predominant mediators (Lucking, et al., 2011; Mills, et al., 2011b). Studies have demonstrated pathophysiologic effects on the CV system following acute exposure to either particle-containing whole diesel exhaust (wDE) (Anselme, et al., 2007; Miller *et al.*, 2009; Mills, et al., 2007) or DE particles alone (Huang, et al., 2010). Likewise, removal of particles by modern DE filters can prevent DE-induced thrombosis and vasoconstriction in healthy humans (Lucking, et al., 2011; Mills, et al., 2011b). Other studies suggest that the gaseous components of DE contribute to the pathophysiologic effects documented in epidemiologic studies. Several reports have shown that particle-free DE exposure promotes acute physiologic alterations that can trigger cardiac dysfunction and injury—including increased blood pressure, vascular plaque formation, cardiac arrhythmia, and enhanced responsiveness to a vasoconstrictor (Campen, et al., 2005; Mills, et al., 2011b). However, neither the dominant constituents nor the primary mechanisms behind DE-induced cardiac toxicity are resolved.

Because the correlations between air pollution and adverse cardiac events are strongest among populations with preexisting CV disease, it is important to model this in animal toxicity studies. We have previously demonstrated that exposures to residual oil PM (Carll, et al., 2010; Farraj *et al.*, 2009; Farraj *et al.*, 2011) the gaseous irritant acrolein, (Hazari *et al.*, 2009), or diesel exhaust (Lamb *et al.*, 2012) cause a number of alterations in cardiac physiology including increased parasympathetic tone, ST depression, and cardiac arrhythmia (e.g., AV block) in rat models of hypertension or heart failure. Because of the continued uncertainty regarding the precise role of specific diesel exhaust constituents in the elicitation of cardiovascular effects, we investigated previously undescribed electrocardiographic and blood pressure effects of acute

exposure to particle-free (gases alone; fDE) and whole DE (particles plus gases; wDE) in heart failure-prone rats. We hypothesized that wDE exposure would provoke greater changes in CV physiology than fDE exposure in Spontaneously Hypertensive Heart Failure (SHHF) rats. Electrocardiogram and blood pressure radiotelemetry were used to monitor autonomic balance (measured by heart rate variability), cardiac arrhythmia, and indicators of altered myocardial conduction, before, during, and after a single whole-body inhalation exposure to either wDE or fDE. Cardiopulmonary injury, inflammation, and oxidative stress were also assessed.

Materials and Methods

Animals and radiotelemetry implantation. Lean male spontaneously hypertensive heart failure (SHHF) rats (*MccCrl-Lepr*^{cp}; n = 20, 9 weeks old; Charles River Laboratories, Kingston, NY) were implanted with radiotelemeters (model TL11M2-C50-PXT; Data Sciences International, St. Paul, MN) capable of transmitting ECG, heart rate (HR), aortic blood pressure (BP), and core body temperature wirelessly to a computer receiver. Telemeter implantation was performed by surgeons at Charles River Laboratory and adhered to preoperative, anesthetic, and surgical procedures described previously (Carll, et al., 2010). Lean male SHHFs acquire cardiac hypertrophy by 3 months of age and transition into dilated cardiomyopathy and heart failure (HF) at 18 months of age as a consequence of hypertension and hyperleptinemia (Carll *et al.*, 2011c). Rats were shipped after a 10-day recovery period to our Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility, housed individually in 42 × 21 × 20-cm Plexiglas cages with pine-shave bedding in a room (22°C ±1°C, 50% ± 5% relative humidity, 12-h light:dark cycle 0600:1800 h), and provided standard Purina rat chow (5001; Brentwood, MO) and water *ad libitum*. All studies conformed to

the guidelines of the US Environmental Protection Agency (EPA) Institutional Animal Care and Use Committee (IACUC). Three days later, rats were transferred to the EPA High Bay facility's satellite animal holding room and maintained under the same conditions as previously stated but in smaller, 33 × 18 × 19-cm Plexiglas cages. Rats were weighed and assigned blindly to one of three exposure groups (clean filtered air, "Air"; whole diesel exhaust, "wDE"; and filtered diesel exhaust, "fDE") while maintaining equivalent mean body weights per group.

Diesel exhaust exposure and generation. Rats were acclimated to exposure conditions in 20 × 12.5 × 17-cm metal wire cages within the clean filtered air exposure chamber for 1 hour at two days preceding exposure. On the exposure day, rats were allowed to acclimate to the chambers for 20 min and then baseline data was recorded for the next 40 min. Rats were then exposed to whole diesel exhaust (wDE, target of 500 µg $PM_{2.5}/m^3$), filtered diesel exhaust (fDE, target of 0 µg $PM_{2.5}/m^3$), or clean filtered air (Air) for 4 hours in whole body exposure chambers. Thereafter, DE exposures were stopped for a 1-hour recovery period in which clean filtered air was circulated through exposure chambers. Rats were returned to home cages immediately after recovery period. DE exposures were at ultrafine PM concentrations comparable to those found in traffic tunnels and (at brief moments) on roadways (Anselme, et al., 2007; Zhu, et al., 2007) and NO_2 and SO_2 concentrations comparable to those observed in traffic tunnels or in cities within the U.S. and Europe (Danzon, 2000; Svartengren *et al.*, 2000).

DE 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 on low sulfur diesel fuel (32ppm) as previously described (Lamb, et al., 2012). Resistance heating elements provided a constant 3 kW load. Engine lubrication oil (Shell Rotella, 15W-40) was changed before each set of exposures. From the engine, the exhaust was mixed with clean air previously

passed through high-efficiency particulate air (HEPA) filters. Air dilution of wDE was adjusted periodically to maintain target $PM_{2.5}$ mass concentration. The diluted DE was delivered to an isolated animal exposure room and was either delivered un-filtered to a Hazelton 1000 (984 L) exposure chamber (wDE) or diverted through a HEPA canister filter and delivered to a similar exposure chamber (fDE). The HEPA canister filter featured a 99.97% removal efficiency standard to 0.3 µm. Although the fDE chamber was relatively absent of PM, its concentrations of diluted combustion gases remained comparable to the unfiltered chamber (Table 2.1). Control animals were placed in a third chamber supplied with the same HEPA-filtered room air as that used to dilute DE. The chambers were operated at the same flow rate (424 L/min; resulting in approximately 25 air exchanges per hour), temperature, and pressure. Integrated 4 h filter samples (14.1 L/min) were collected daily from each chamber and analyzed gravimetrically to determine particle concentrations. Chamber concentrations of PM, oxygen (O_2), carbon monoxide (CO), nitrogen oxides (NO and NO_2), and sulfur dioxide (SO_2) were measured as previously described (Lamb, et al., 2012). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges.

Radiotelemetry data acquisition and analysis. Radiotelemetry was used to track changes in CV and thermoregulatory function by continuously monitoring core body temperature, blood pressure, ECG, and activity in awake, unrestrained rats beginning at 1 day before inhalation exposure and continuing through exposure until euthanasia 24 hours after exposure. Data was monitored by remote receivers (Model RPC-1; Data Sciences International, Inc.) positioned under the home cages within the animal facility, and beside cages within exposure chambers. Arterial blood pressures (mean, systolic, diastolic, and pulse), heart rate, and QA interval were derived from pressure and ECG waveforms collected at a sample rate of 1000 Hz for 2 min of

every 10 min and automatically analyzed by computer software (DataART 3.01; Data Sciences International) as previously described (Carll, et al., 2010). QA interval provides an index of contractility determined by a measure of the aortic pre-ejection period. Specifically, QA interval is the delay between onset of left ventricular depolarization and ejection, which are respectively indicated by the initializations of the R-wave and the following increase in aortic pressure (Cambridge & Whiting, 1986). Averages were calculated for blood pressures on an hourly basis over the 4 hours of exposure (mid-inhalation, within exposure chambers), the roughly 40-minute baseline and 1-hour recovery periods (within exposure chambers), the 4-hour periods in home cages immediately pre- and post- exposure, and a home cage period from the day preceding exposure that was time-matched with exposure.

ECG waveforms were analyzed with computer software (ECGauto 2.5.1.35; EMKA Technologies, Falls Church, VA) that enabled visual arrhythmia identification and automated RR interval and HRV measures using an RR-only analysis platform. Additionally, ECG morphologic traits (duration, area, and amplitude of intervals and waves within each P-Q-R-S-T beat) were measured through this software's ECG analysis platform. ECG landmarks (P, Q, R, S, and T waves) were identified through application of a library of 58 representative waveforms, which were collected and marked manually during a survey of each rat ECG within the present study. Several parameters were determined for each ECG waveform: PR interval; Q and R wave amplitudes; QRS duration; ST interval, amplitude, and area (negative area starting from S until intersection with iso-electric line); T wave amplitude and area; raw QT interval (from Q to peak of T); QTe interval (from onset of the Q wave to end of T wave); heart rate-corrected QT interval (using both Fridericia and Bazett's corrections); interval from peak of T to end of T wave; and RR interval. The equation for Fridericia correction was $QTc = QT \div \sqrt[3]{RR}$), whereas the

equation for Bazett's correction was $QTc = QT \div \sqrt{RR}$). We present Fridericia-corrected QT interval as "QTc". In both QT interval and QTc intervals, peak of T wave was used because it was more consistently detected by software than end of the T wave.

HRV analysis generated heart rate (HR) and time-domain measures, including mean time between adjacent QRS-complex peaks (RR interval), standard deviation of the RR interval (SDNN), square root of the mean of squared differences of adjacent RR intervals (RMSSD), triangular index, and percent of adjacent normal RR intervals differing by ≥15 ms (pNN15). pNN15 is a measure of parasympathetic tone comparable to pNN50 in humans. SDNN and triangular index represent overall HRV, whereas RMSSD represents parasympathetic influence over heart rate (Rowan *et al.*, 2007). HRV analysis also provided frequency-domain parameters, including low frequency (LF: 0.200-0.750 Hz) and high frequency (HF: 0.750-2.00 Hz), and the ratio of these two frequency-domains (LF/HF). For frequency-domain analysis, the signal was analyzed with a Hanning window for segment lengths of 512 samples with 50% overlapping. LF is generally believed to represent a combination of sympathetic and parasympathetic tone, whereas HF indicates cardiac vagal (parasympathetic) tone, and LF/HF serves as an index of sympathovagal balance (Rowan, et al., 2007).

Arrhythmias were verified from time-matched blood pressure, and identified (while blinded to treatment group) as ventricular, supraventricular, junctional, and atrial premature beats, sinoatrial blocks, or AV blocks using the Lambeth Conventions (Walker *et al.*, 1988) as a guideline and according to additional, more specific criteria (Carll, et al., 2010). Each AV block Mobitz II arrhythmia was marked by a non-conducted P-wave that lacked the following four features: (i) an RR interval less than twice the average of the preceding 3 RR intervals, (ii) progressive PR interval prolongation in the preceding three PQRST complexes, (iii) PR

shortening in the first subsequent PQRST complex, and (iv) PP interval shortening immediately prior to the dropped R wave. To facilitate statistical analysis of each arrhythmia type and allow the data to converge under the Poisson distribution, zero-values for each arrhythmia type within a sample interval were converted to 0.1. Arrhythmia frequencies were calculated over specific periods in home cages (pre-exposure and post-exposure, 6 hours each) as well as in exposure chamber (baseline, mid-exposure, recovery), normalized to adjust for time differences between periods and gaps in data, and presented as number of events per-hour of theoretically continuous ECG waveforms.

HRV and ECG morphologic analyses were conducted on ECG waveforms collected while rats resided in home cages at pre-exposure and post-exposure periods (both 2pm-8pm), which were time-matched to control for physiologic effects of circadian rhythm. ECG data collected within the exposure chamber (5 h 10 min total) was also analyzed according to the following periods: baseline (8:50am-9:30am), exposure (9:30am-1:30pm), and recovery (1:30pm-2:00pm). All 2-min ECG streams with less than 10 seconds of identifiable conduction cycles were excluded from calculation. For HRV analysis, thorough visual inspection was conducted to identify and exclude arrhythmias, artifacts, and 2-min ECG waveforms with less than 60 seconds of distinguishable R-waves.

Tissue collection and analysis. At approximately 24 hours after onset of the 4-hour inhalation exposure, 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). Whole blood was collected from the descending abdominal aorta in serum separator tubes and microcentrifuge tubes containing either buffered sodium citrate or K2EDTA (Becton, Dickinson, and Company, Franklin Lakes, NJ) as previously described (Carll *et al.*, 2011a). Hearts were

excised, trimmed free of arterial tissue and fat, and weighed. Right tibia length was measured by caliper for heart weight normalization. The trachea was cannulated, and the lungs were lavaged with a total volume of 20 ml/kg of Ca^{+}, Mg^{+}, and phenol red-free Dulbecco's phosphate-buffered saline (SAFC Biosciences, Lenexa, MD) that was divided into two equal aliquots and processed for cell differentials (Carll, et al., 2010). Lavage, serum, and plasma samples were collected, centrifuged, stored, and subsequently analyzed according to previously published procedures for the following biomarkers: lavage supernatants were analyzed for albumin, lactate dehydrogenase, N-acetyl-b-d-glucosaminidase, total antioxidant status, and total protein; supernatants from serum were analyzed for creatine kinase, C-reactive protein, total protein, and glutathiones peroxidase, reductase, and –S-transferase; and supernatants from plasma were assayed for angiotensin converting enzyme, albumin, blood urea nitrogen, creatinine, and total protein (Carll, et al., 2011a). Serum was also analyzed for α-hydroxybutyrate dehydrogenase, glucose, total cholesterol, and triglycerides (Sigma-Aldrich, St. Louis, MO) as well as alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase-1, myoglobin, high and low density lipoprotein cholesterol, and sorbitol dehydrogenase according to previous procedures (Carll, et al., 2010), while commercially available kits were used in analysis of serum for D-Dimer, ferritin, and insulin (Kamiya Biomedical Company, Seattle, WA), lipoprotein (a), superoxide dismutase (SOD), manganese SOD, and copper-zinc SOD (Randox Life Sciences, Antrim, United Kingdom), and lipase (Genyzme Diagnostics, Framingham, MA). Lavage was also analyzed for gamma-glutamyl transferase (Fisher Diagnostics, Middletown, VA).

Statistics. The statistical analyses for all data in this study were performed using Prism version 4.03 (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) with Tukey post-hoc test was used to detect significant differences between groups in

biochemical endpoints and tissue weight. Repeated measures two-way ANOVA with Bonferroni post-hoc test was performed on (I) arrhythmia frequency data, which were collected at pre-, mid-, and post-exposure periods (spanning approximately 6, 4, and 6 hours, respectively) and normalized by sampling duration; (II) HRV, ECG morphology, blood pressure, and blood pressure parameters during the exposure period, including exposure hours 1-4, baseline (40 min), and recovery periods (30 min); and (III) HRV, ECG morphology, and blood pressure to analyze for intra-group differences between time-matched periods (separated by exactly 24 hours) collected on the exposure day and on the previous day. Two-way ANOVA with Bonferroni post-test was also used to analyze for inter-group differences in percent change in HRV and ECG parameters at post-exposure relative to the day before exposure. Blood pressure data from the day of exposure were analyzed both by treatment period and by individual hour for significant time-matched inter-group differences by two-way ANOVA with Bonferroni post-hoc test. $P < 0.05$ was considered statistically significant. Linear regressions were performed to test for correlations between various physiologic endpoints.

Results

Physiological Responses during Inhalation Exposure.

Cardiac arrhythmia. There were no changes in the frequencies of any arrhythmia type during exposure when compared to other groups (during exposure) or to each group's own pre-exposure values (in home cages).

Heart rate and heart rate variability. There were no group differences in heart rate or heart rate variability at baseline (Table 2.2). All groups were upright and active at the beginning of the exposure and became recumbent and inactive during exposure to Air or DE. As would be expected with decreased activity, heart rate decreased from baseline at multiple hours of

exposure for the Air, fDE, and wDE groups ($P < .05$; Figure 2.1). LF/HF paralleled changes in heart rate, significantly declining from baseline for the Air (hours 2-3), fDE (hour 2), and wDE (hours 2-4) groups ($P < .05$). In contrast to Air or wDE, fDE significantly increased triangular index during hour 4 of exposure relative to baseline ($P < .05$). During the recovery period, while all rats remained in exposure chambers and were breathing clean filtered air, only the wDE-exposed rats had a significant change in RMSSD, which decreased by 24% relative to baseline ($P < .05$). Simultaneously, LF/HF for only the DE-exposed groups significantly rebounded from their mid-exposure values (exceeding hours 1-4 for fDE and hours 2-4 for wDE; $P < .05$).

Hemodynamics and thermoregulation. There were no group differences in blood pressure at baseline (Table 2.2). At hour 2 of exposure, fDE decreased systolic BP relative to baseline (Figure 2.2; -8.5 mmHg, $P < .05$). In contrast, the Air group had increased pressure at hour 4 relative to baseline (+9.0 mmHg in MAP, +9.5 mmHg in diastolic BP; $P < .05$) and the fDE-exposed group (+12.2 mmHg in MAP, +13.2 mmHg in systolic BP, +11.7 mmHg in diastolic BP; $P < .05$). During the recovery period when all groups were provided clean filtered air within exposure chambers, the Air group still had significantly increased BP relative to baseline (+8.8 mmHg in MAP, $P < .05$). Meanwhile, the wDE group also exceeded its own baseline diastolic pressure during recovery (+7.0 mmHg, $P < .05$). There was no such increase in the fDE-exposed group. There were no significant changes in pulse pressure, QA interval (an index of contractility), or core body temperature during exposure ($P > .05$).

ECG Morphology. There were no group differences in ECG morphology at baseline (Table 2.2). During hour 3 of exposure, fDE increased PR interval by 2.7 msec and decreased T-wave amplitude by 25% relative to baseline, while wDE also prolonged PR by 2.0 msec from baseline (Figure 2.3; $P < .05$). At hour 4, fDE exposure prolonged PR interval relative to baseline

(+3.8 msec; $P < .05$) and relative to Air at the same time (+3.3 msec; $P < .05$). During recovery the fDE-exposed group had a significant increase in corrected QT interval (QTc) relative to its own baseline (+5.0 msec) as well as relative to Air (+6.9 msec), while it also continued to have significantly prolonged PR relative to baseline (+2.4 msec; all comparisons $P < .05$). Notably, among all rats, changes from baseline in PR during hour 4 and recovery correlated positively with the subsequent rate of post-exposure Mobitz II AV block in home cages (vs. hour 4 PR r^2: 0.41, $P = 0.003$; and vs. recovery PR r^2: 0.22, $P = 0.042$). As well, the change from baseline in QTc during recovery significantly correlated with the change from baseline in PR during hour 4 of exposure (r^2: 0.42, $P = 0.006$) and with the change from baseline in T-amplitude during recovery (r^2: 0.51, $P = 0.002$) and over the entire exposure period (r^2: 0.35, $P = .016$).

Physiologic Responses after Exposure (while in Home Cages)

Cardiac arrhythmia. Over the 6-hours following exposure while the animals were in home cages, the fDE group had an increased frequency of second degree Mobitz Type II AV block relative to (1) itself during the same time on the previous day while in home cages, (2) itself during the exposure period within chambers, and (3) the wDE group during the same post-exposure period (Figure 2.4; $P < .05$). Notably, 77% of the fDE group's post-exposure bradyarrhythmias occurred within the first 2 hours of this 6 hour period. After the first 6 hours of post-exposure, AV block arrhythmias were rare for all groups.

Heart rate and heart rate variability. Within the first hour after animals were returned to home cages for post-exposure monitoring, LF/HF increased in each group relative to the corresponding hour on the prior day ($P < .05$). Meanwhile heart rate also had a trend of an increase for all groups ($P > .05$; Table 2.3). At hour 4 of post-exposure, both DE-exposed groups

decreased in heart rate from the preceding day, but only the wDE group's change was statistically significant (wDE -38 beats/min, $P < .05$; fDE -37 beats/min, $P > .05$; Table 2.3). There was an apparent difference in heart rate between the DE groups and the Air group at hour 4 of the prior day (Table 2.3; $P = 0.22$), but the post-exposure decreases in heart rate were roughly 2-3 times larger than these preexisting differences. LF/HF also appeared to decrease for both DE groups at hour 4 post-exposure, but these changes were not statistically significant (fDE: -32% and wDE: -31% from prior day; $P > .05$).

Hemodynamics, ECG morphology, and thermoregulation. In the first post-exposure hour in home cages, the Air group had an increased MAP relative to its own time-matched values from the prior day (+17.4 mmHg, $P < .05$) while the wDE group had a trend of similarly increased MAP (+17.3 mmHg; $P > .05$), which the fDE group lacked (+8.5 mmHg; $P > .05$). At 4 hours post-exposure, no group significantly differed in BP from itself on the prior day. There were no significant post-exposure changes in pulse pressure, QA interval (an index of contractility), or body temperature. There were no significant post-exposure changes in ECG morphology.

Biochemical markers of cardiopulmonary and circulatory injury, oxidative stress, and inflammation. HDL cholesterol increased in the fDE-exposed group by 25% ($P < .05$; Table 2.4). There were no significant effects of DE inhalation on pulmonary inflammatory cells, pulmonary and circulating anti-oxidants, or cardiopulmonary markers of injury. There were trends of decreased plasma and serum glutathione S- transferase with wDE exposure (-29% and -21% from Air, respectively; $P > .05$), increased lactate dehydrogenase-1with exposure to fDE or wDE (+81% and +101% from Air, respectively; $P > .05$), and increased serum ferritin with fDE or wDE exposure (+23% and +12% from Air, respectively; $P > .05$).

Discussion

We present evidence that a single four-hour exposure to diesel exhaust (DE) in a rat model of hypertension and mild (pre-heart failure) cardiomyopathy differentially alters cardiac rhythmicity, blood pressure, and autonomic modulation of the heart based on the DE constituents present. DE caused a decrease in blood pressure and concomitant PR prolongation during exposure regardless of the presence of particles, and these effects remained with exposure to DE gases alone. Only the filtered DE (fDE) group had increased HRV triangular index and T-wave flattening during exposure, as well as post-exposure QT prolongation and increased Mobitz II AV block arrhythmias. Collectively, changes in PR and BP and a unique increase in HRV and bradyarrhythmias within the fDE group suggest that DE gases cause parasympathetic (vagal) dominance. This was further evidenced by a post-exposure bradycardia in both DE groups. Meanwhile, the whole DE group's less overt responses during exposure and significantly fewer arrhythmias thereafter (relative to fDE group) suggest that these specific effects of DE gases are partly inhibited either by physico-chemical interactions with DE particles or by competing autonomic impacts of the two constituents. In further support of the latter, HRV decreased and diastolic BP increased immediately after wDE exposure, indicating sympathetic excitation.

Filtered DE caused a 3-hour PR prolongation upon exposure and a unique increase in second degree Mobitz type II AV block arrhythmias thereafter, indicating markedly impaired AV conduction. In contrast, wDE caused only a 1-hour PR prolongation and did not elicit any arrhythmia. There was a correlation between mid-exposure PR prolongation and post-exposure AV block arrhythmias, indicating that PR prolongation may by several hours precede (or perhaps even predict) air pollutant-induced AV block arrhythmias and that DE gases may promote spontaneous bradyarrhythmia through impaired conduction along the AV pathway. Although the

capacity for DE to elicit this specific type of arrhythmia in humans is unclear, these findings suggest that DE exposure may increase vulnerability to spontaneous cardiac arrhythmia.

Increased vagal tone has been shown to prolong PR, cause nitric oxide-mediated vasodilation (Hotta *et al.*, 2009; Katz, 2006), increase HRV triangular index (Kouidi *et al.*, 2002), provoke AV block Mobitz II arrhythmia (Castellanos *et al.*, 1974; Hotta, et al., 2009; Massie *et al.*, 1978), decrease heart rate, and inhibit sympathetic-mediated norepinephrine release and vasoconstriction (Katz, 2006; Vanhoutte & Levy, 1980). We have previously shown that, relative to wDE, fDE causes PR prolongation and bradycardia, a more pronounced increase in HRV, and greater or equal susceptibility to provocation of arrhythmia, ventricular fibrillation, and cardiac arrest by a pro-arrhythmic drug (Hazari *et al.*, 2011; Lamb, et al., 2012). As well, Campen and colleagues found that fDE and wDE cause equally marked bradycardia in mice (2005). Fittingly, others have demonstrated by way of increased HRV that DE may cause parasympathetic dominance (Mills, et al., 2011a; Peretz, et al., 2008a). Meanwhile, one study has found that DE decreases HRV in a rat model of advanced heart failure (Anselme, et al., 2007), while another provides evidence that ultrafine PM may mediate this effect (Chuang *et al.*, 2005). The present study expands the body of research suggesting that gaseous exhaust can mediate several of DE's effects on cardiac rhythmicity and autonomics (Table 2.5). Additional inhalation studies that examine the effect of DE particles alone (currently beyond the capacity of our inhalation facilities) are needed to disentangle the various effects of DE constituents.

Exposure to filtered DE altered the ECG (i.e., QTc and T-wave amplitude), indicating changes in the spatiotemporal pattern of ventricular repolarization. These findings are not unprecedented; acute exposure to *particle-free* DE gases has been shown to decrease T-wave amplitude in two separate studies involving atherosclerotic mice and hypertensive rats, but not in

healthy controls (Table 2.5) (Campen, et al., 2005; Lamb, et al., 2012). In humans, air pollutant exposure has also been associated with prolonged QTc and/or decreased T-wave amplitude (Henneberger *et al.*, 2005; Liao *et al.*, 2010). Ventricular repolarization may be impeded by parasympathetic dominance (Conrath & Opthof, 2006; Katz, 2006; Murakawa *et al.*, 1992), inflammation (Zhang *et al.*, 2011), and myocardial ischemia (Channer & Morris, 2002). The inhalation of *particle-free* DE or one of its primary gases (NO_2) has been shown to promote inflammation and pro-ischemic vascular effects (Campen *et al.*, 2010; Channell *et al.*, 2012). Others have found that non-particulate components may mediate vehicular emissions-induced aortic remodeling in entirety (Lund *et al.*, 2007) or pulmonary inflammatory signaling in part (Elder *et al.*, 2004). On the other hand, some have demonstrated that particle filtration inhibits vascular effects of DE (Lucking, et al., 2011; Mills, et al., 2011b). These discrepancies may point to the divergent vascular and cardiac effects of specific constituents of DE.

Both DE groups had decreased blood pressure during exposure, suggesting mediation by DE gases. While the specific gases mediating this effect remain unclear, there is mounting evidence that inhaled NO remains in the blood up to 2 hours post-exposure (Knuckles, et al., 2011). NO decreases blood pressure through vasodilation and facilitates parasympathetic control over cardiac function (Conlon & Kidd, 1999; Yabe *et al.*, 1998). In turn, parasympathetic activation inhibits stress-induced catecholamine release (Katz, 2006; Vanhoutte & Levy, 1980). Conversely, clean air exposure increased blood pressure, likely due to stress-induced catecholamine release, which has been demonstrated under conditions similar to our control exposure (e.g., confinement) (Morimoto *et al.*, 2000). Incidentally, increased catecholamines decrease HDL cholesterol (Kjeldsen *et al.*, 1992), while parasympathetic activation inhibits this effect (Benthem *et al.*, 2001), perhaps explaining why air-exposed rats had decreased HDL (and

fDE-exposed rats had normal HDL) relative to naïve un-confined SHHF rats from a previous study (Carll, et al., 2011a). We have previously found 'increases' in HDL concomitant with similar parasympathetic-associated effects from PM (Carll, et al., 2010). The mechanisms underlying the hemodynamic and biochemical effects of DE gases may bear important implications for the cardiovascular risks of DE.

Ultimately, our findings of altered CV function identify potentially disparate responses to the gaseous and particulate components of DE with equally relevant implications toward CV risk and autonomic balance. Increased sympathetic tone is a major putative mechanism of PM-induced CV pathogenesis (Brook, 2008). Consistent with this prevailing hypothesis, inclusion of particles in DE caused a decrease in HRV and diluted the parasympathetic-associated effects of DE gases (AV conduction delay, Mobitz II AV block, HRV increase, BP decrease), suggesting increased sympathetic tone. There is some evidence that oxidative stress mediates particulate-induced sympathetic excitation and decreased HRV (Rhoden *et al.*, 2005), but this mechanism remains underexplored. Regardless, it is important to note that our data neither suggest inherently protective nor prove directly autonomic effects of DE particles. Meanwhile, the stimulation of pulmonary irritant receptors (e.g., C-fibers, TRPA1) is known to cause parasympathetic activation (and resulting cardiovascular reflexes, including bradycardia and hypotension) (Widdicombe & Lee, 2001). Although there is evidence that whole DE (Hazari, et al., 2011; Wong *et al.*, 2003) and several of its components, including SO_2 (Wang *et al.*, 1996) and particles (Deering-Rice *et al.*, 2011) stimulate pulmonary irritant receptors, it remains unclear to what extent each component factors into physiologic reflexes. Our findings imply a predominant role for DE gases in pulmonary irritant reflexes and a potentially divergent mode for PM-induced autonomic effects; however, mechanistic studies are required to discern whether

the effects of fDE and wDE result from differing autonomic influences of components or other factors, such as physico-chemical interactions between particles and gases. For instance, particles could alter the dynamics of gas deposition, thereby modifying the stimulation of irritant receptor subgroups, which can mediate opposing physiologic reflexes (Widdicombe & Lee, 2001).

The SHHF strain is derived from the SH rat, which we have recently shown has enhanced susceptibility to DE exposure (Lamb, et al., 2012). Yet, the effects of fDE that we report here on AV block arrhythmia and HRV were not seen in our companion study involving SH rats (Lamb, et al., 2012). Thus, the SHHF appears to be even more sensitive to the cardiophysiologic impacts of DE exposure, potentially stemming from its underlying pathology including more severe hypertension than the SH rat and more rapid myocardial remodeling (Carll, et al., 2011c). The 10-week old SHHF rats used in the present study had a significant cardiac hypertrophy (tibia-normalized heart weight 15% greater than 19-week old normotensive Wistar Kyoto [WKY] rats; $P < .01$) that was equivalent to 23-week old SH rats (Lamb, et al., 2012); Carll, *unpublished data*). Additionally, AV conduction rate (PR) seems to be slower in the SHHF than the SH rat, which itself has a longer PR interval than age-matched WKYs (Hazari, et al., 2009). Notably, PR duration correlates with age and arterial stiffness (Gosse *et al.*, 2011). Despite being less than half the age of the SH rats used in our companion study (Lamb, et al., 2012), the SHHF rats herein had a baseline PR interval that was 5.2 msec longer. Further investigation is required to determine if aspects of cardiac and vascular remodeling mediate the SHHF's heightened sensitivity to air pollutant exposure. Nevertheless, the parallels between the SHHF and hypertensive, hypertrophic humans, combined with our findings of enhanced responses in the SHHF relative to SH and WKY rats, further indicate that cardiovascular disease confers sensitivity to the effects of air pollution on cardiac conduction.

In conclusion, the present findings demonstrate that DE gases trigger immediate cardiovascular responses in the rat (decreased BP, prolonged PR interval, increased HRV, altered repolarization, and AV block arrhythmia) suggesting mediation by increased parasympathetic tone. Inclusion of DE particles in whole diesel exhaust largely attenuated these responses, potentially stemming from atmospheric interactions of gases and particles and/or their opposing autonomic influences. Thus, toxic effects of concurrent exposure to two or more air pollutants may not follow conventional dose-response relationships. Collectively, our findings demonstrate that a single 4-hour diesel exhaust inhalation causes cardiovascular dysfunction, with differential effects between filtered and whole diesel exhaust.

Tables

Table 2.1. Inhalation exposure characterization.

	Air	fDE	wDE
$PM_{2.5}$ ($\mu g/m^3$)	-	3 ±2	472 ±2
$PM_{2.5}$ number (n/cm^3)	-	$1.4x10^3$ ±9	$2.1x10^6$ $±3x10^3$
Number median diameter of PM (nm)	34 ±3	21 ±3	61 ±0
Volume median diameter of PM (nm)	124 ±0	125 ±0	91 ±0
O_2 (%)	21.0 ±0.2	20.6 ±0.0	20.6 ±0.2
CO (ppm)	*BDL*	9.7 ±0.7	9.5 ±4.2
NO (ppm)	0.1 ±0.0	10.0 ±0.8	10.3 ±1.9
NO_2 (ppm)	0.0 ±0.0	0.4 ±0.1	0.3 ±0.2
SO_2 (ppm)	*BDL*	0.6 ±0.1	0.4 ±0.2

Data represent mean values ± standard error (SE) generated from measurements made either continuously (concentrations of O_2, CO, NO, NO_2, SO_2), once ($PM_{2.5}$ mass concentration), six times (wDE $PM_{2.5}$ number), or four times (fDE $PM_{2.5}$ number) per exposure. Number median diameter was based on exposure day particle size distributions ± SE. Volume diameter was calculated from number-based mobility diameters and assumes spherical particles. Air indicates filtered air; fDE, filtered diesel exhaust; wDE, whole diesel exhaust; $PM_{2.5}$, fine particulate matter; BDL, below detectable limit.

Table 2.2. Cardiovascular physiology during baseline period within exposure chamber.

Heart Rate Variability		Air	fDE	wDE
Heart Rate	(beats/min)	327 (6)	329 (8)	334 (8)
RMSSD	(msec)	3.2 (0.2)	3.0 (0.2)	3.4 (0.4)
Tri. Index		1.11 (0.06)	1.12 (0.07)	1.12 (0.06)
LF/HF		2.84 (0.60)	2.54 (0.21)	2.75 (0.24)
Aortic Pressure				
MAP	(mmHg)	168 (4)	163 (6)	162 (3)
Systolic	(mmHg)	201 (5)	193 (7)	192 (5)
Diastolic	(mmHg)	139 (4)	134 (5)	135 (2)
ECG				
PR	(msec)	49.0 (0.7)	49.4 (1.8)	51.3 (1.0)
T amplitude	(mV)	0.139 (0.021)	0.125 (0.008)	0.115 (0.010)
QTc	(msec)	65.0 (1.1)	63.1 (1.6)	64.6 (1.0)

Data represent mean values and standard error (SE, in parentheses). Parameters were measured over 40 minutes of baseline while all rats were exposed to filtered air within exposure chambers and after a 20-minute acclimation period. No significant differences were found between groups. QTc: Fridericia-corrected QT-interval.

Table 2.3. Time-matched comparison of heart rate before and after exposure.

	Air		fDE		wDE	
Hour	Before	After	Before	After	Before	After
1	299 (7)	325 (8)	307 (7)	334 (9)	335 (14)*	354 (9)
2	287 (2)	299 (5)	305 (5)	311 (7)	316 (12)	308 (5)
3	304 (8)	303 (8)	323 (13)	305 (8)	311 (6)	303 (4)
4	331 (5)	320 (14)	352 (12)	315 (5)	344 (6)	306 (7)**
5	320 (10)	319 (15)	338 (15)	354 (8)	326 (10)	330 (7)
6	369 (12)	364 (10)	366 (15)	387 (6)	365 (14)	362 (6)

Heart rate at post-exposure was compared to the time-matched period 24 hours before exposure (while in home cages). * denotes significant difference from Air group ($P < .05$). ** denotes a single group's significant decrease from itself on prior day ($P < .05$). Values represent means (SEM)

Table 2.4. Circulating endogenous antioxidants and markers of cardiovascular risk and injury

		Air		fDE		wDE	
HDL cholesterol	(mg/dl serum)	18.7	±1.7	23.4	±1.1 **	21.3	±0.7
ferritin	(ng/ml serum)	194.7	±13.9	239.6	±15.0	218.5	±18.2
lactate dehydrogenase-1	(U/l serum)	88.9	±19.2	161.1	±32.4	178.8	±49.4
glutathione S-transferase	(IU/µl plasma)	7.29	±0.84	6.83	±0.60	5.14	±0.70

Means ± S.E. HDL: high-density lipoprotein. ** denotes significant difference from Air group ($P < .05$). See *Methods* section for other markers of cardiopulmonary toxicity, risk, inflammation, and antioxidants measured in serum, plasma, and bronchoalveolar lavage fluid. All measures were unaffected by exposure. U and IU denote units and international units, respectively.

Table 2.5. Prior evidence linking DE gases to alterations in cardiac rhythmicity, repolarization, and autonomic balance.

Study	Disease Model	Exposures (PM conc.)	Findings	Interpretations
Campen et al., 2005	atherosclerosis: $ApoE^{-/-}$ mice on high fat diet)	fDE and wDE ("low" - 500 µg/m^3 "high" - 3,600 µg/m^3)	fDE and wDE equally ↓heart rate & ↓T-wave area during exposure.	filtration did not alter mid-exposure effects of DE on cardiac rhythm and repolarization.
Hazari et al., 2011	hypertension: Spontaneously Hypertensive rats	fDE and wDE ("low" - 150 µg/m^3 "high" - 500 µg/m^3)	1 day post-exposure: low fDE ↑RMSSD & ↑SDNN; high fDE ↑LF/HF. wDE did not affect HRV. fDE ↑ sensitivity to drug-induced ventricular fibrillation and cardiac arrest, but wDE did not. fDE ↑ sensitivity to drug-induced ventricular tachycardia equal to wDE.	1 day after exposure, filtered DE exclusively caused autonomic imbalance and increased sensitivity to drug-induced fatal arrhythmia.
Lamb et al., 2012	hypertension: Spontaneously Hypertensive rats	fDE and wDE ("low" - 150 µg/m^3 "high" - 500 µg/m^3)	fDE but not wDE ↓heart rate, ↑PR, ↓ST area and ↓T-wave area during exposure.	filtered DE exclusively caused parasympathetic activation, slowed AV conduction, and altered repolarization

Figures

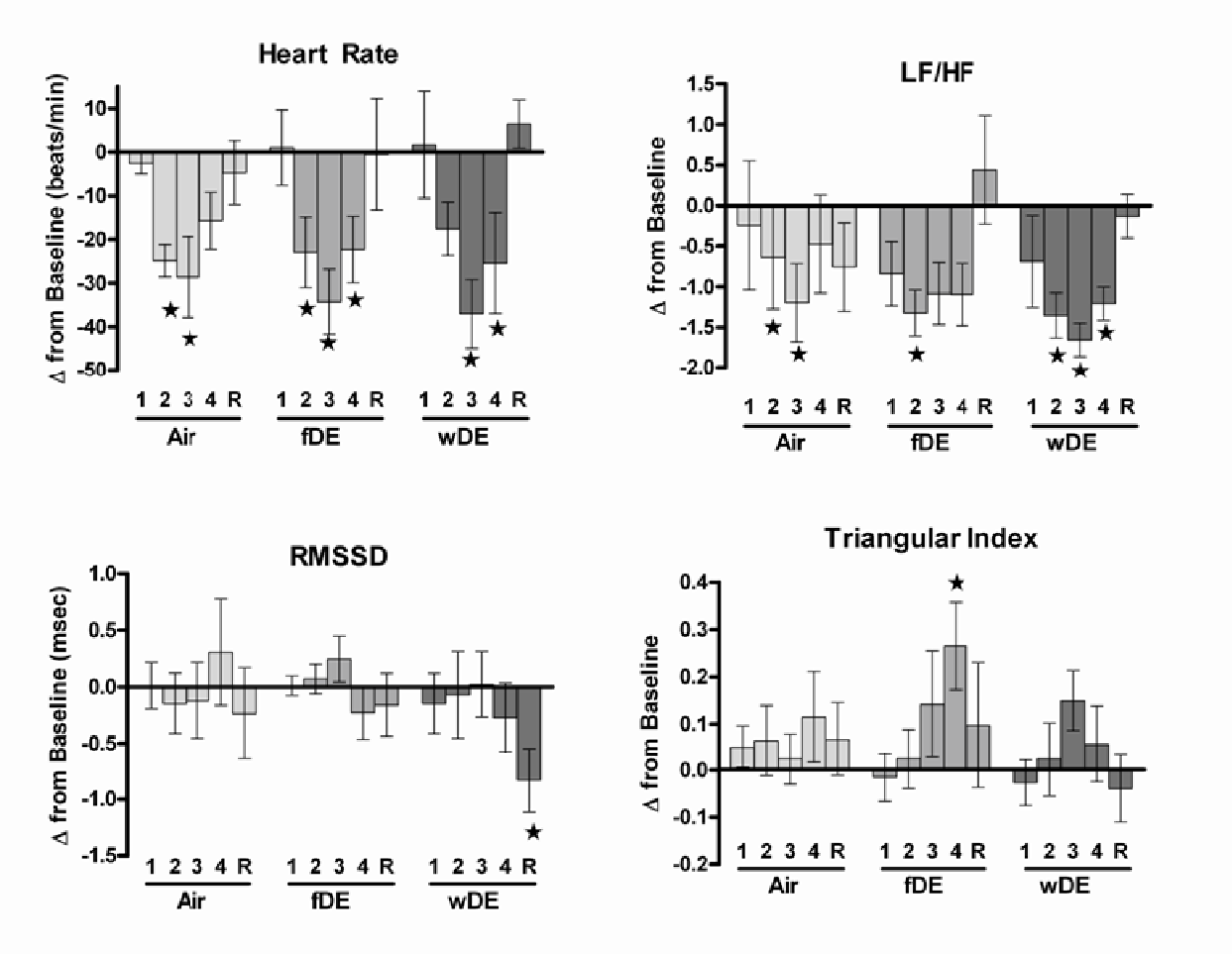
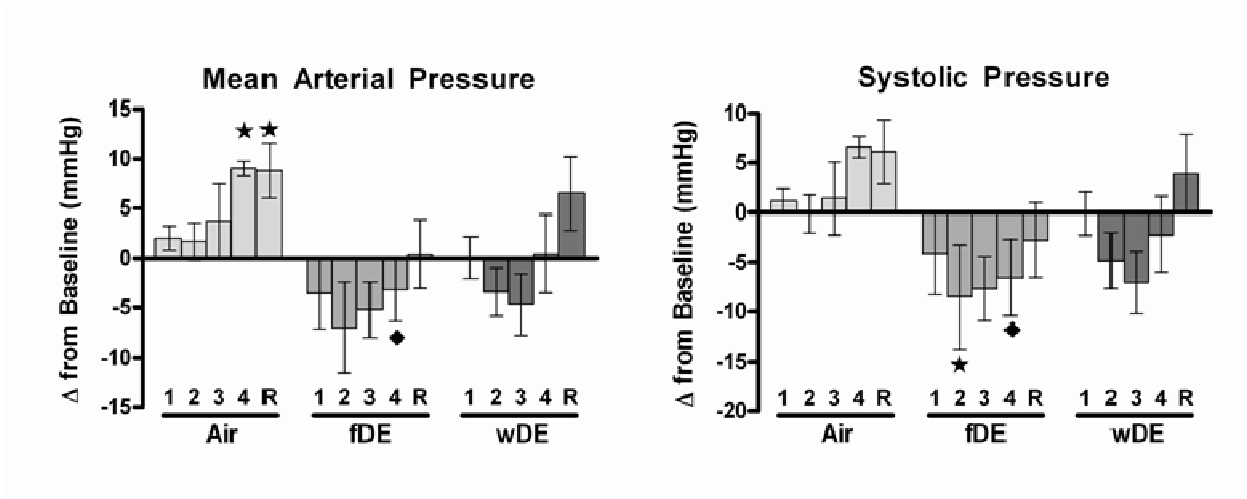


Figure 2.1 Change from baseline in heart rate and heart rate variability endpoints (mean + SE) during whole body exposure. 1, 2, 3, 4, and R represent hours 1-4 of exposure and post-exposure Recovery within the chamber, respectively. All measurements were taken from conscious rats temporarily housed within exposure chambers. Stars and diamonds respectively mark significant differences from baseline (in chambers) and the Air group (at the same hour), respectively ($P < .05$). See Table 2.2 for baseline values.



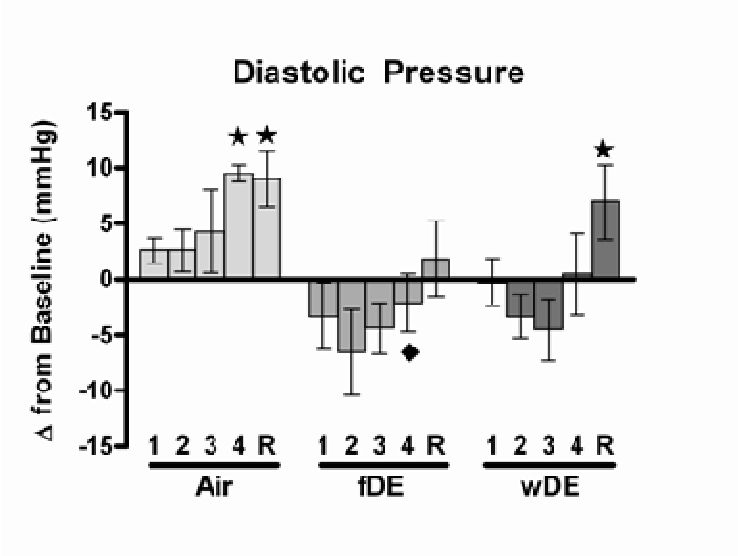
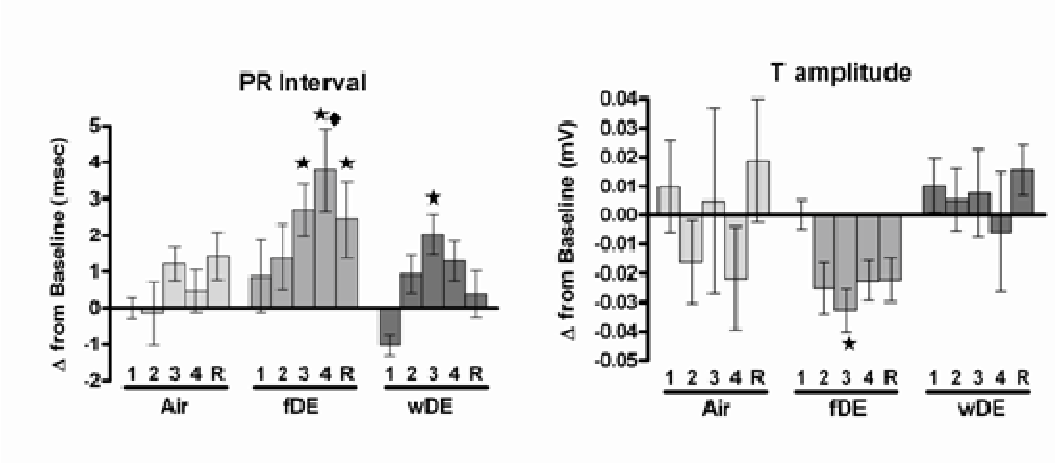


Figure 2.2 Change from baseline in blood pressure during whole body exposure (mean + SE). 1, 2, 3, 4, and R represent hours 1-4 of exposure and post-exposure Recovery within the chamber, respectively. All measurements, including baseline, were taken from conscious rats temporarily housed within exposure chambers. Stars and diamonds respectively mark significant differences from baseline (in chambers) and the Air group (at the same hour), respectively ($P < .05$). See Table 2.2 for baseline values.



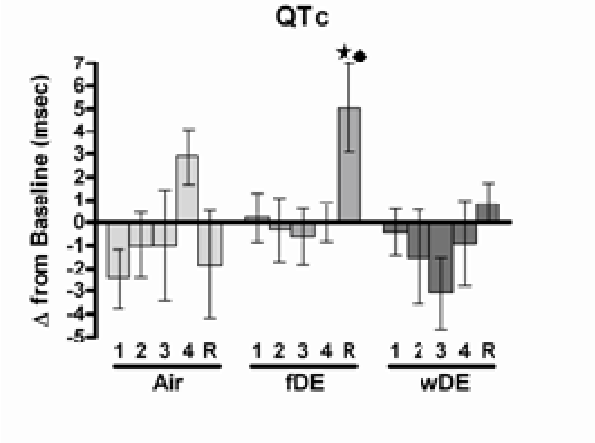


Figure 2.3 Change from baseline in ECG endpoints during whole body exposure (mean + SE). 1, 2, 3, 4, and R represent hours 1-4 of exposure and post-exposure Recovery within the chamber, respectively. All measurements were taken from conscious rats temporarily housed within exposure chambers. Significant differences are indicated by stars (relative to baseline) and diamonds (relative to Air group at the same hour; $P < .05$). See Table 2.2 for baseline values. QTc: Fridericia-corrected QT-interval.

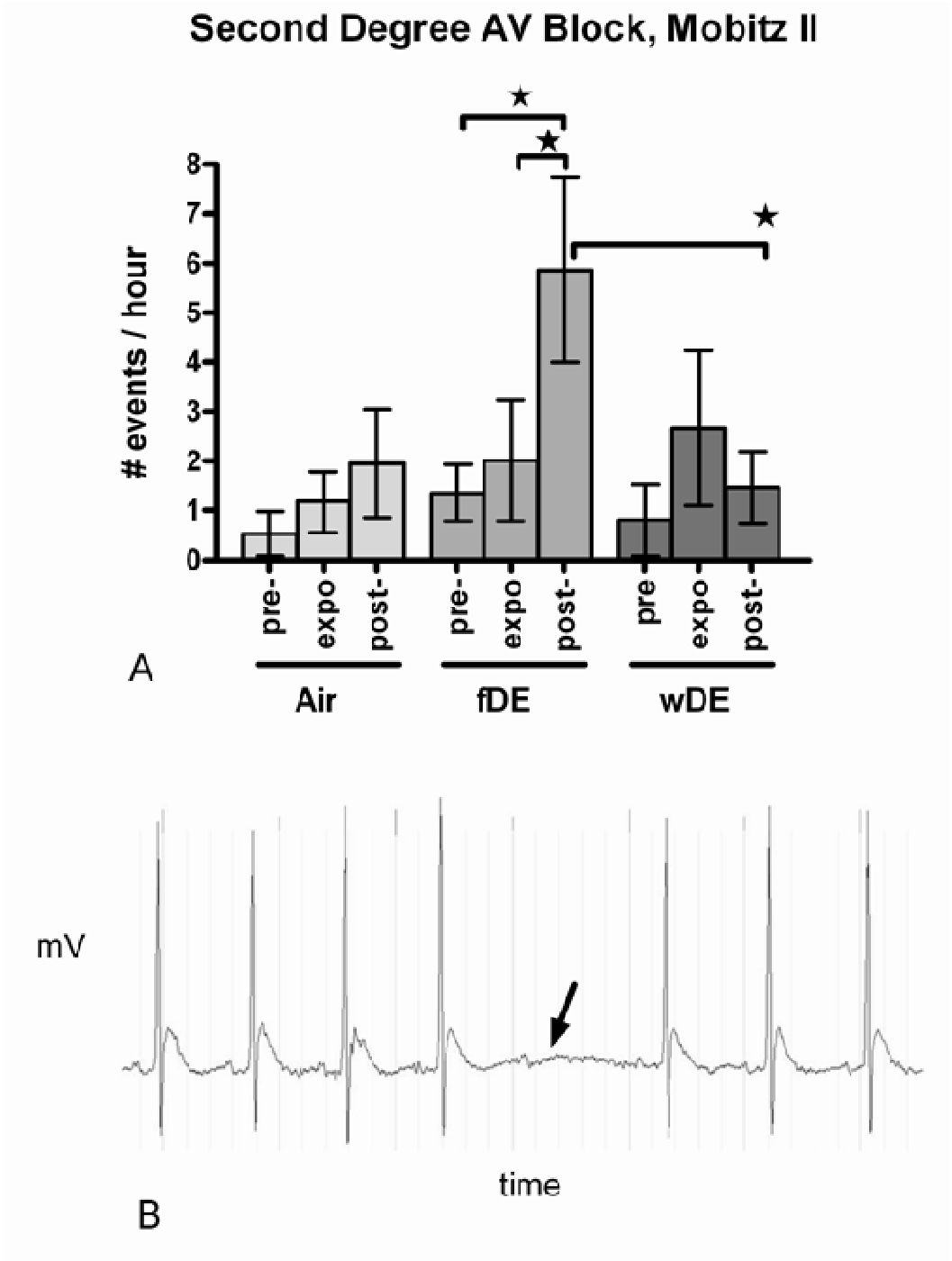


Figure 2.4 Filtered diesel exhaust induced AV block Mobitz II arrhythmia. Hourly rate of Mobitz II AV block per rat, mean ± SE (A); ECG waveform with representative second degree AV block Mobitz II arrhythmia following fDE exposure (B). "pre-", "expo-", and "post-" represent periods of ECG analysis conducted before (6 hours), during (4 hours), and after (6 hours) inhalation exposure, respectively. Stars and brackets above standard error bars indicate significant differences between periods or groups ($P < .05$). Arrow indicates individual arrhythmia. Vertical grey lines behind ECG waveform indicate time in 50 msec intervals.

CHAPTER 3

DIESEL EXHAUST INHALATION INCREASES CARDIAC OUTPUT, BRADYARRHYTHMIAS, AND PARASYMPATHETIC TONE IN AGED HEART FAILURE-PRONE RATS

Overview

Acute air pollutant inhalation is linked to adverse cardiac events and death, and hospitalizations for heart failure. Diesel exhaust (DE) is a major air pollutant suspected to exacerbate preexisting cardiac conditions, in part, through autonomic and electrophysiologic disturbance of normal cardiac function. To explore this putative mechanism, we examined cardiophysiologic responses to DE inhalation in a model of aged heart failure-prone rats without signs or symptoms of overt heart failure. We hypothesized that acute DE exposure would alter heart rhythm, cardiac electrophysiology, and ventricular performance and dimensions consistent with autonomic imbalance, while increasing biochemical markers of toxicity. Spontaneously Hypertensive Heart Failure rats (SHHF, 16 months) were exposed once to whole DE (4 h, target $PM_{2.5}$ concentration: 500 $\mu g/m^3$) or filtered air. DE increased multiple heart rate variability (HRV) parameters during exposure. In the 4 h after exposure, DE increased cardiac output, left ventricular volume (end diastolic and systolic), stroke volume, HRV, and atrioventricular (AV) block arrhythmias while increasing electrocardiographic measures of ventricular repolarization (i.e., ST- and T-amplitudes, ST area, Tpeak-Tend duration). DE did not affect heart rate relative to Air. Changes in HRV positively correlated with post-exposure changes in bradyarrhythmia frequency, repolarization, and echocardiographic parameters. At 24 hours post-exposure, DE-

exposed rats had increased serum C-reactive protein and pulmonary eosinophils. This study demonstrates that cardiac effects of DE inhalation are likely to occur through changes in autonomic balance associated with modulation of cardiac electrophysiology and mechanical function, and may offer insights into the adverse health effects of traffic related air pollutants.

Introduction

Exposure to combustion-derived air pollutants has been linked to adverse cardiovascular health outcomes, especially in individuals with preexisting cardiac disease. Epidemiological studies suggest the involvement of multiple air pollutants, including fine and ultra-fine particulate matter ($PM_{2.5}$ and UFP, with diameters < 2.5 µm and < 0.1 µm, respectively), nitrogen dioxide (NO_2), carbon monoxide (CO), and sulfur dioxide (SO_2) (Brook *et al.*, 2004). Diesel engine exhaust (DE) is a major urban source of these pollutants, as well as volatile and semi-volatile organics, and carbonyls. Moreover, DE is an important contributor to vehicular emissions attendant to adverse clinical outcomes near roadways. For instance, ischemic heart disease hospitalizations in eight European cities have been attributed to DE exposure (Le Tertre, et al., 2002), and increases in mortality have been shown to parallel increased traffic particle levels (Maynard *et al.*, 2007). Clinical studies indicate that DE may impart toxicity by adversely altering cardiovascular function. Recent research has demonstrated that acute DE inhalation increases systolic blood pressure, impairs vasodilation, and/or enhances vasoconstriction in humans (Cosselman *et al.*, 2012; Mills, et al., 2011b). In addition, Mills *et al.* (2007) found that DE exposure increased exercise-induced electrocardiographic ST depression (indicative of myocardial ischemia or altered cardiac repolarization) in volunteers with known coronary heart disease and prior myocardial infarction.

Investigations into the adverse health effects of short-term air pollutant exposure indicate that individuals with heart failure are a particularly susceptible subgroup (Dominici *et al.*, 2006; Goldberg *et al.*, 2003; Pope *et al.*, 2008). For example, elevations in daily particulate matter (PM) concentrations have been associated with increased heart failure-related hospitalizations (Dominici *et al.*, 2006; Pope *et al.*, 2008) and mortality (Goldberg *et al.*, 2000), and rising PM_{10} levels increase the rate of new heart failure diagnoses and deaths in survivors of myocardial infarction (Zanobetti & Schwartz, 2007). The multiple biochemical and physiological responses demonstrated within susceptible subgroups such as heart failure patients highlight several candidate mechanisms of toxicity, including changes in autonomic balance, electrophysiological properties, vascular function, hemostasis and thrombosis, and systemic inflammation (Anselme, et al., 2007; Brook, 2008; Campen, et al., 2005; Lucking, et al., 2011; Mills, et al., 2007; Peretz, et al., 2008a). Yet, the mechanisms accounting for increased vulnerability of the failing heart to the effects of air pollution remain unclear.

Evidence demonstrating that DE inhalation can alter cardiac dimensions and performance (key indices of normal mechanical function) is limited. Yan *et al.* (2008) and Huang et al. (2010) found suggestions of such effects in rodents, but these studies were restricted to DE particles and used intra-tracheal instillation rather than inhalation. Others demonstrated in rats with post-infarct heart failure that DE-induced spontaneous arrhythmia was associated with decreased heart rate variability (HRV) (Anselme, et al., 2007), indicating possible autonomic mediation of effects. In contrast, DE exposure of younger healthier rats with minimal cardiac hypertrophy caused only modest changes in HRV and electrocardiography (Carll *et al.*, 2012), demonstrating the importance of modeling susceptibility in animal studies. To further elucidate the impact of short-term DE exposure on cardiac function in heart failure and its relation to

autonomic nervous regulation, we examined the effects of a single inhalation exposure to DE on cardiac performance, ventricular chamber dimension, arrhythmia, repolarization, and HRV measures of autonomic modulation, as well as pulmonary and systemic injury and inflammation in an aged rat model prone to heart failure.

Materials and Methods

Animals and radiotelemetry implantation. Lean male spontaneously hypertensive heart failure (SHHF) rats (*MccCrl-Lepr*^{cp}; n = 20) were shipped at 6 weeks of age from Charles River Laboratories, (Kingston, NY) to the U.S. EPA's AAALAC-approved animal facility, housed in pairs in 42 × 21 × 20-cm Plexiglas cages with pine-shave bedding in a room (22°C ±1°C, 50% ± 5% relative humidity, 12-h light:dark cycle 0600:1800 h), and provided standard Purina rat chow (5001; Brentwood, MO) and water *ad libitum*. At 15 months of age, 16 rats were implanted with radiotelemeters (model TA11CTA-F40; Data Sciences International, St. Paul, MN) for the purpose of recording ECG, heart rate (HR), core body temperature, and activity wirelessly as previously described (Lamb, et al., 2012). Lean male SHHFs acquire cardiac hypertrophy by 3 months of age and transition into dilated cardiomyopathy and heart failure at 18 months of age (Carll, et al., 2011b). All studies conformed to the guidelines of the US EPA Institutional Animal Care and Use Committee. Three weeks after surgery, rats were weighed, monitored by ECG, and omitted from telemetric monitoring if they differed from the mean body weight by more than 1 standard deviation (n=2) or if their cardiac electrograms were unsuitable for HRV analysis due to frequent arrhythmias (n=1) or a displaced lead (n=1). All rats were then habituated to 3 min of manual restraint on two consecutive days in preparation for echocardiographic assessments. All animals were assigned semi-blindly to one of two exposure

groups (clean filtered air, “Air”; or whole diesel exhaust, “DE”) while maintaining equivalent mean body weights per group.

Diesel exhaust exposure and generation. Rats were acclimated twice to exposure conditions within the clean filtered air chamber for 1 h on two separate days before exposure. On the exposure day, rats were allowed to acclimate to the chambers for 20 min and then baseline data were recorded for the next 30 min. Rats were then exposed to DE (target $PM_{2.5}$ of 500 $\mu g/m^3$) or Air for 4 h in whole body exposure chambers. Thereafter, DE exposures were stopped for a 30-min recovery period in which clean filtered air was circulated through exposure chambers. Rats were returned to home cages immediately thereafter. DE exposures were at UFP and NO_2 concentrations comparable to those found in traffic tunnels and roadways within the U.S. and Europe (Anselme, et al., 2007; Svartengren, et al., 2000; Zhu, et al., 2007). DE 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 on low sulfur diesel fuel (32ppm) at a constant load of 3 kW as previously described (Carll, et al., 2012). From the engine, the exhaust was mixed with clean air previously passed through high-efficiency particulate air (HEPA) filters. Air dilution of DE was adjusted periodically to maintain target $PM_{2.5}$ mass concentration. The diluted DE was delivered to a Hazelton 1000 (984 L) exposure chamber. Control animals were placed in a second chamber supplied with the same HEPA-filtered room air as that used to dilute DE. The chambers were operated at the same temperature, pressure and flow rate (424 L/min; approximating 25 air exchanges per hour). Chamber concentrations of PM, O_2, CO, NO, NO_2, and SO_2 were measured as previously described (Carll et al., 2012). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges (< 80 dB, 30-70%, and 73° ± 5° F).

Left ventricular (LV) echocardiography. One hour after the second exposure acclimation (1 day before DE or Air exposure), rats were held supine at 45° for no more than 3 minutes while being measured for baseline LV function and dimensions by trans-thoracic echocardiography (Nemio 30, Toshiba; Duluth, GA) using a 14-MHz linear array transducer (PLM 1204AT). Measures were repeated on the day of inhalation exposure, 1.5-2 h after cessation of DE exposure (1-1.5 h after removal from exposure chambers). Handling, measurements, and data analyses were performed in random order with laboratory personnel blinded to treatment groups. Two-dimensional long-axis images of the LV were obtained in parasternal long- and short-axis views with M-mode recordings at the mid-ventricular level in both views. At least three consecutive LV contraction cycles were used to determine heart HR, fractional shortening (FS), ejection fraction (EF), cardiac output, stroke volume (SV), internal LV diameter at the ends of diastole (EDD) and systole (ESD), and thickness of the posterior wall and the inter-ventricular septum (IVS). End diastolic and end systolic volumes (EDV & ESV) were determined using the area-length method as validated previously (Joho *et al.*, 2007). LV dimensions were used to calculate FS ([EDD-ESD]/EDD), SV (EDV-ESV), and EF (SV/EDV).

Radiotelemetry data acquisition and analysis. Radiotelemetry was used to track changes in cardiovascular and thermoregulatory function by continuously monitoring ECG, core body temperature, and activity in awake, unrestrained rats beginning at 3 days before inhalation exposure and continuing through exposure until euthanasia 24 h after exposure. Data were obtained as described previously (Carll, et al., 2012) and ECG waveforms were sampled at a rate of 1,000 Hz in 2-min streams every 10 min within home cages, and 1-min streams every 5 min within exposure chambers (DataART 3.01; Data Sciences International, Inc.). ECG waveforms were analyzed with computer software (ECGauto 2.5.1.35; EMKA Technologies, Falls Church,

VA) that enabled user identification and exclusion of arrhythmias and artifacts from automated analysis of HRV and ECG morphology parameters as previously detailed (Carll, et al., 2012). ECG morphology parameters were based on P, Q, R, S, and T waves and included the following: intervals of PR, QRS, ST, QT, QTe (onset of Q to end of T), QTc (heart-rate corrected QT, using Fridericia correction), T-peak to T-end (Tp-Te), and amplitudes of Q, R, T-peak, and ST (mean amplitude between S and T-peak), relative to the iso-electric line (15 ms before Q). ST area was calculated as total negative area underneath the isoelectric line from S-nadir to T-peak.

HRV analysis generated HR and time-domain measures, including mean time between adjacent QRS-complex peaks (RR interval), standard deviation of the RR interval (SDNN), square root of the mean of squared differences of adjacent RR intervals (RMSSD), triangular index, and percent of adjacent normal RR intervals differing by ≥15 ms (pNN15). pNN15 is a measure of parasympathetic tone. SDNN and triangular index represent overall HRV, whereas RMSSD represents parasympathetic influence over HR (Rowan, et al., 2007). HRV analysis also provided frequency-domain parameters, including low frequency (LF: 0.200-0.750 Hz) and high frequency (HF: 0.75-2.00 Hz), and the ratio of these two frequency-domains (LF/HF). For frequency-domain analysis, the signal was analyzed with a Hanning window for segment lengths of 512 samples with 50% overlapping. LF is generally believed to represent a combination of sympathetic and parasympathetic tone, whereas HF indicates cardiac vagal (parasympathetic) tone, and LF/HF serves as an index of sympathovagal balance (Rowan, et al., 2007).

Arrhythmias were identified while blinded to treatment group according to previously described criteria (Carll, et al., 2012). To facilitate statistical analysis of each arrhythmia type and allow the data to converge under the Poisson distribution, zero-values for each arrhythmia type within a sample interval were converted to 0.1. Arrhythmia frequencies were calculated

over specific periods in home cages (pre-exposure and post-exposure, 7 hours each) as well as in exposure chamber (baseline, mid-exposure, recovery), normalized to adjust for time differences between periods and gaps in data, and presented as number of events per-hour of theoretically continuous ECG waveforms. Each premature beat was counted individually as a single event (e.g., 1 bigeminy = 2 ventricular premature beat [VPB] events), whereas atrioventricular (AV) or sino-atrial block arrhythmias were counted as one event regardless of duration or neighboring events.

HRV and ECG morphologic analyses were conducted on ECG waveforms collected while rats resided in home cages at 3 days pre-exposure and immediately post-exposure (both 1pm-8pm), which were time-matched to control for physiologic effects of circadian rhythm. The post-exposure period of 1:30pm-2:30pm was excluded from HRV and arrhythmia analyses to allow animals to recover from handling during echocardiographic measurements. To adjust for this gap in sampling, data collected at 1:00pm-1:30pm and 2:30-3:00pm were used to represent hour 1 and hour 2 post-exposure, respectively. ECG data collected within the exposure chamber (5 h total) was also analyzed according to the following periods: baseline (7:50am-8:20am), exposure (8:20am-12:20pm), and recovery (12:20pm-12:50pm). All ECG streams with less than 10 seconds of identifiable conduction cycles were excluded from ECG parameter calculation and streams with less than 30 seconds of identifiable RR intervals were excluded from HRV analysis. Thorough visual inspection was conducted to identify and exclude arrhythmias and artifacts.

Tissue collection and analysis. At approximately 24 hours after onset of the 4-hour inhalation exposure, rats were deeply anesthetized with an intraperitoneal injection of a sodium pentobarbital / phenytoin solution. Tissue samples of blood, lung lavage fluid, heart, and lungs, were collected, processed, and analyzed as previously described (Carll, et al., 2012). Heart

weight was normalized by right tibia length. To examine for indications of cardiopulmonary inflammation, injury, oxidative stress, and risk, multiple biochemical markers were assayed. Lavage supernatants were analyzed for albumin, gamma-glutamyl transferase, lactate dehydrogenase, N-acetyl-b-d-glucosaminidase (NAG), total antioxidant status, and total protein (Carll, et al., 2010). Serum supernatants were analyzed for creatine kinase, C-reactive protein (CRP), total protein, and glutathione peroxidase, reductase, and -S-transferase; and supernatants from plasma were assayed for angiotensin converting enzyme (ACE), albumin, blood urea nitrogen, creatinine, and total protein (Carll, et al., 2011a). Serum was also analyzed for α-hydroxybutyrate dehydrogenase, D-dimer, ferritin, glucose, insulin, lipoprotein (a), total cholesterol, triglycerides, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase-1, lipase, high and low density lipoprotein cholesterol, myoglobin, sorbitol dehydrogenase, superoxide dismutase (SOD), manganese SOD, and copper-zinc SOD according to previous procedures (Carll, et al., 2012; Carll, et al., 2010).

Statistics. The statistical analyses for all data in this study were performed using Prism version 4.03 (GraphPad Software, Inc., San Diego, CA). One-way analysis of variance (ANOVA) with Tukey post-hoc test was used to detect significant differences between groups in biochemical endpoints and tissue weight. Repeated measures two-way ANOVA with Bonferroni post-hoc test was performed on (I) arrhythmia frequency data, which were collected at pre-, mid-, and post-exposure periods (spanning approximately 4 hours each) and normalized by sampling duration; (II) HRV and ECG morphology parameters during the exposure period, including exposure hours 1-4, baseline (30 min), and recovery periods (30 min); and (III) HRV and ECG morphology to analyze for intra-group differences between time-matched periods (separated by exactly 24 hours) collected on the exposure day and on the previous day. Two-way ANOVA

with Bonferroni post-test was also used to analyze for inter-group differences in change in HRV and ECG parameters at post-exposure relative to the day before exposure. $P < 0.05$ was considered statistically significant. Linear regressions were performed to test for correlations between various physiologic endpoints.

Results

Physiological Responses to Exposure by Inhalation.

Heart rate, heart rate variability, and ECG morphology. At pre-exposure and baseline no significant differences emerged between the groups for HR, HRV parameters, or ECG morphology (Table 3.2). All rats in each group were active at the beginning of the exposure and became inactive during exposure to Air or DE. As expected with decreased activity, HR decreased for both the Air and DE groups over the exposure period (Figure 3.1). Also, HR increased for both groups during baseline measurements after transfer of animals to the exposure chamber (Table 3.2). Only DE exposure altered HRV parameters significantly during exposure relative to baseline. SDNN, RMSSD, triangular index, LF, HF, and pNN15 increased at hours 3 and 4, and recovery, consistent with parasympathetic activation ($P < 0.05$). The groups did not differ from each other in HR at any time during exposure; yet, DE exposure increased pNN15 (at hour 4) and triangular index (at recovery) relative to the Air group (all $P < 0.05$). Exposure to DE did not affect any measures of ECG morphology relative to baseline or the Air group during the exposure period.

Cardiac arrhythmia. During exposure, the DE and Air groups did not differ from each other in their rates of second-degree AV block events or premature beats, including VPBs ($P =$

NS). The rate of VPBs increased during mid-exposure to Air when compared to pre-exposure, baseline, and recovery (Supplemental Figure 3.1, $P < 0.05$).

Physiological Responses after Exposure by Inhalation

Heart rate and heart rate variability. There were notable differences in HRV over the first four hours after animals were returned to home cages after inhalation exposure. The DE group exceeded the Air group in change in triangular index and LF from pre-exposure (Figure 3.2; all $P < 0.05$). Change in SDNN was also higher in the DE group relative to Air during hours 1-4 of post-exposure (Figure 3.2; $P = 0.06$).

ECG morphology. Relative to air exposure, DE caused several differences in ECG morphology over the first four hours of post-exposure (in home cages). The DE group exceeded the Air group in change from pre-exposure ST area, T amplitude, and Tp-Te (Figure 3.2; all $P < 0.05$). DE also exceeded Air in ST amplitude change (mean ±SE—2.0 ±7μV Air vs. 24.2 ±7μV DE; $P = 0.04$). These alterations in T-wave and S-wave area, amplitude, and duration (Figure 3.3) indicate that DE altered repolarization. No other aspects of ECG morphology were affected by DE in the hours following exposure. All four measures of ventricular repolarization at post-exposure (ST amplitude, ST area, T amplitude and Tp-Te) positively correlated with mid-exposure HRV (Table 3.3; all $P < 0.05$), indicating a possible relationship between changes in repolarization and preceding autonomic imbalance. Post-exposure triangular index also correlated with post-exposure Tp-Te ($P = 0.03$), and had a near-significant correlation with ST-amplitude (Supplemental Figure 3.2; $P = 0.07$).

Cardiac Arrhythmia. DE exposure increased the rate of Mobitz type II second-degree AV block events over the first 4 hours after removal of animals from exposure chambers. The DE group's rate of Mobitz II AV block events was increased relative to itself at all prior periods,

as well as relative to the Air group at post-exposure (Figure 3.4; $P < 0.05$). Among the eight individual Mobitz II AV block events observed at post-exposure in the DE-exposed group, four happened among four rats within 1 h of return to home cages (1.5 h post-exposure), and the remaining half occurred in a single rat 4 h into the post-exposure period within home cages. Because most DE-exposed rats (4 of 6) had an AV block arrhythmia at hour 1 of post-exposure, we looked at this time point for associations with changes in HRV and ECG morphology. At hour 1 of post-exposure, AV block events among all rats positively correlated with change in RMSSD and SDNN, whereas AV block negatively correlated with heart rate (Supplemental Table 3.1; $P < 0.05$).

Echocardiography. At pre-exposure LV systolic function in both groups was normal as indicated by mean ejection fraction (90%) and fractional shortening (54%). DE exposure altered several measures of LV diameter, volume, and wall thickness concomitant with an increase in cardiac output (Figure 3.5). At post-exposure, end-diastolic and end-systolic volumes (EDV and ESV) increased in the DE group relative to pre-exposure (178 µl and 34 µl, respectively), leading to an increase in stroke volume (144 µl) and cardiac output (39.4 ml/min) (Figure 3.5, $P < 0.05$). These increases in LV volumes and output corresponded with LV wall thinning, including decreased posterior wall thickness at diastole (-13%) and systole (-10%) and decreased interventricular septal thickness at diastole (-11%) and systole (-10%) relative to pre-exposure (all $P < 0.05$). At post-exposure, DE also increased stroke volume, cardiac output (body weight-normalized and raw), and EDV while decreasing posterior wall thickness relative to Air (all $P < 0.05$). DE did not affect HR during echocardiographic measures (332 ± 14 bpm Air vs. 339 ± 7 bpm DE; P = NS), but both groups decreased in HR relative to their own pre-exposure values

(Figure 3.5, $P < 0.05$). All of the cardiac parameters that were affected by DE exposure also correlated with changes in HRV during the exposure period (Table 3.4, $P < 0.05$).

Pulmonary and Systemic Markers of Inflammation and Injury

The air-exposed aged SHHF rats in this study had cardiac hypertrophy relative to 10-week-old SHHF, 19-week-old WKY, and 15-month-old WKY rats (Figure 3.6; $P < 0.05$). Exposure to DE did not alter cardiac or lung weights (Supplemental Table 3.2). DE exposure increased pulmonary eosinophils in aged SHHF rats (Supplemental Table 3.2, $P < 0.05$). Relative to the Air group, the DE group increased serum CRP (+6.5%; $P = 0.01$) and plasma total protein (+7%; $P = 0.05$). Decreases in NAG and lipase of uncertain significance were evident in the DE group relative to Air control ($P < 0.05$). There were no other significant changes in circulating or pulmonary biochemical or cellular endpoints.

Discussion

In the present study, a single four-hour exposure to diesel exhaust (DE) by inhalation altered multiple cardiac endpoints in aged heart failure prone rats with cardiac hypertrophy but without overt signs or symptoms of heart failure. Principal among these was DE-induced LV dilation, which may correspond with myocardial stretch and attendant electrophysiologic effects (Franz *et al.*, 1992) and changes in cardiac repolarization. This effect may bear particular implications for the mechanisms underlying air pollutant-induced hospitalizations for heart failure. In the 16-month-old SHHFs of our current study, the absence of congestive symptoms at baseline, maintenance of normal LV ejection fraction (90%) and fractional shortening (relative to (Tamura, et al., 1999)), and elevated cardiac weight, indicate a state of compensated LV hypertrophy prior to exposure. Fractional shortening at baseline (FS: 53%) and dimensional

changes after DE exposure compared closely to conscious measurements in terminally senescent mice exposed to carbon black particles (Tankersley, et al., 2008). In these mice, a 4-day inhalation exposure ($PM_{2.5}$ = 400 $\mu g/m^3$) caused LV dilation and evidence of myocardial stretch (increased gene expression of natriuretic peptides), while decreasing FS and activating proteases responsible for myocardial remodeling. Similarly, others have demonstrated decreased contractility accompanied by increased LV volume and pressure after a single, high, 1-2 mg/kg intra-tracheal dose of DE particles (Huang et al., 2010; Yan et al., 2008). Importantly, LV dilation can increase filling pressures and wall stress (Tkacova *et al.*, 1997), which promotes parasympathetic reflexes (Wang *et al.*, 1995), cardiac arrhythmia (Huang *et al.*, 2009), and signaling pathways for LV remodeling (Force *et al.*, 2002). Given our observations, it seems increasingly plausible that repeat exposures could cause LV remodeling.

The mechanisms that underlie DE-induced increases in cardiac output remain uncertain but may involve alterations in venous tone. The increase in SV and LV volume concurrent with normal HR, EF, and FS indicate that DE increased ventricular preload. Accordingly, Knuckles and colleagues (2008) previously found that DE enhances endothelin-1-induced constriction of veins (but not arterioles) through uncoupling of nitric oxide synthase (NOS). Tankersley *et al.* (2008) found in restrained, conscious mice that PM-induced LV dilation was mediated by NOS uncoupling. Importantly, physical restraint causes acute stress, which stimulates endothelin-1 release (Treiber *et al.*, 2000) and could trigger DE-enhanced venoconstriction. Although we found no effects of DE on ACE, the observations of others (Ghelfi *et al.*, 2010) implicate the renin-angiotensin system (RAS) as a mediator of DE-induced increases in cardiac output. Our findings merit further investigation to determine their mechanistic origins.

DE exposure in aged hypertrophic SHHFs within the current study led to robust increases

in HRV both during and after exposure. These results are in contrast to our recent findings in similarly exposed young adult SHHF, normotensive Wistar Kyoto (WKY), and hypertensive (SH) rats (Carll et al., 2012; Lamb et al, 2012), in which there was little evidence of HRV effects. Although this is our strongest evidence to date that DE exposure can cause a relative parasympathetic dominance over cardiac function, we have seen similar HRV effects with ozone inhalation (Farraj *et al.*, 2012). Likewise, Tankersley et al. (2004) saw marked increases in HRV with repeated (4-day) inhalation exposure to carbon particles in terminally senescent mice. Previous studies by us and others have shown exposure to DE or DE particles causes pulmonary irritant receptor activation (Deering-Rice, et al., 2011; Hazari, et al., 2011; Wong, et al., 2003), which is known to cause parasympathetic reflexes (Widdicombe & Lee, 2001). The multiple effects of whole DE on arrhythmia and HRV in the aged SHHF rat vs. the minimal effects of this same exposure on young SHHF rats (Carll et al., 2012) supports epidemiologic evidence that age and progression of cardiac disease heighten susceptibility to air pollutant exposure (Brook *et al*., 2004). Moreover, these effects indicate that parasympathetic reflexes may factor into this susceptibility. Increased parasympathetic neural input to the heart can provoke second-degree AV block (Drici *et al.*, 2000; Massie, et al., 1978). We found correlations between post-exposure second-degree AV block events and changes in HRV (Supplemental Table 3.1) that correspond with previous demonstrations of parasympathetic-mediated AV block (Castellanos, et al., 1974; Drici, et al., 2000; Massie, et al., 1978). Beyond this effect, the clinical health implications of enhanced parasympathetic tone in response to DE exposure remain uncertain and unexplored.

The effects of DE that we observed on autonomic balance, bradyarrhythmia due to AV block, ventricular repolarization and LV dilation may interrelate (Figure 3.7). Changes in HRV

and LV volume correlated, consistent with the known link between parasympathetic excitation and the activation of myocardial stretch receptors (Crawford, 2003). Parasympathetic activation provokes a K^+ channel (I_{KACh}) that augments repolarizing currents, decreases spontaneous depolarization, increases HRV, and promotes AV block (Drici, et al., 2000; Moreno-Galindo *et al.*, 2011). Likewise, the changes in spatiotemporal heterogeneity of repolarization that we observed may have resulted from several factors, including myocardial stretch (Tan *et al.*, 2004; Xian Tao *et al.*, 2006), autonomic mechanisms (Drici, et al., 2000; Kanda *et al.*, 2011; Moreno-Galindo, et al., 2011), inflammation (Zhang *et al.*, 2011) or changes in heart rate, electrolyte balance, and metabolism (Channer & Morris, 2002). Ultimately, our findings of altered repolarization, increased HRV, LV dilation, and AV block collectively suggest an important role for parasympathetic mechanisms in the adverse cardiophysiologic effects of DE.

Our findings of DE-induced ECG changes correspond with prior observations and bear notable implications for the health effects of DE exposure. Rich and colleagues (2012) recently found in patients with prior coronary events that Tp-Te (a measure of the heterogeneity of transmural depolarization (Castro Hevia *et al.*, 2006)]) increased with exposure to fine mode particles. We similarly observed an increase in Tp-Te in the first 4 hours after DE exposure, suggesting that DE may desynchronize repolarization between the different regions or cell types of the ventricular myocardium. Tp-Te prolongation has been demonstrated to correlate with post-infarct LV remodeling (Szydlo *et al.*, 2010) as well as ventricular tachycardia and sudden cardiac death in patients with hypertrophic cardiomyopathy (Shimizu *et al.*, 2002). Similarly, the effects of DE on ST-area and ST- and T-wave amplitudes were not unprecedented. Both elevation and depression of the ST-interval and T-wave may indicate myocardial ischemia (Channer & Morris, 2002). Several researchers have reported decreased T-wave or ST-

amplitudes with air pollutant exposure. Such findings occurred with exposure to ambient particles in ischemic heart disease patients (Henneberger, et al., 2005), inhalation of DE in exercised coronary artery disease patients (Mills *et al.*, 2007) and sedentary atherosclerotic mice (Campen *et al.*, 2005), and inhalation of particle-free DE gases in young adult SH and SHHF rats (Lamb *et al.*, 2012; Carll *et al.*, 2012). While these changes in repolarization may result from ischemia, they may also stem from alterations in transmembane K^+ balance by myocardial stretch and parasympathetic activation as previously mentioned.

Unlike our previous findings in young-adult WKY, SH, and SHHF rats (Carll et al., 2012; Lamb et al., 2012), DE increased markers of cardiopulmonary inflammation in aged SHHF rats suggesting that age and advancement toward heart failure mediate enhanced proclivity to these effects. The DE-induced increased in serum CRP is consistent with the findings of Rich and associates (2012), who along with the aforementioned observations of Tp-Te prolongation, recently noted positive correlations between CRP and particle concentrations within the preceding 2-4 days. The increase in pulmonary eosinophils was unexpected; however, similar effects of DE have been observed in healthy humans (Ghio *et al.*, 2012). The relationship between these findings and the changes in cardiac physiology require further study.

In summary, a single inhalation exposure to DE in aged heart failure-prone rats without evidence of heart failure caused LV dilation and changes in cardiac repolarization. In light of our prior study (Carll et al., 2012), our findings demonstrate that age in a heart failure-prone rat strain confers overt susceptibility to the effects of air pollutant exposure on the occurrence of bradyarrhythmia, repolarization, and HRV. Most of the observed physiologic changes correlated with increased HRV markers of parasympathetic influence, suggesting autonomic modulation played an important role in the observations. The mechanism by which increased

parasympathetic influence may relate to such effects requires further study. Taken together, these findings may provide new insight on the health effects of traffic related air pollutants.

Tables

Table 3.1. Inhalation Exposure Characterization

	Air	DE
$PM_{2.5}$ ($\mu g/m^3$)	21	515
$PM_{2.5}$ number (n/cm^3)	$9.7x10^3$	$2.3x10^6$
Number median diameter of PM (nm)	96	58
Volume median diameter of PM (nm)	184	89
O_2 (%)	20.7 ±0.0	20.2 ±0.0
CO (ppm)	0.0 ±0.1	16.6 ±1.4
NO (ppm)	0.06 ±0.00	15.9 ±1.2
NO_2 (ppm)	0.07 ±0.00	0.66 ±0.09
SO_2 (ppm)	*BDL*	*BDL*

Data represent mean values ± standard deviation (SD) generated from measurements made either continuously (concentrations of O_2, CO, NO, and NO_2), once ($PM_{2.5}$ mass concentration), or six times (DE $PM_{2.5}$ number) per exposure. Number median diameter was based on exposure day particle size distributions ± SD. Volume diameter was calculated from number-based mobility diameters and assumed spherical particles. Air indicates filtered air; DE, diesel exhaust; $PM_{2.5}$, fine particulate matter; BDL, below detectable limit.

Table 3.2. Heart rate variability and ECG morphology parameters prior to exposure to diesel exhaust (DE).

	Pre-Exposure (home cages)		Baseline (exposure chamber)	
	Air	DE	Air	DE
HRV	mean (SE)	mean (SE)	mean (SE)	mean (SE)
HR (beats/min)	277 (7.5)	282 (5)	355 (13)	334 (6)
SDNN (msec)	10.5 (0.7)	9.9 (0.9)	8.1 (0.5)	8.0 (0.6)
RMSSD (msec)	5.7 (0.9)	5.7 (1.4)	3.8 (0.8)	4.1 (1.1)
Tri. Index	1.25 (0.04)	1.18 (0.04)	1.20 (0.08)	1.20 (0.05)
LF/HF	0.92 (0.15)	1.15 (0.32)	1.42 (0.33)	1.17 (0.11)
LF ($msec^2$)	1.64 (0.58)	2.35 (0.74)	0.66 (0.16)	0.68 (0.21)
HF ($msec^2$)	2.30 (0.75)	2.8 (1.3)	0.78 (0.34)	0.93 (0.47)
pNN15 (%)	5.7 (0.9)	5.7 (1.4)	1.6 (1.5)	2.3 (2.2)
ECG morphology				
PR (msec)	61.0 (1.8)	59.7 (2.3)	57.7 (1.9)	56.3 (1.7)
T amplitude (mV)	0.058 (0.011)	0.063 (0.089)	0.074 (0.021)	0.100 (0.013)
QTc (msec)	80.1 (3.3)	78.3 (0.8)	83.3 (2.1)	80.9 (2.0)
ST area (mV*msec)	-0.92 (0.4)	-0.93 (0.19)	-0.55 (0.45)	-0.72 (0.18)
Tp-Te (msec)	28.9 (3.1)	27.7 (1.7)	30.3 (4.1)	30.3 (2.0)

Pre-exposure values represent an average over 4 hours time-matched with the first 4 h of post-exposure within home cages. Baseline values are averages from ECGs collected 30 min to 1 h after placement in exposure chamber but before initiating DE generation. QTc: Fridericia-corrected QT interval. N = 5-6/group.

Table 3.3. Correlations between mid-exposure change in HRV and post-exposure change in ECG measures of ventricular repolarization. ECG values were limited to hours 1-4 of post-exposure.

HRV parameter	Exposure hour	ECG parameter	Post-expo. hour	r	P-value
SDNN	3	Tp-Te	1	0.70	0.03
	Recovery	Tp-Te	2	0.66	0.04
		ST area	2	0.62	0.06
Triangular Index	4	Tp-Te	1-4	0.67	0.03
		ST amplitude	1-4	0.60	0.06
		Tp-Te	4	0.74	0.01
		T amplitude	4	0.69	0.03
		ST amplitude	4	0.66	0.04
	Recovery	T amplitude	1-4	0.63	0.05
		Tp-Te	1-4	0.61	0.06
		T amplitude	2	0.67	0.03
		ST area	4	0.66	0.04
		Tp-Te	4	0.62	0.05

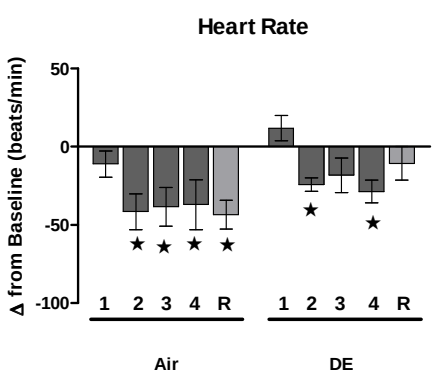
Change in HRV during individual hours of exposure (relative to baseline) significantly correlated with a subsequent change in ECG during hours 1-4 of post-exposure. "r" indicates Pearson correlation coefficient. "Post-expo." indicates post-exposure, when animals were monitored in home cages. N = 5-6/group.

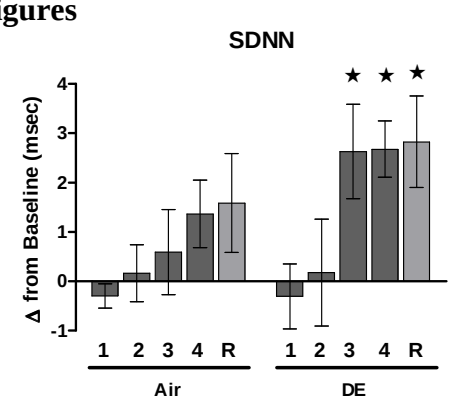
Table 3.4. Correlations between mid-exposure change in HRV and post-exposure change in left ventricular function and dimensions.

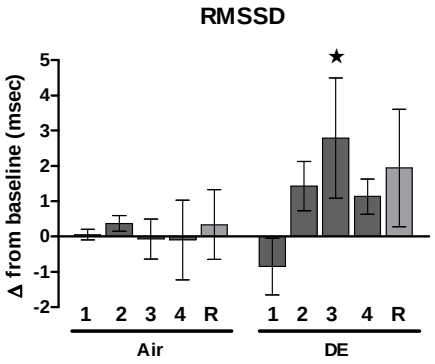
Echo Parameter	HRV parameter	Exposure hour	r	P-value
PW (D)	RMSSD	3	-0.60	0.05
		4	-0.82	< 0.01
		R	-0.64	0.04
	SDNN	3	-0.76	< 0.01
		4	-0.74	< 0.01
	pNN15	4	-0.65	0.04
PW (S)	SDNN	3	-0.71	0.02
		4	-0.76	< 0.01
	RMSSD	4	-0.82	< 0.01
EDV	RMSSD	3	0.74	< 0.01
	HF	3	0.63	0.04
	pNN15	4	0.84	< 0.01
ESV	pNN15	4	0.73	0.02
SV	RMSSD	3	0.77	< 0.01
	pNN15	4	0.71	0.02
	HF	3	0.70	0.02
	HF	R	0.66	< 0.01
Cardiac output	RMSSD	3	0.65	0.03
	pNN15	4	0.65	0.04

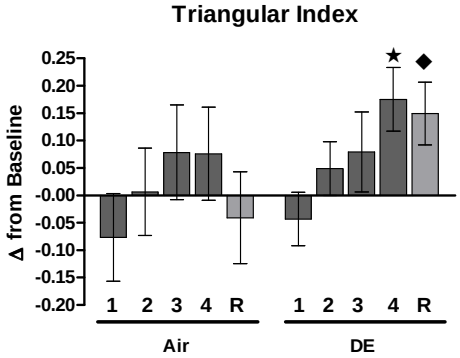
Changes in echocardiographic measures after the exposure period correlated with changes in HRV during exposure. Change was calculated as difference from baseline. N = 6/group. 3, 4, and R represent the third and fourth hour of exposure and the recovery hour immediately following inside exposure chambers. "r" indicates Pearson correlation coefficient. PW (D) and PW (S), posterior wall thickness at end diastole and end systole, respectively; EDV, end diastolic volume; ESV, end systolic volume; SV, stroke volume.

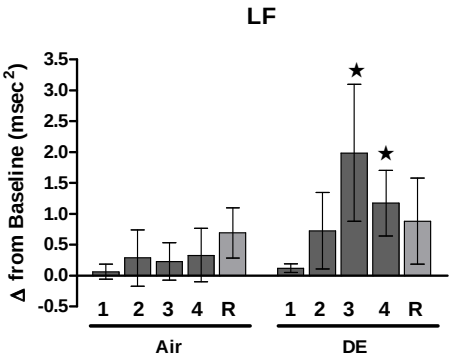
Figures

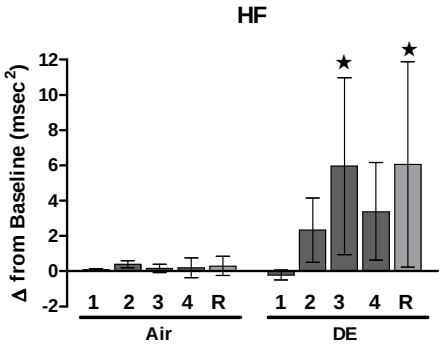

Heart Rate
Δ from Baseline (beats/min)
50
0
-50
-100
1 2 3 4 R 1 2 3 4 R
Air
DE

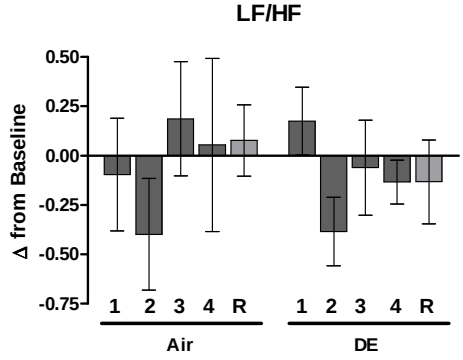

SDNN
Δ from Baseline (msec)
4
3
2
1
0
-1
1 2 3 4 R 1 2 3 4 R
Air
DE


RMSSD
Δ from baseline (msec)
5
4
3
2
1
0
-1
-2
1 2 3 4 R 1 2 3 4 R
Air
DE


Triangular Index
Δ from Baseline
0.25
0.20
0.15
0.10
0.05
-0.00
-0.05
-0.10
-0.15
-0.20
1 2 3 4 R 1 2 3 4 R
Air
DE


LF
Δ from Baseline (msec2)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
1 2 3 4 R 1 2 3 4 R
Air
DE


HF
Δ from Baseline (msec2)
12
10
8
6
4
2
0
-2
1 2 3 4 R 1 2 3 4 R
Air
DE


LF/HF
Δ from Baseline
0.50
0.25
0.00
-0.25
-0.50
-0.75
1 2 3 4 R 1 2 3 4 R
Air
DE

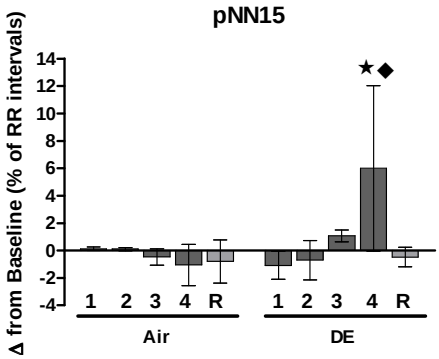

pNN15
Δ from Baseline (% of RR intervals)
14
12
10
8
6
4
2
0
-2
-4
1 2 3 4 R 1 2 3 4 R
Air
DE

Figure 3.1. Change from baseline in heart rate and HRV endpoints (mean +/- SE) during whole body exposure. 1, 2, 3, 4, and R represent hours 1 through 4 of exposure and Recovery (post-exposure) within the chamber. All measurements were taken from un-restrained conscious rats temporarily housed within exposure chambers. Stars and diamonds mark significant differences from baseline (in chambers) and the Air group (at the same hour), respectively ($P < 0.05$). See Table 3.2 for baseline values. N = 6/group.

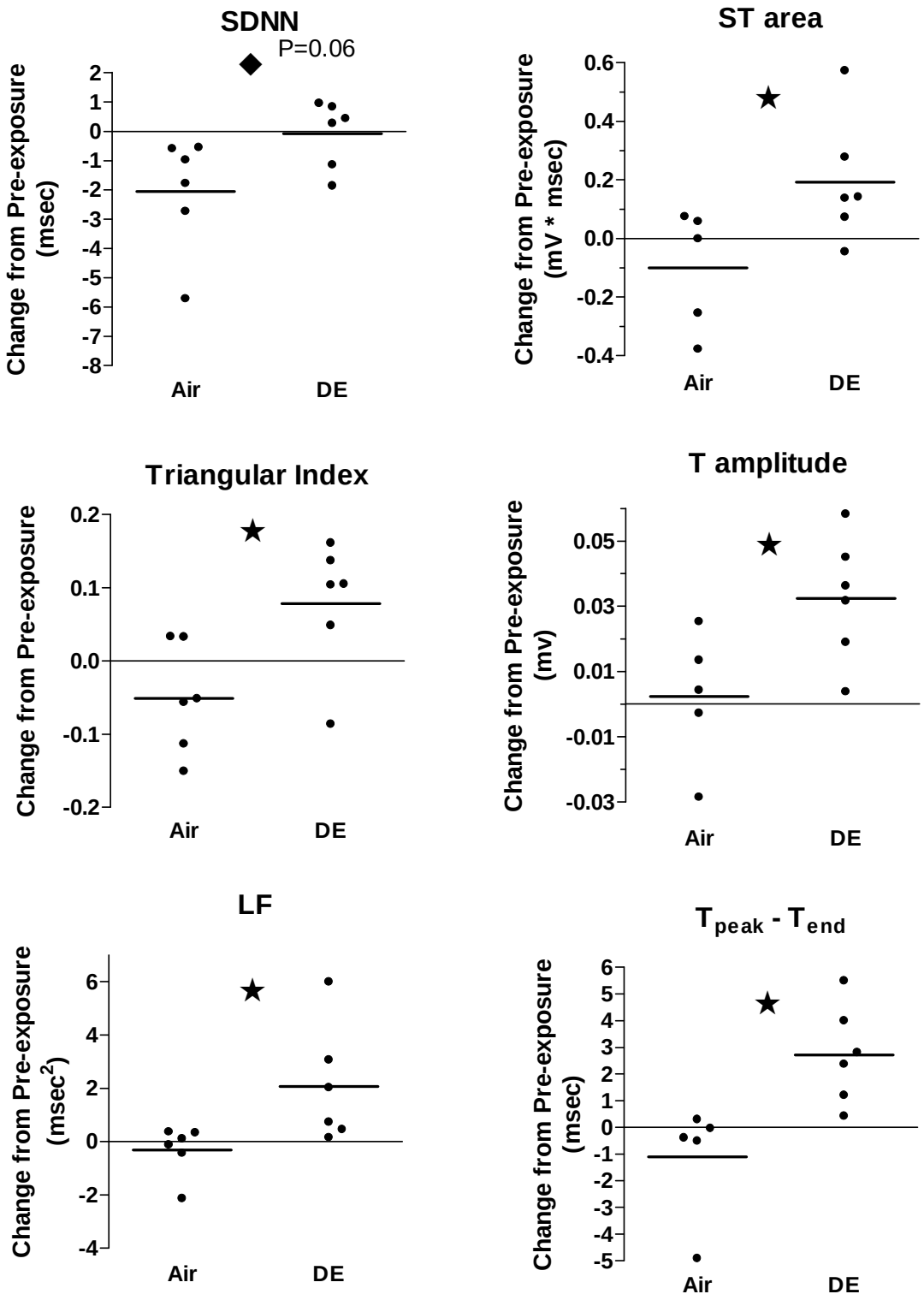


Figure 3.2. Change in HRV (left column) and ECG (right column) over the first 4 hours after removal from exposure chambers. Data were collected within home cages at hours 1-4 of post-exposure, excluding the first 30 minutes after echocardiography. Stars and diamonds indicate P < 0.05 and P < .10, respectively. N = 5-6 / group.

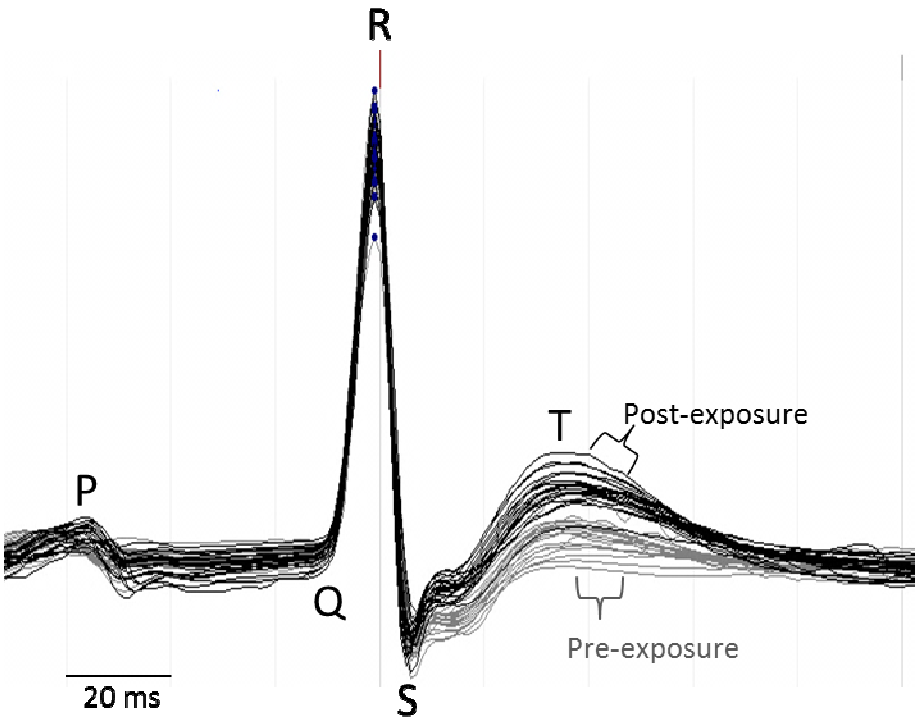


Figure 3.3. ECG waveforms before (gray) and after (black) DE exposure in a single rat. Each waveform represents the average cardiac electrogram from a 2-min ECG sampled at 10-min intervals. Data were collected from an individual rat within its home cage at hours 1-4 of post-exposure, excluding the first 30 minutes after echocardiography. Horizontal time mark indicates 20 ms interval.

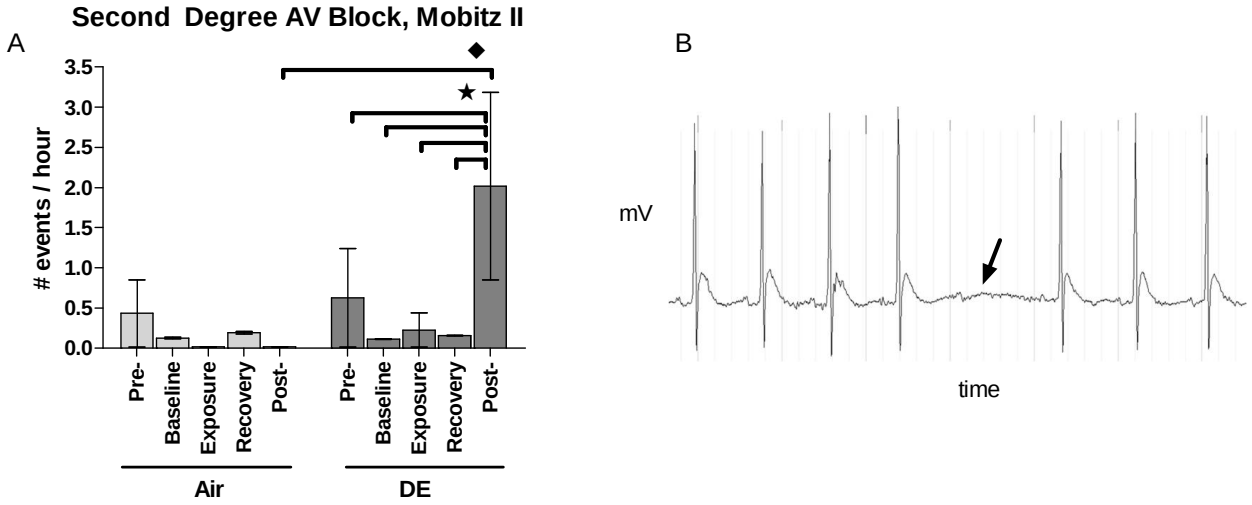


Figure 3.4. Diesel exhaust increased hourly rate of Mobitz II AV block per rat (mean +/- SE) at post-exposure (A). ECG waveform with representative second degree AV block Mobitz II arrhythmia (arrow) following DE exposure (B). Baseline, Exposure, and Recovery were all measured within exposure chambers. Stars and diamonds indicate significant differences between periods and groups, respectively ($P < 0.05$). Vertical grey lines behind ECG waveform indicate time in 50 msec intervals. N = 6/group.

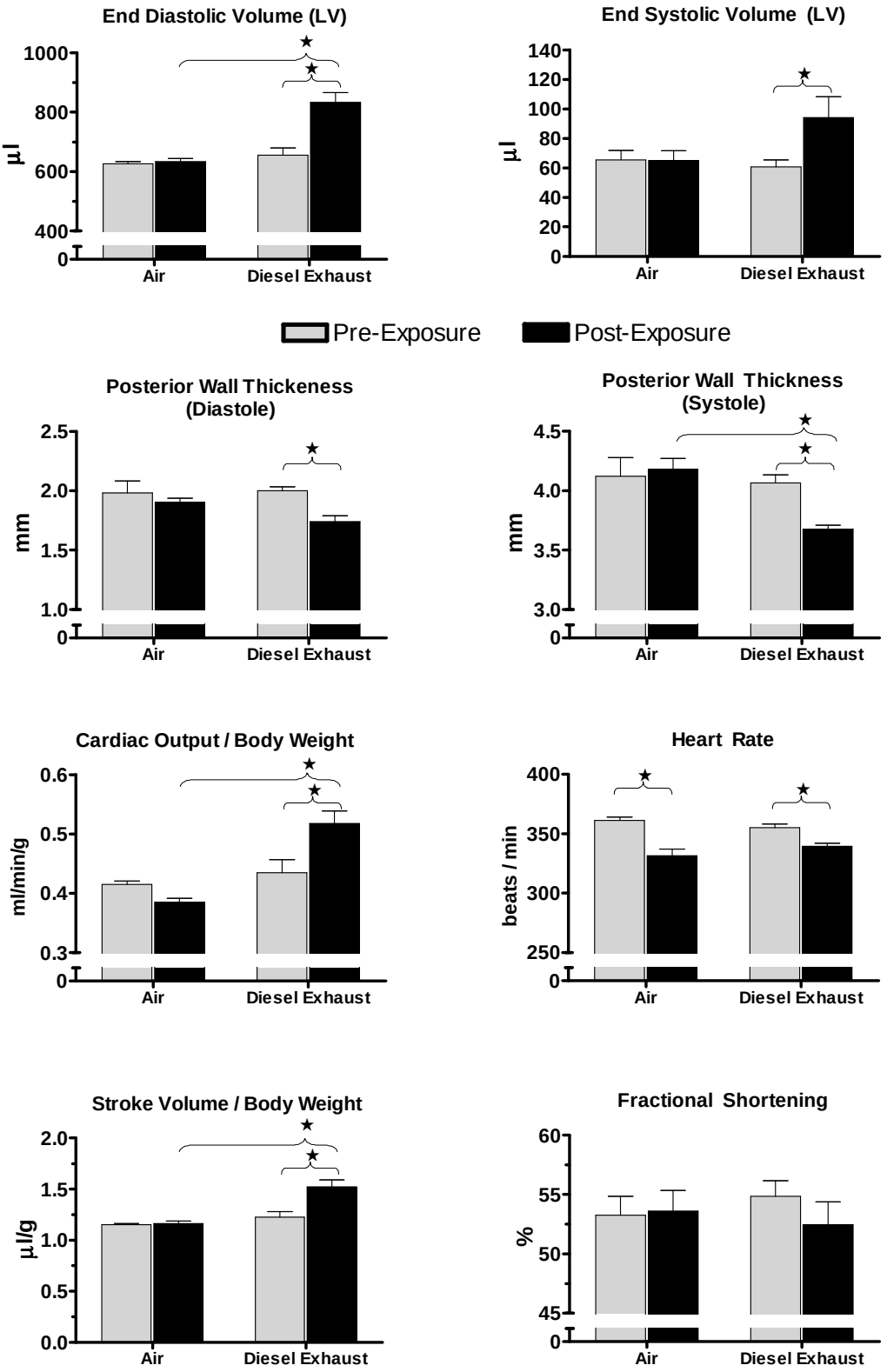


Figure 3.5. A single 4-hour exposure to diesel exhaust (500 µg/m^3) increased left ventricular (LV) chamber volume, stroke volume, and cardiac output, and decreased LV wall thickness in aged SHHF rats. The groups did not differ from each other in heart rate before or after exposure.

Echocardiographic measures of LV volume, thickness, and function were performed on conscious rats before and after exposure to filtered air or diesel exhaust. Stars and brackets indicate significant differences ($P < 0.05$). N = 6 to 8 / group.

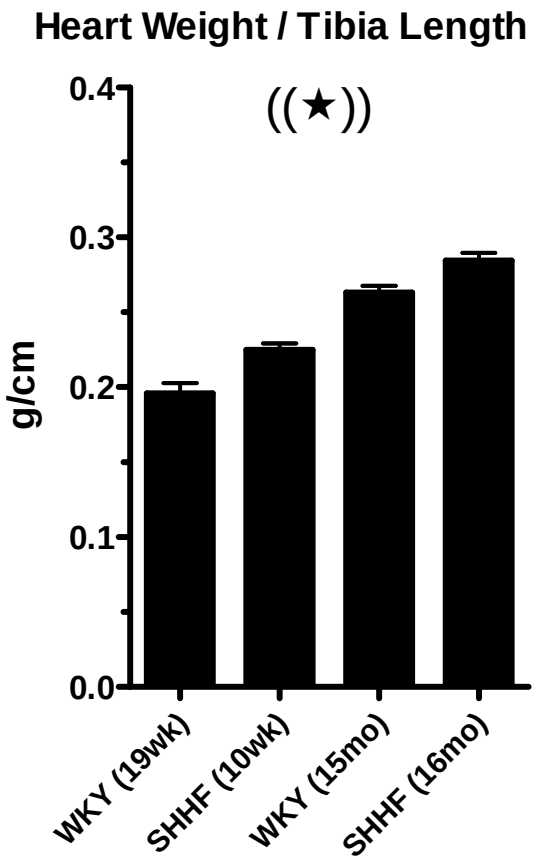


Figure 3.6. Age and strain-dependent cardiac hypertrophy in SHHF rats. Mean (+ SE) heart weight to tibia length ratio of 16-month-old air-exposed SHHF rats in this study compared to air-exposed animals in similar studies within our laboratory (Carll et al., *in press*; Lamb et al., 2012). Star and parentheses indicate all groups are significantly different from each other ($P < 0.05$).

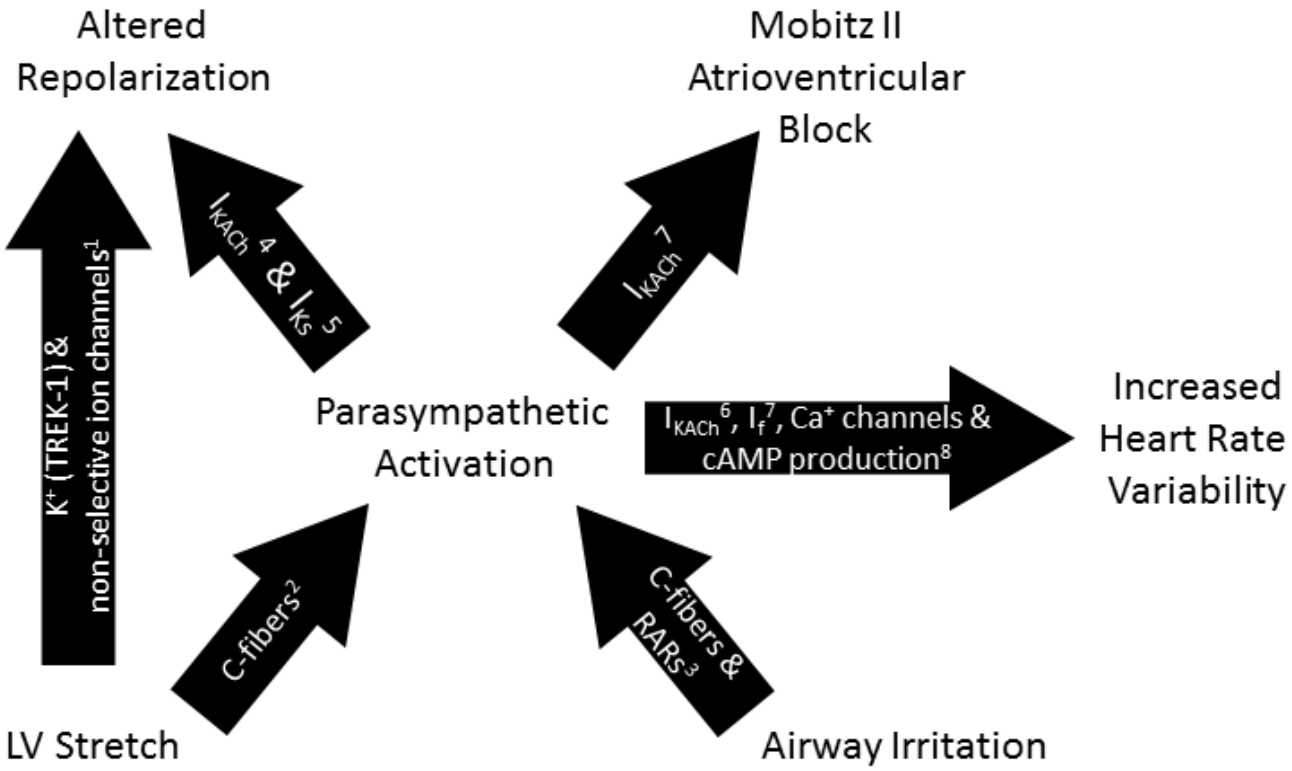


Figure 3.7. Proposed pathways accounting for electrophysiological effects of DE. cAMP: cyclic adenosine monophosphate, I_f: hyper-polarization-activated current; I_{KACh}: acetylcholine-sensitive K^+ channel; I_{Ks}: delayed rectifier K^+ channel, RARs: rapidly activated receptors, TREK-1: two-pore-domain potassium channel. 1—Tan et al., 2004; Li et al., 2006; 2—Crawford MH, 2003; Wang et al., 1995; 3—Widdicombe & Lee, 2001; 4—Moreno-Galindo et al., 2011; 5—Kanda et al., 2011; 6—Wickman et al., 1998; 7—Drici et al., 2000; 8—Harvey and Belevych, 2003.

Supplemental Material

Supplemental Table 3.1. Correlations between changes in AV block Mobitz II events and HRV or HR at hour 1 of post-exposure.

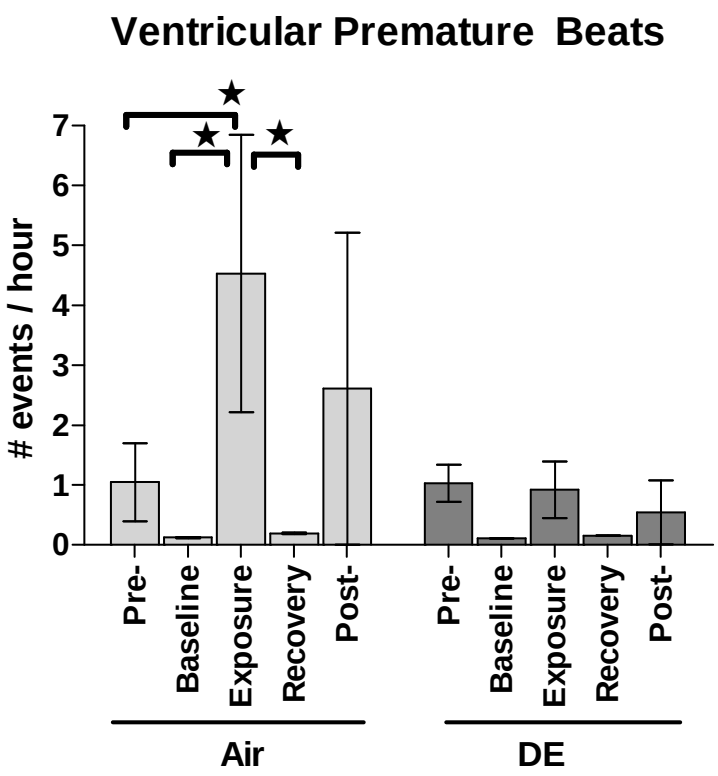
parameter	slope	r^2	P-value
RMSSD (msec)	3.2	0.45	0.01
SDNN (msec)	5.1	0.44	0.02
heart rate (beats / min)	-55.8	0.44	0.02

Linear regressions were based on mean HRV and total AV block events for each rat over the first hour of post-exposure within home cages (prior to echocardiography). N = 5-6 / group.

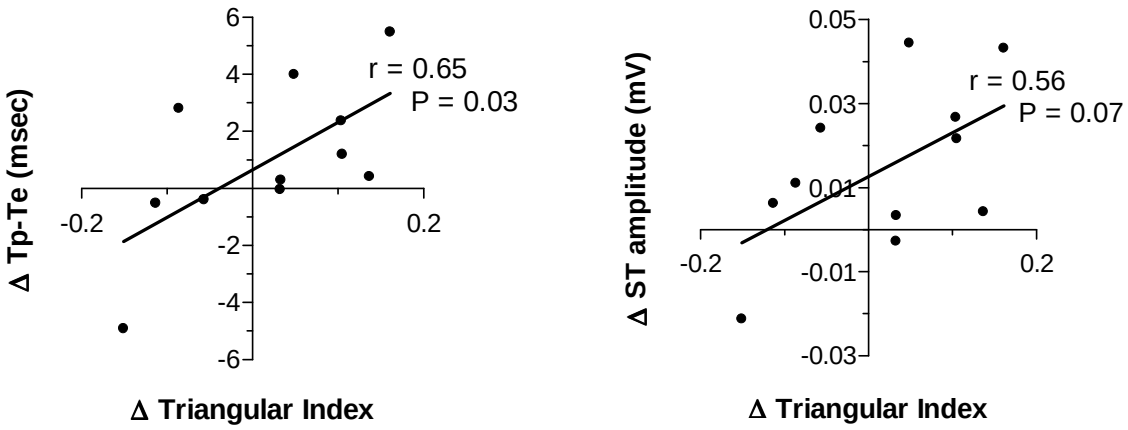
Supplemental Table 3.2. Endogenous anti-oxidants and markers of cardiovascular risk and injury.

	Air	DE	P-value
Broncho-alveolar lavage fluid			
eosinophils (#/ml)	0 (0)	45 (21)*	0.03
neutrophils (#/ml)	707 (160)	651 (211)	0.84
lymphocytes (#/ml)	789 (122)	687 (206)	0.69
macrophages (#/ml)	11,282 (772)	12,431 (1,268)	0.46
N-acetyl glucosaminidase	5.9 (0.3)	5.0 (0.2)*	0.03
total anti-oxidant status (µg/L)	113 (13)	144 (13)	0.11
Serum			
C-reactive protein (µg/L)	216 (3)	230 (3)*	0.01
lipase (U/L)	267 (23)	195 (18)*	0.02
ALT (U/L)	107 (8)	152 (20)	0.06
AST (U/L)	220 (12)	274 (32)	0.15
GST (IU/L)	49 (4)	92 (26)	0.14
Plasma			
total protein (g/dl)	4.7 (0.2)	5.1 (0.1)*	0.05
TIMP-1 (pg/ml)	4,264 (941)	7,109 (899)	0.06#
Organ Weights			
heart/tibia (g/cm)	0.285 (0.005)	0.277 (0.006)	0.35
heart/body (g/kg)	3.37 (0.05)	3.32 (0.02)	0.34
caudal lung lobe/tibia (g/cm)	0.081 (0.003)	0.085 (0.003)	0.44

Values presented as means (SE). 4 of 9 DE-exposed rats had pulmonary eosinophilia. See *Methods* section for other markers of cardiopulmonary toxicity, risk, inflammation, and antioxidants measured in serum, plasma, and bronchoalveolar lavage fluid. U and IU denote units and international units, respectively. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GST, glutathione S-transferase; TIMP-1, tissue inhibitor of metalloproteinase 1. * indicates significant difference from Air group. N = 8-9 / group, except # indicates N = 4/group.



Supplemental Figure 3.1. Hourly rate of ventricular premature beats. Stars indicate significant differences between periods or time-matched values of other group ($P < 0.05$). N = 6/group.



Supplemental Figure 3.2. Linear regression of HRV triangular index and ECG measures of ventricular repolarization after exposure. Data are presented as mean change over hours 1-4 after exposure relative to change from pre-exposure (72 hours prior) for each rat, regardless of exposure and excluding the first 30 minutes after echocardiography. N = 5 to 6 / group.

CHAPTER 4

DIESEL EXHAUST-INDUCED CARDIAC DYSFUNCTION IS MEDIATED BY AUTONOMIC IMBALANCE IN HEART FAILURE-PRONE RATS

Overview

Short-term exposure to vehicular emissions is strongly associated with adverse cardiac events. Diesel exhaust (DE) is a ubiquitous air pollutant hypothesized to provoke adverse cardiac events, in part, through defective co-ordination of the sympathetic and parasympathetic branches of the autonomic nervous system. To investigate this putative mechanism, we examined cardiophysiologic responses to a single DE inhalation exposure (500 μg/m^3, 4 h, whole-body) in young adult heart failure-prone rats and incorporated autonomic challenges and inhibition. These included post-DE sympathetic agonism (dobutamine, 320 μg/kg/min x 2 min *i.v.*) with and without parasympathetic ablation (vagotomy) and, separately, treadmill exercise and pretreatment with a sympathetic or parasympathetic inhibitor (atenolol or atropine; 5 mg/kg *i.p.* each). Measures of cardiac function by left ventricular (LV) pressure, autonomic balance by heart rate (HR) and HR variability (HRV), electrocardiogram, and aortic pressure were performed. Upon exercise recovery at 4 h post-exposure, HRV and HR changes indicated that DE increased parasympathetic influence. At 21 h post-exposure, DE increased sympathetic influence during exercise recovery only in saline-pretreated rats. DE impaired-contractility and decreased systolic blood pressure relative to Air-exposed rats during exercise recovery at 21 h post-exposure, and this effect was prevented only by sympathetic inhibition. Intra-cardiac

pressures indicated DE impaired systolic and diastolic function and altered diastolic and chronotropic responses to dobutamine partly through impaired parasympathetic regulation. Thus, altered autonomic regulation of the heart, characterized by an early parasympathetic dominance and a delayed sympathetic dominance, mediates adverse cardiac effects of air pollution exposure.

Introduction

Exposure to combustion-derived air pollution has been consistently linked to near-road adverse clinical outcomes, especially in those with preexisting cardiac disease (Bell, et al., 2009; Brook, 2008; Chiusolo, et al., 2011; Mann, et al., 2002; Pope *et al.*, 2008). Multiple pollutants have been implicated in these observations, including fine and ultra-fine particulate matter ($PM_{2.5}$ and UFP, with diameters < 2.5 μm and < 0.1 μm, respectively), nitrogen dioxide (NO_2), carbon monoxide (CO), and sulfur dioxide (SO_2). Diesel engine exhaust (DE) is a major urban source of these pollutants, as well as volatile organics, and carbonyls, and it may thus contribute largely to pollutant-induced adverse cardiovascular outcomes (Krivoshto, et al., 2008; Peretz, et al., 2008a). For instance, ischemic heart disease hospitalizations have been attributed, in part, to short-term DE exposure in eight European cities (Le Tertre, et al., 2002). The physiologic and biochemical responses observed with DE exposures have highlighted several candidate mechanisms of toxicity, including autonomic imbalance, myocardial ischemia, and electrophysiological dysfunction—among others.

Epidemiological studies have linked exposure to components of air pollution to autonomic imbalance and ischemia as reflected by alterations in heart rate variability (HRV) and ST-interval amplitude among other changes in the electrocardiogram (ECG). Our group and others have shown limited DE-induced changes in these endpoints in animal models of

cardiovascular disease (Anselme, et al., 2007; Campen, et al., 2005; Carll, et al., 2012; Carll *et al., in review;* Lamb, et al., 2012). The most profound ischemic effects of DE exposure reported to date were measured during exercise stress tests in coronary artery disease patients (Mills, et al., 2007). Because physical exertion increases oxygen demand and provokes autonomic compensatory reflexes (including sympathetic activation during exercise and parasympathetic activation thereafter), exercise stress tests are common clinical tools for unmasking latent myocardial ischemia and autonomic imbalance as reflected within the ECG (Froelicher & Myers, 2006; Goldberger *et al.*, 2006). Myriad studies have demonstrated that, upon exercise stress test, increased cardiac arrhythmia and abnormal responses in heart rate (HR), HRV, and ECG correlate with cardiovascular disease and risk of cardiovascular death (Beckerman *et al.*, 2005; Dewey *et al.*, 2007; Jae *et al.*, 2006; Watanabe *et al.*, 2001). Meanwhile, cardiac dysfunction during and after exercise tests has been shown to predict adverse outcomes in patients with hypertrophic cardiomyopathy (Pelliccia *et al.*, 2007).

In addition to using treadmill stress tests, exercise can be mimicked using sympathetic agonists to unmask latent effects of exposure. Our lab recently found in hypertensive rats that DE inhalation enhanced sympathetic, ischemic, and arrhythmic responses to dobutamine (Hazari *et al.*, 2012). It is unclear whether dobutamine-induced changes in cardiac function, specifically left ventricular pressure, cardiac contractility and lusitropy (cardiac relaxation), are also modified by DE exposure. In addition, little is known about the contribution of the autonomic nervous system (ANS) to the potential adverse effects of DE. Thus, the following hypotheses were tested in the present study in heart failure-prone rats: 1) determine if DE exposure modifies the physiologic response to treadmill exercise, 2) determine if DE exposure alters dobutamine-induced changes in cardiac function, and 3) by incorporating pharmacologic inhibitors of the

ANS during treadmill challenge as well as vagotomy during dobutamine challenge, determine if imbalance of the ANS mediates the adverse physiologic effects of DE exposure. HR, HRV, arrhythmia, repolarization, left ventricular pressure, cardiac contractility and lusitropy, and blood pressure were all measured to assess the effects of exposure.

Materials and Methods

Diesel Exhaust Exposure and Generation. All animals were exposed to either whole diesel exhaust (DE, target of 500 µg $PM_{2.5}/m^3$) or filtered air (Air) under conditions previously described (Carll, et al., 2012). DE exposures were at ultrafine PM and NO_2 concentrations comparable to those found in traffic tunnels and roadways in the U.S. and Europe (Anselme, et al., 2007; Svartengren, et al., 2000; Zhu, et al., 2007). DE 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 on low sulfur diesel fuel (16 ppm) at a constant load of 3 kW as previously described (Carll, et al., 2012). The exhaust was diluted with clean room air previously passed through high-efficiency particulate air (HEPA) filters adjusted periodically to maintain target $PM_{2.5}$ mass concentration. The diluted DE was delivered to a Hazelton 1000 (984 L) exposure chamber. Control animals were placed in a second chamber supplied with HEPA-filtered room air. The chambers were operated at the same temperature, pressure and flow rate (424 L/min; approximating 25 air exchanges per hour). Chamber concentrations of PM, O_2, CO, NO, NO_2, and SO_2 were measured as previously described (Carll et al., 2012). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges (< 80 dB, 30-70%, and 73° ± 5° F).

DE Exposure Study 1—Left Ventricular (LV) Pressure, Dobutamine Stress Test, & Vagotomy.

Lean male rats of the spontaneously hypertensive heart failure strain (SHHF *MccCrl-Lepr^{cp}*, Charles River Laboratories) were acquired (n=10; 7 weeks old). These rats acquire cardiac hypertrophy by 2 months of age and transition into dilated cardiomyopathy and overt heart failure (HF) at 18 months (Carll et al., 2011; Carll et al., 2012). Rats (13.5 weeks old) were exposed by whole-body inhalation to either filtered air or DE (target $PM_{2.5}$ concentration: 500 μg/m^3). At 20-24 h after exposure, rats were anesthetized with urethane (2 mg/kg *i.p.*, Sigma) and prepared for LV pressure measurement by right carotid arterial catheterization with a 2-French transducer (SPR-320, Millar Instruments**)**. The left jugular vein was cannulated in preparation for cardiac stress test by administration of a sympathomimetic (dobutamine). 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. Rats were observed for a 2-min aortic pressure baseline, the transducer was advanced into the LV for a 4 min baseline, and the rats were then infused for 2 min (Infusion A) with freshly diluted dobutamine hydrochloride (320 μg/kg/min *i.v.*, dissolved in 0.9% NaCl saline at a concentration of 640 μg/ml). Rats were observed for 12 min after infusion cessation, which pilot studies revealed as adequate time for recovery to resting heart rate and dP/dt_{max}. Animals then received bilateral vagotomy by suture occlusion of both right and left vagus nerves followed by a stabilization period (3 min), a second 2-min dobutamine infusion at the same dose (Infusion B), and a post-infusion observation period (2.5 min). The transducer was then retracted for measurement of aortic pressure (2 min), after which the rats were euthanized by exsanguination. For details, see Supplemental Material. Acquisition software (LabChart Pro version 7.3.2, ADInstruments) generated LV pressure parameters at end diastole (EDP) and end systole (ESP)

and the maximum upslope (dP/dt_{max}) and minimum downslope (dP/dt_{min}) of LV pressure per beat, indicative of contractility and relaxation rate (lusitropy), respectively.

DE Exposure Study 2—Treadmill Stress Test & Pharmacologic Autonomic Inhibition

Radiotelemetry implantation. Lean male SHHF rats were implanted with radiotelemeters transmitting ECG, aortic blood pressure (BP), and core body temperature (*n* = 24, 8 weeks old, telemeter model TL11M2-C50-PXT, Data Sciences International) by surgeons at Charles River Laboratory adhering to preoperative, anesthetic, and surgical procedures as described previously (Carll, et al., 2010). Rats were shipped after a 10-day recovery period to our AAALAC International-approved animal facility. Additional SHHF rats (*n* = 15, 11-12 weeks old) were implanted in our AAALAC International-approved animal facility at the U.S. Environmental Protection Agency (EPA) laboratory with radiotelemeters equipped for ECG, HR, and core body temperature measurements (model TA11CTA-F40, Data Sciences International) while adhering to preoperative, anesthetic, and surgical procedures described previously (Lamb et al., 2012). All rats were housed individually in 42 × 21 × 20-cm Plexiglas cages with pine-shave bedding in an animal holding room (22°C ± 1°C, 50% ± 5% relative humidity, 12-h light:dark cycle 0600:1800 h), and provided standard Purina rat chow (5001; Brentwood, MO) and water *ad libitum*. All studies conformed to the guidelines of the US EPA Institutional Animal Care and Use Committee (IACUC). After $\geq$ 10 days of surgical recovery, rats were transferred to a satellite facility and maintained under the same conditions as previously stated but in 33 × 18 × 19-cm Plexiglas cages.

Autonomic Inhibition and Treadmill Challenge. Rats were weighed and assigned blindly to one of six treatment groups (Air-Saline, Air-Atropine, Air-Atenolol, DE-Saline, DE-Atropine,

and DE-Atenolol) while maintaining equivalent mean body weights between groups. All animals were trained for treadmill challenge (Treadmill Simplex II, Columbus Instruments) on two subsequent days before telemeter data were collected for baseline treadmill challenges. Each challenge involved an initial 4-min run at 0° incline (Run A), a 20-min resting period, and a subsequent 5-min run at 25° incline (Run B) with a mild electric stimulus (1.47 mA at 2.9 Hz) at the rear of the treadmill to encourage consistent movement. For Run B, peak belt speed and incline were set to optimize ECG signal clarity and approach a peak heart rate response of 500 beats per min (BPM) based on pilot study observations.

Rats were placed in exposure chambers for a 2 h acclimation and returned 2 days later for a 5 h exposure to filtered air (baseline) with telemetry monitoring. At 3-5 h and 20-22 h after end of baseline, animals were subjected to treadmill challenges. Inhalation exposures began 48 h after baseline exposure. At 1 h before inhalation exposure, atropine and atenolol were each dissolved into saline, twice sonicated and vortexed for 2 min each, maintained at 38° C. Rats (12-15 weeks old) were then weighed and injected *i.p.* with saline vehicle (0.9% NaCl, Sigma Inc.), atropine (5 mg/kg, Sigma), or atenolol (5 mg/kg, Sigma) at a volume of 2.5 ml/kg body weight, placed in exposure chambers immediately thereafter, and allowed 30 min to equilibrate. Animals were exposed whole body for 4 h to either filtered Air or whole DE (target $PM_{2.5}$ concentration of 500 $\mu g/m^3$), followed by a 1 h wash-out period for both groups in which clean filtered air was circulated through exposure chambers. Treadmill challenges were repeated at 3-5 h and 20-22 h after cessation of exposures. For details, see Supplemental Material.

Radiotelemetry data acquisition and analysis. Radiotelemetry was used to track changes in cardiovascular and thermoregulatory function by continuously monitoring ECG, BP, core body temperature, and activity in awake, unrestrained rats beginning at 3 days before inhalation

exposure and continuing through exposure until euthanasia 24 h after exposure. Arterial BP (mean, systolic, diastolic, and pulse), heart rate, and aortic pre-ejection period (PEP) were derived from pressure and ECG waveforms sampled at a rate of 1,000 Hz in 2-min streams every 10 min within home cages, and 1-min streams every 5 min within exposure chambers. Treadmill ECG and BP waveforms were sampled continuously at 1,000 Hz. Parameters were automatically calculated using software (DataART 3.01; DSI) as previously described (Carll, et al., 2010). The aortic PEP (also referred to as QA interval) provides an index of contractility measured by the delay between onset of LV depolarization and ejection, which are respectively indicated by the initializations of the R-wave and the increase in aortic pressure (Cambridge & Whiting, 1986).

ECG waveforms were analyzed with computer software (ECGauto 2.8.1.26; EMKA Technologies, Falls Church, VA) that enabled user identification and exclusion of arrhythmias and artifacts from automated HRV and ECG morphology analysis as previously detailed (Carll, et al., 2012). HRV analysis generated HR and time-domain measures, including mean time between adjacent QRS-complex peaks (RR interval), standard deviation of the RR interval (SDNN), square root of the mean of squared differences of adjacent RR intervals (RMSSD), and percent of adjacent normal RR intervals differing by ≥15 ms (pNN15). pNN15 is a measure of parasympathetic tone. SDNN represents overall HRV, whereas RMSSD represents parasympathetic influence over HR (Rowan, et al., 2007). HRV analysis also provided frequency-domain parameters, including low frequency (LF: 0.200-0.750 Hz) and high frequency (HF: 0.75-3.50 Hz), and the ratio of these two frequency-domains (LF/HF). For frequency-domain analysis, the signal was analyzed with a Hanning window for segment lengths of 512 samples with 50% overlapping. LF is generally believed to represent a combination of sympathetic and parasympathetic tone, whereas HF indicates cardiac vagal (parasympathetic)

tone, and LF/HF serves as an index of sympathovagal balance (Rowan, et al., 2007).

Measures of ECG, BP, and arrhythmia frequency were obtained during and after treadmill challenge occurring after sham air exposure and after subsequent exposure to either DE or Air. Arrhythmias were analyzed and identified while blinded to treatment group according to previously described criteria (Carll, et al., 2012; Carll, et al., *in review*). HRV and ECG morphologic analyses were conducted on ECG waveforms collected during treadmill challenges. All 30-sec ECG streams with less than 5 identifiable conduction cycles were excluded from ECG parameter calculation and streams with less than 20 sec of identifiable RR intervals were excluded from HRV analysis. Thorough visual inspection was conducted to identify and exclude arrhythmias and artifacts from HRV and ECG analyses.

Tissue collection and analysis. At approximately 24 h after termination of the 4-h inhalation exposure, rats were deeply anesthetized with an intraperitoneal injection of a sodium pentobarbital / phenytoin solution. Tissue samples of blood, lung lavage fluid, heart, and lungs, were collected, processed, and analyzed as previously described (Carll, et al., 2012). Heart weight was normalized by right tibia length. To examine for indications of cardiopulmonary inflammation, injury, oxidative stress, and risk, multiple biochemical markers were assayed. Lavage supernatants were analyzed for albumin, gamma-glutamyl transferase, lactate dehydrogenase, N-acetyl-b-d-glucosaminidase, total antioxidant status, and total protein (Carll, et al., 2010). Serum supernatants were analyzed for creatine kinase, C-reactive protein (CRP), total protein, and glutathione peroxidase, reductase, and -S-transferase; and supernatants from plasma were assayed for angiotensin converting enzyme (ACE), albumin, blood urea nitrogen, creatinine, and total protein (Carll, et al., 2011a). Serum was also analyzed for α-hydroxybutyrate dehydrogenase, D-dimer, ferritin, glucose, insulin, lipoprotein (a), total

cholesterol, triglycerides, alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase-1, lipase, high and low density lipoprotein cholesterol, myoglobin, sorbitol dehydrogenase, superoxide dismutase (SOD), manganese SOD, and copper-zinc SOD according to previous procedures (Carll, et al., 2012; Carll, et al., 2010).

*Statistics. S*tatistical analyses of all data were performed using Prism version 4.03 (GraphPad Software, Inc., San Diego, CA). Repeated measures two-way ANOVA with Bonferroni post-hoc test was performed on LV pressure data for specific moments: immediately before Infusion A (Pre-Infusion), 12 min after Infusion A (Recovery), 2-3 min after bilateral vagotomy (Post-Vagotomy), the final 10 sec of Infusion A, and last 10 sec of Infusion B. As with autonomic inhibitors, brief bouts of exercise alter autonomic regulation of cardiovascular physiology (Chen *et al.*, 2009). To control for these effects and test for effects of DE exposure, changes in physiologic parameters from pre-exposure treadmill to post-exposure treadmill were compared between groups pretreated with the same autonomic inhibitor (or saline) by two-tailed t-test. To control for potential physiologic effects following containment in exposure chambers, treadmill data at 3-5 h and 20-22h after inhalation exposure were compared to challenges at corresponding times relative to sham exposure. One-way analysis of variance (ANOVA) with Tukey post-hoc test was used to detect significant differences between groups in biochemical endpoints and tissue weight. For all analyses, $P < 0.05$ was considered statistically significant.

Results

Study 1. Effects of DE Inhalation on LV Pressure and Autonomic Modulation.

Pre-Infusion. At 20-24 h after inhalation exposure, DE increased LV end diastolic pressure (DE 4.4±0.9 vs. Air 0.4±1.6 mmHg, $P < 0.05$), decreased contractility (dP/dt_{max}, P =

0.056; Figure 4.1-A), and slowed LV relaxation (dP/dt_{min}, $P < 0.05$; Figure 4.1-C). DE did not affect heart rate, arterial pressure, or LV systolic pressure before infusion (all $P > 0.05$).

Dobutamine Infusion A. Dobutamine equally increased dP/dt_{max} for both groups (Figure 4.1-B) such that the groups no longer differed in dP/dt_{max} at recovery (Figure 4.1-A). Dobutamine infusion abolished the difference between the Air and DE groups in dP/dt_{min} at recovery (Figure 4.1-C) by disproportionately increasing this parameter in the Air group during infusion ($P < 0.05$; Figure 4.1-D). Interestingly, DE-inhalation at Pre-Infusion had a similar effect on diastolic function as did dobutamine infusion in the Air group at Recovery (Figure 4.1-C). Heart rate (HR) increased by approximately 150 BPM for both groups during the first 90 sec of infusion, but the Air exposed rats had a decline in HR shortly before the end of infusion such that the DE group exceeded the Air group at the last 10 sec of infusion ($^{+}44$ BPM vs. Air, $P < 0.05$; Figure 4.1-F).

Vagus Nerve Ablation and Dobutamine Infusion B. The Air and DE groups did not differ from each other in dP/dt_{min}, dP/dt_{max}, or HR during the recovery period after Infusion A (Figure 4.1; $P > 0.05$). HR and dP/dt_{max} returned to near-baseline values for both groups during this recovery period (Figure 4.1-A & 4.1-E), whereas the Air group continued to have elevated dP/dt_{min} relative to its own baseline ($P < 0.05$, Figure 4.1-C). Vagotomy restored the difference between the Air and DE groups in dP/dt_{min} ($P < 0.05$; Figure 4.1-C) and increased HR and dP/dt_{max} for both groups ($P < 0.05$ each vs. Pre-Infusion and Recovery; Figure 4.1-A & 4.1-E). Notably, in contrast to the effects of Infusion A, dobutamine after vagotomy had equivalent effects on dP/dt_{min} and HR in the Air and DE groups (Figure 4.1-D & 4.1-F). There were no significant differences between groups in HR or dP/dt_{max} during or after Infusion B, nor in

arterial pressure after infusion; nevertheless, the DE group appeared to recover from peak dP/dt_{max} more slowly than the Air group (see Supplemental Figure 4.4).

Study 2. Effects of DE inhalation on Cardiovascular Responses to Treadmill Exercise.

Prior to treatment, treadmill challenge caused a peak HR of approximately 500 BPM in all groups at both Run A and Run B, indicating a robust response relative to the normal resting heart rate in conscious rats of this strain and phenotype (roughly 325 BPM [Carll et al., 2012]). At treadmill challenge 3-5 h after exposure, the DE and Air groups differed from each other in their changes in HRV from pre-exposure during recovery from treadmill Run B (Figure 4.3); the DE-Atenolol group's change in HR was 67 BPM less than Air-Atenolol ($P < 0.05$). At this same point, the DE-Saline group exceeded the Air-Saline group in change from pre-exposure SDNN by 4.4 msec ($P < 0.05$). Both the DE-Saline and DE-Atropine groups had a change in recovery RMSSD that exceeded their respective air controls (respectively, 1.22 and 0.63 msec greater, $P < 0.05$ each).

At treadmill challenge 20-22 h post-exposure, the DE- and Air- groups significantly differed in their HRV and/or HR responses only during the recovery period for Run A (Figure 4.4). Specifically, the DE-Saline group's changes in HR and LF/HF from pre-exposure treadmill were 32 BPM and 0.99 units greater than those of the Air-Saline group ($P < 0.05$). As well, the DE-Saline group's change in RMSSD was lower than that of the Air-Saline group, but this difference was only marginally significant ($P = 0.07$). In saline-treated rats, DE inhalation prolonged PEP relative to Air ($P < 0.05$). At this same time point, the DE-Atropine group had a significantly lower change in systolic BP than Air-Atropine (Figure 4.5), and the saline-pretreated groups had a similar trend ($P = 0.12$). In contrast, atenolol pretreated rats did not have

any significant changes in HRV, BP, or PEP at 24 h post-exposure. There were no significant effects of DE or Air exposure on arrhythmia frequency, HR-increase, or HR-decrease during treadmill challenges. There were no clear effects of DE or autonomic inhibitors on biochemical measures of cardiopulmonary injury, inflammation, or oxidative stress.

Discussion

We demonstrate that a single inhalation exposure to an environmentally relevant concentration of DE impairs cardiac performance, in part, through altered autonomic balance. DE exposure in hypertensive heart failure-prone rats caused changes in intra-cardiac pressures indicative of LV systolic and diastolic dysfunction at rest. DE exposure decreased contractility approximately one day after exposure as evidenced by changes in two indicators: decreased dP/dt_{max} during LV pressure assessments and, separately, increased pre-ejection period (PEP) after treadmill challenge. This decrement was in part mediated by sympathetic dominance, as evidenced by inhibition of DE-induced PEP prolongation and HRV decrements with a sympathetic antagonist (atenolol). In addition, DE increased end diastolic pressure and impaired LV relaxation (dP/dt_{min}). Impairments in LV ejection and relaxation and increased filling pressure can promote pulmonary edema and heart failure (Katz, 2006). As such, these effects alone offer insight into epidemiological findings that short-term air pollution exposure increases heart failure-related hospitalizations and deaths (Dominici, et al., 2006; Goldberg, et al., 2000; Pope, et al., 2008) and complement our recent observation that DE causes LV dilation in aged SHHF rats (Carll, et al., *in review*). DE changed diastolic and HR responses to sympathomimetic administration and altered autonomic reflexes to exercise recovery, suggesting an impaired ability to compensate to physiologic stress. Exercise challenges can unmask cardiac pump

dysfunction and autonomic imbalance with strong predictive ability of adverse cardiovascular outcomes and death (Dewey, et al., 2007; Duncker *et al.*, 2005; Pelliccia *et al.*, 2007). The effects of DE indicate an early enhancement of parasympathetic activation shortly after exposure followed by increased sympathetic influence one day later. Notably, sympathetic antagonism in DE-exposed rats prevented the DE-induced decline in contractility as evidenced by the reversal of the effects on PEP and systolic BP after exercise. Thus, the data reveal that short-term DE exposure induces an early increase in parasympathetic influence followed by a late sympathetic dominance that may mediate contractile and diastolic dysfunction. Ultimately, this study indicates that a predominance of sympathetic influence over the heart may cause air pollutant-induced cardiac dysfunction, and that β-adrenergic blockade bears important therapeutic potential for mitigating these effects.

The relative responses of DE- and air-exposed rats to dobutamine infusion and vagotomy indicated that DE caused a loss of parasympathetic modulation of cardiac function. DE exposure impaired pre-dobutamine LV lusitropy comparable to the effects of dobutamine in air-exposed rats at infusion recovery. Vagotomy (occlusion of the nerve fibers responsible for parasyampathetic cardiovascular regulation) did not alter the DE group's lusitropy, whereas it restored the Air group to pre-infusion dP/dt_{min}. Finally, vagotomy caused both the Air and DE groups to have the same responses in lusitropy to dobutamine infusion, indicating their prior differences may have been vagal-mediated. Further supporting this, and in concordance with our recent findings (Hazari, et al., 2012), DE exposure abolished inhibitory chronotropic responses to dobutamine infusion that were otherwise evident in the Air group. The absence of similar reflexes in air-exposed rats following vagotomy appears to confirm that this effect was parasympathetic in origin. Thus, responses to both dobutamine and vagotomy demonstrated that

DE impaired normal parasympathetic function, thereby enabling increased sympathetic influence and impeding cardiovascular function.

Several aspects of our study indicate a major role for the parasympathetic branch in DE-induced autonomic imbalance. DE-induced decrements in contractility at 1 day post-exposure were preceded by an increase in parasympathetic tone shortly after exposure. The changes in HRV and HR that we observed at 3-5 h post-DE accord with our previous evidence of parasympathetic dominance during acute exposure to DE (Carll, et al., *in review*), residual oil fly ash PM (Farraj, et al., 2011) or ozone (Farraj et al, 2012) in rat models of hypertension or heart failure. Atropine's inability to prevent parasympathetic dominance at treadmill challenge 3-5 h post-exposure (8-10 h post-injection) may relate to the drug's relatively short half-life (2-4 h) (Gyermek, 1998) and does not rule out the possibility of parasympathetic antagonism occurring *during* exposure, when vagal responses to DE are usually most pronounced (Carll, et al., 2012; Carll, et al., *in review*). Regardless, atropine inhibited sympatho-excitation at 1 day post-DE, suggesting that air pollutant-induced parasympathetic activation may later lead to sympathetic dominance.

We observed changes in LV function and HRV that suggest a central role for the autonomic nervous system and may stem from the complex relationships between oxidative stress, nitric oxide (NO), and autonomic balance. Anti-oxidant treatment can prevent PM-induced changes in HRV (Rhoden, et al., 2005), whereas air pollutant exposure has been repeatedly associated with systemic and cardiac oxidative stress and increased sympathetic influence (Brook, 2008). Sympathetic activation increases the release of catecholamines, which promote oxidative stress and mediate cardiac disease progression (Dhalla *et al.*, 2000). Interestingly, both sympathetic and parasympathetic inhibition have been found to decrease air

pollutant-induced cardiac oxidative stress (Rhoden, et al., 2005), which is a key cause of contractile dysfunction and cardiomyocyte injury and death (Dhalla, et al., 2000). Air pollutant exposure may also promote cardiac dysfunction through NO synthase uncoupling (Knuckles, et al., 2008; Tankersley, et al., 2008), which impairs NO homeostasis and promotes oxidative stress via superoxide production. NO is a presynaptic modulator of parasympathetic neurotransmission that can suppress cardiomyocyte contractile responses to β-adrenergic receptor (βAR) activation. When increased in the brain's autonomic regulatory site, NO causes short term parasympathetic-associated physiological reflexes later followed by evidence of sympatho-excitation among hypertensive rats, the latter of which may be mediated by superoxide production (Danson & Paterson, 2006). Further studies are necessary to disentangle the interactions between oxidative stress, NO, and autonomic balance in air pollutant cardiotoxicity.

DE exposure caused an early vagal dominance that may relate to the triggering of pulmonary irritant receptors, including the transient receptor potential ankyrin 1 (TRPA1) channel, which activate sensory nerves (C-fibers) (Hazari, et al., 2011), thereby causing acute parasympathetic cardiovascular reflexes (Widdicombe & Lee, 2001). We have previously shown that both (A) the inhibition of TRPA1 channels prior to DE exposure and (B) the administration of a sympathetic antagonist 1 day after DE exposure prevent DE-enhanced sensitivity to a pro-arrhythmic drug (Hazari, et al., 2012). Meanwhile, others have found that a 3-day $PM_{2.5}$ inhalation exposure in mice decreases cardiac vagal neuron excitability and HRV, indicating that air pollutant exposure can compromise the parasympathetic counterbalance to sympatho-excitation through induced neuroplasticity (Pham *et al.*, 2009). Importantly, the parasympathetic branch suppresses sympathetic influence over the heart through a number of mechanisms, including presynaptic inhibition of sympathetic neurons, inhibition of

catecholamine release, and decreased firing rates of the sino-atrial and atrioventricular pacemaker nodes (Katz, 2006). In contrast, the parasympathetic branch has relatively minimal effects on vascular tone, which is disproportionately mediated by sympathetic input in humans (Chong & Michel, 2012) and Spontaneously Hypertensive rats (Friberg *et al.*, 1988). Our observation that DE decreased both HRV and systolic BP suggests that sympathetic dominance may have resulted from diminished parasympathetic regulation of the heart rather than increased sympathetic cardiovascular regulation. Decreased vagal tone and increased sympathetic influence over the heart have been found to correspond with heart failure exacerbation and predict arrhythmia and sudden cardiac death in humans (La Rovere *et al.*, 1994; Nolan *et al.*, 1998). Thus, a deterioration of parasympathetic influence bears critical implications for cardiac health.

In summary, our findings here demonstrate that DE-induced cardiac dysfunction is mediated in part by autonomic imbalance. These findings highlight the utility of treadmill exercise tests and dobutamine infusion as tools to unmask latents effects of air pollution exposure and imply that evidence toward the putative mechanism of autonomic-mediated air pollution cardiotoxicity may elude conventional measures due to their dependency upon uniform physiological conditions or autonomic stimuli. Additionally, our study indicates that β-adrenergic blockade can prevent DE-induced sympatho-excitation and LV dysfunction, perhaps by accommodating a deterioration in parasympathetic function. Ultimately, the potential for β-adrenergic blockade to mitigate the adverse cardiac effects of air pollutant exposures deserves further investigation and should be factored into clinical and epidemiological studies.

Tables

Table 4.1. Inhalation Exposure Characterization

	Air	DE
$PM_{2.5}$ ($\mu g/m^3$)	1.9 (0.3)	502 (2.8)
Volume median diameter of PM (nm)	184	89
O_2 (%)	20.9 (0.0)	20.3 (0.1)
CO (ppm)	< 0.5	33.1 (2.3)
NO (ppm)	< 0.5	23.4 (1.8)
NO_2 (ppm)	< 0.5	3.8 (0.22)
SO_2 (ppm)	< 0.5	< 0.5
Temperature (°F)	73.9 (1.3)	71.7 (0.8)
Humidity (%)	43.8 (2.6)	56.1 (1.2)

Data represent mean values (standard error in parentheses) generated from measurements made daily either continuously (concentrations of O_2, CO, NO, and NO_2), once ($PM_{2.5}$ mass concentration), or six times (DE $PM_{2.5}$ number) per exposure over 4 exposure days per group. Volume diameter was calculated from number-based mobility diameters and assumed spherical particles. Air indicates filtered air; DE, diesel exhaust; $PM_{2.5}$, fine particulate matter.

Figures

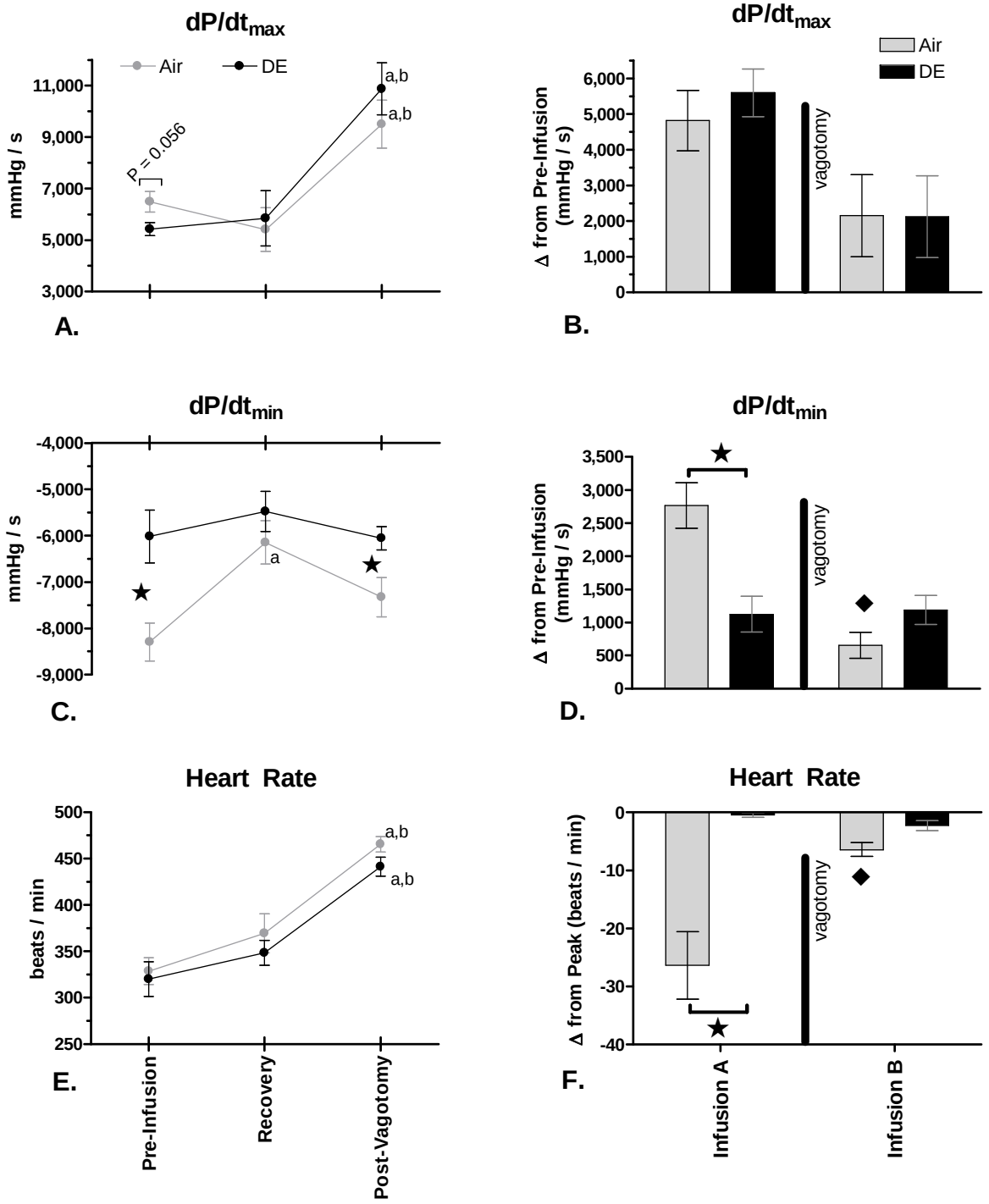


Figure 4.1. LV pressure measurements of contractility (dP/dt_{max}), lusitropy (dP/dt_{min}), and heart rate 1 day after DE exposure. Panels A, C, and E: raw 10-sec means (±SE) before dobutamine (Pre-Infusion), 12-min after termination of initial infusion (Recovery), and after vagus nerve occlusion (Post-Vagotomy). Panels B and D: change from pre-infusion to final 10 sec of infusion. Panel E: change from peak heart rate during infusion to final 10 sec of infusion. Stars indicate differences between Air and DE groups, letters indicate differences from Pre-Infusion (a) or Recovery (b), and diamonds indicate differences from Infusion A ($P < 0.05$). N = 5/group.

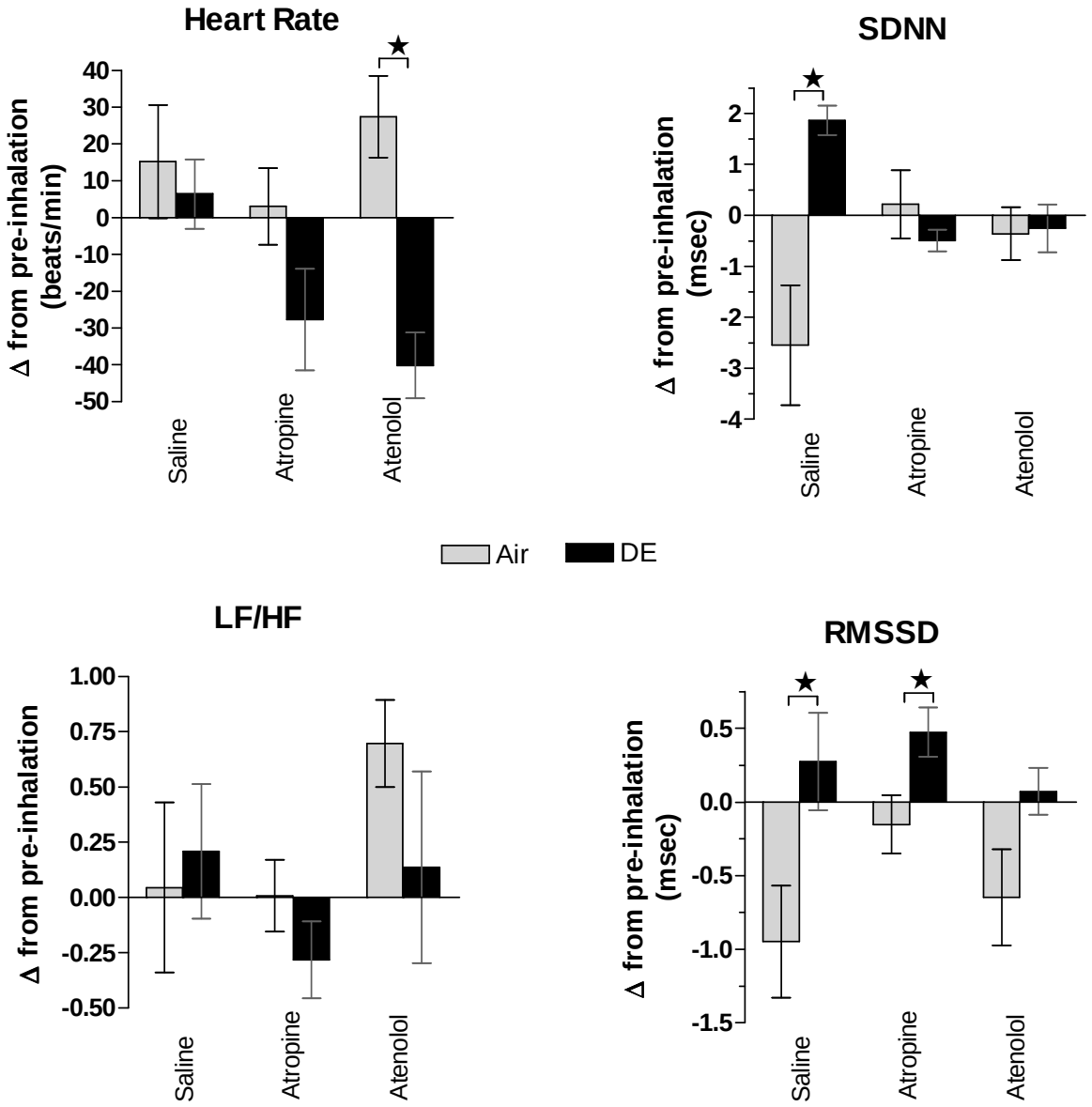


Figure 4.2. Changes in HR and HRV during recovery from treadmill run B at 3-5 h post-exposure relative to run B at 3-5 h post-sham exposure. Stars indicate differences between Air and DE groups ($P < 0.05$). Values represent group means over 3 min, N = 5-6 / group.

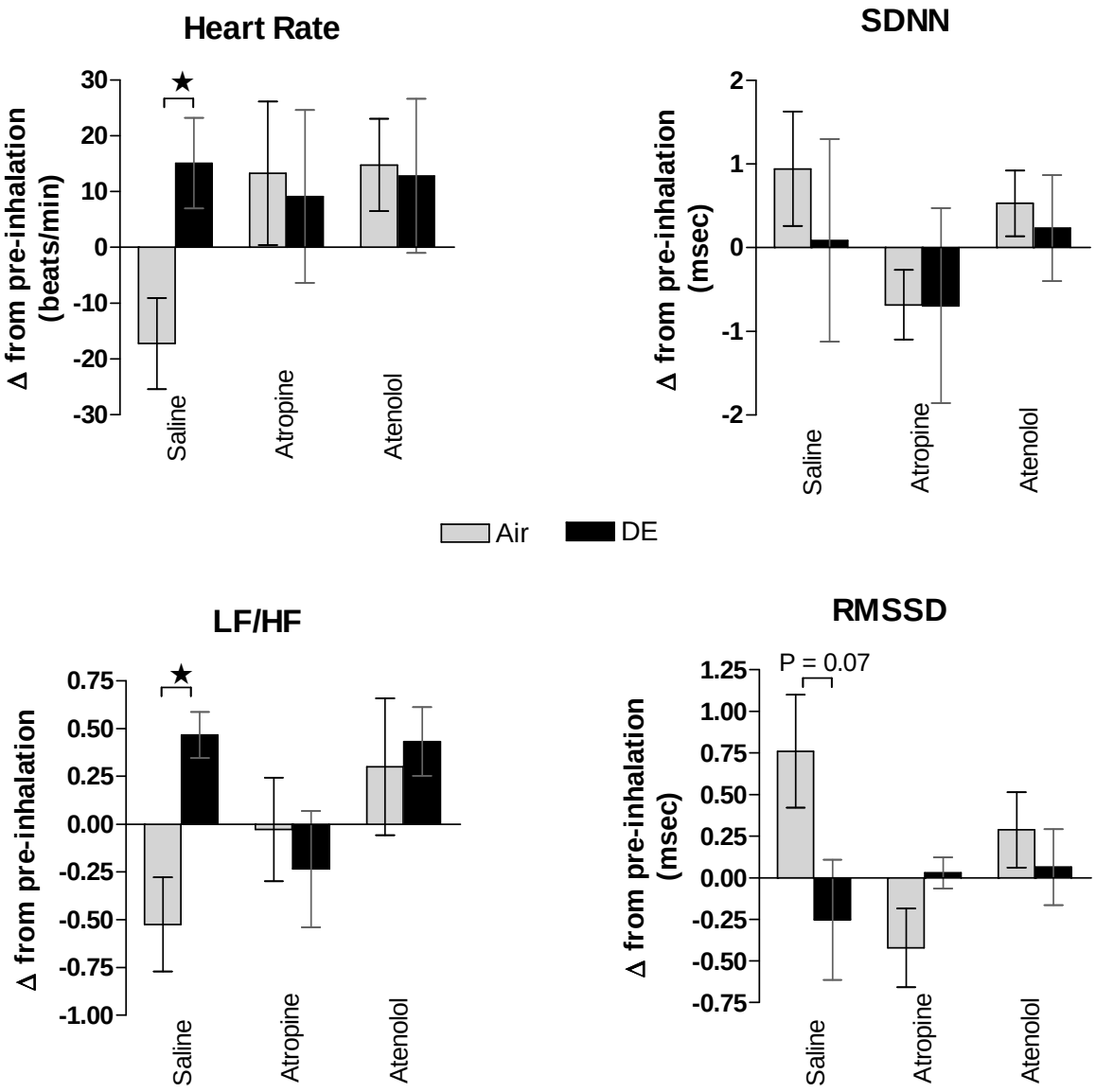


Figure 4.3. Changes in HR and HRV during recovery from treadmill run A at 20-22 h post-exposure relative to run A at 20-22 h post-sham exposure. Stars indicate differences between Air and DE groups ($P < 0.05$). Values represent group means over 2 min, N = 5-6 / group.

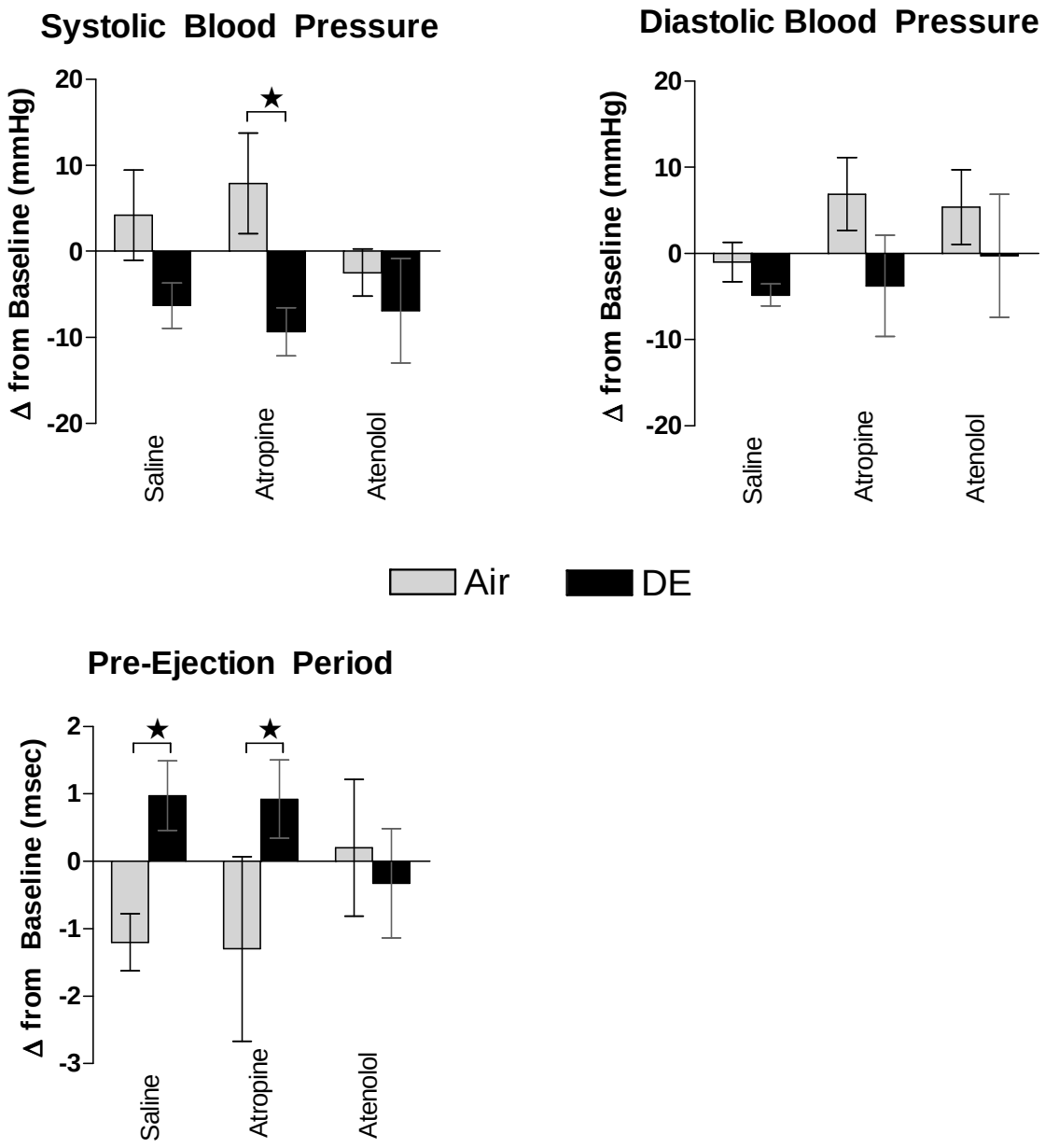
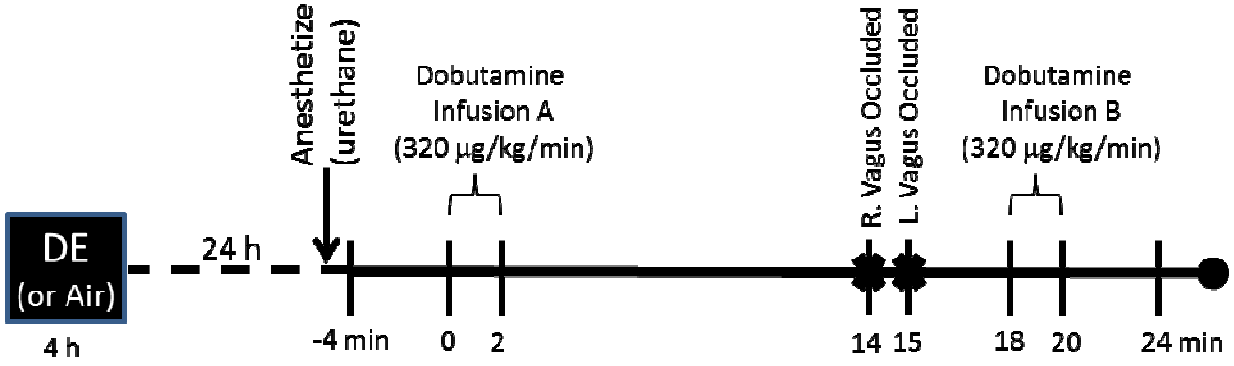
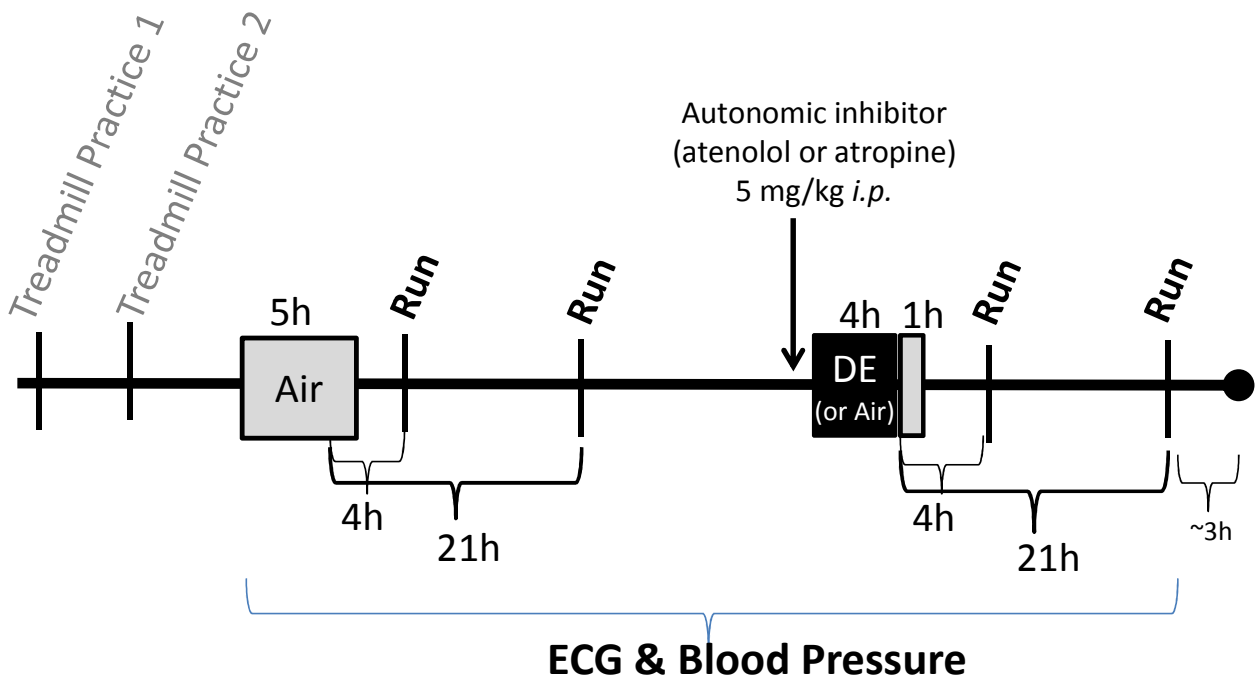


Figure 4.4. Changes in systolic and diastolic aortic pressures and pre-ejection period (an index of contracitility) during recovery from treadmill run B at 20-22 h post-exposure relative to run B at 20-22 h post-sham exposure. Stars indicate differences between Air and DE groups ($P < 0.05$). Values represent group means over 3 min, N = 4 / group.

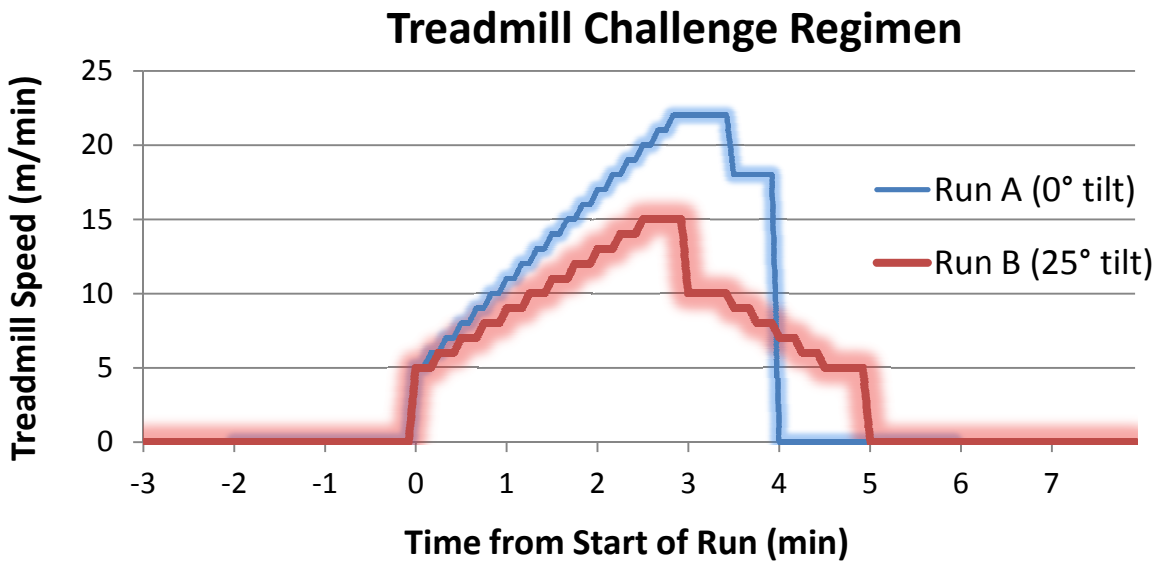
Supplemental Material



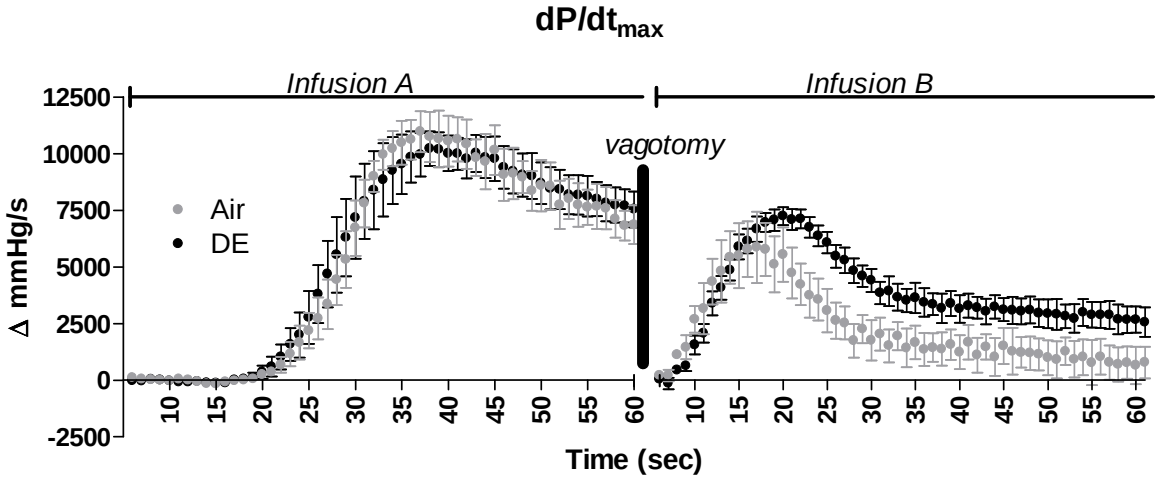
Supplemental Figure 4.1. Study 1 Regimen. The Effects of DE Inhalation on Cardiovascular Responses to Dobutamine Infusion and Vagotomy. Left ventricular pressure was measured from -4 min until 22.5 min. Animals were exposed whole-body to DE at a PM concentration of 500 $\mu g/m^3$ and challenged 1 day later.



Supplemental Figure 4.2. Study 2 Regimen. Effects of DE Inhalation on Cardiovascular Responses to Treadmill Exercise. ECG and blood pressure were measured by radiotelemetry. Animals were pre-treated with an autonomic inhibitor or saline control 30 min before whole-body exposure to clean air or DE at a PM concentration of 500 μg/m^3 and challenged 3-5 h and 20-22 h later. Sham exposure to Air for both groups occurred 3 days before actual exposure.



Supplemental Figure 4.3. Treadmill challenge regimen. For Run A, belt speed increased by 1 m/min in 10 sec increments from 5 to 22 m/min, followed by 30 sec periods at 22 m/min and then 18 m/min, after which the treadmill was stopped for a 2-min recovery, and animals were returned to home cages for 10 min. Run B regimen involved a 3-min "pre-run" stationary period, subsequent belt speed increases by 1 m/min every 15 seconds from 5 m/min to a 30-sec 15 m/min period, and then speed was decreased to 10, 9-8, 7-6, and 5 m/min in the ensuing 30-sec intervals. Belt speeds and inclines were set to optimize ECG signal clarity at peak heart rate responses $\geq$ 500 bpm based on pilot study observations.



Supplemental Figure 4.4. Changes in an index of contractility during first 60 sec of dobutamine infusions A and B. Points indicate 1-sec averages ± standard error.

CHAPTER 5

IMPLICATIONS AND CONCLUSION

Implications

This research was conducted to investigate the toxicological mechanisms underlying epidemiologic findings that acute exposure to ambient air pollution increases adverse cardiac events and related mortality, especially in humans with preexisting cardiac conditions (Brook et al., 2008). Heart failure-prone rats of the SHHF strain were incorporated into several studies to determine (1) if short-term exposure to diesel exhaust (DE) promotes changes in autonomic balance and cardiac function, (2) if physiologic stress tests can unmask latent cardiac and autonomic effects of DE exposure, (3) whether autonomic changes are associated with adverse cardiac effects, and (4) if changes in autonomic regulation of cardiac function mediate adverse cardiac effects of DE exposure.

Whole and particle-free diesel exhausts differentially affect cardiac electrophysiology, blood pressure, and autonomic balance in heart failure-prone rats.

The initial characterization study in young adult SHHF rats revealed that acute DE inhalation of either particle-free DE or whole-DE can affect cardiovascular physiology during or shortly after exposure by (i) altering ventricular repolarization, (ii) impeding atrioventricular (AV) nodal conduction, (iii) increasing the frequency of spontaneous AV block bradyarrhythmias, (iv) increasing heart rate variability (HRV) and/or decreasing heart rate (HR),

and (v) decreasing blood pressure. Alone, these effects are consistent with dominance of the parasympathetic nervous system and indicate a potential for adverse cardiac events, including fatal arrhythmia and myocardial ischemia. In comparison between whole DE and filtered DE groups, the effects appeared to be primarily mediated by DE gases and may have been partly counteracted by the presence of particulate matter, as the whole DE-exposed group had fewer significant responses and had HRV indications of a transient sympathetic excitation. The increased adverse effects of particle-free DE compared to whole DE deserves additional investigation, particularly given the increasing use of exhaust filters on new diesel-burning on-road vehicles. Although the public health implications of these results are unclear, the data provide evidence that air pollution alters autonomic regulation of cardiac function and promotes arrhythmia.

Diesel Exhaust Inhalation Increases Cardiac Output, Bradyarrhythmias, and Parasympathetic Tone in Aged Heart Failure-Prone Rats

To further investigate the cardiophysiologic effects of DE and associated changes in autonomic influence, an additional study was conducted in aged adult SHHF rats with normal cardiac function at pre-exposure. Echocardiography in conscious rats 1.5 h after inhalation exposure revealed that DE caused left ventricular (LV) dilation and increased cardiac output. Additionally, electrocardiographic (ECG) data indicated that DE altered ventricular repolarization, increased AV block bradyarrhythmias, and markedly increased several HRV measures of parasympathetic cardiac regulation during and/or shortly after exposure. These effects in aged SHHF rats were more pronounced than observed in the prior study involving whole DE exposure in young adult SHHFs, suggesting that age and progression toward heart failure confer added susceptibility to the cardiovascular impacts of air pollutant exposure. If

sustained, increased venous return, LV dilation, and increased cardiac output can promote myocardial remodeling and eventual heart failure through volume overload. Appropriately, myriad epidemiologic studies have demonstrated positive correlations between heart failure morbidity and mortality and exposure to air pollution (Bell, et al., 2009; Chiusolo, et al., 2011; Colais *et al.*, 2012; Dominguez-Rodriguez *et al.*, 2011; Dominici, et al., 2006; Goldberg, et al., 2003; Mann, et al., 2002; Pope, et al., 2008). The echocardiographic evidence of LV dilation and increased cardiac output indicate potential increases in LV pressure and complement the findings of Tankersley and associates (2004 & 2008), which collectively involved PM-induced LV dilation, impaired systolic function, molecular evidence of increased myocardial stretch, and parasympathetic activation in a mouse model of terminal senescence. The observations also accord with recent findings that DE inhalation enhances venoconstriction in mice (Knuckles et al., 2008), which can acutely augment venous return of blood to the heart thereby increasing cardiac output and LV volumes. In addition, this study introduced a new putative mechanism of air pollutant-induced parasympathetic activation and altered cardiac repolarization, as LV dilation can activate myocardial stretch receptors that cause parasympathetic reflexes (Crawford, 2003; Wang, et al., 1995) and alter ion channels directly responsible for repolarization (Tan, et al., 2004; Xian Tao, et al., 2006). Thus, the findings justify further investigations into air pollutant-induced LV dilation, including its causes, its mechanistic link to the autonomic effects of air pollutants, and its downstream effects on cardiac function.

Treadmill Stress Test after Diesel Exhaust Particulate Intra-tracheal Instillation Reveals a Time-dependent Shift from Parasympathetic to Sympathetic Dominance—a pilot study.

The prior studies revealed that the aged (16 month-old) SHHF rat was a more useful model than young adult SHHFs for demonstrating autonomic-associated cardiovascular effects

of DE exposure. Nevertheless, the aged SHHF rat was difficult to reliably obtain for further mechanistic investigation. Thus, a pilot study was performed to determine if physiologic stress tests in young adult SHHFs could reveal autonomic and cardiovascular effects of DE exposure that were unapparent in sedentary rats. These rats were intra-tracheally instilled with diesel exhaust particles (DEP, n=4) or saline vehicle (n=4) and observed both at rest and during treadmill challenge. At rest, young adult SHHFs had increased HRV in the first 10 hours after exposure (Appendix Figure 4 & 5). These effects were recapitulated during treadmill challenge at 3 hours post-exposure (Appendix Figure 2), indicating that DEP causes a short-term parasympathetic dominance. At 1 day post-instillation, DEP tended to decrease HRV during treadmill challenge, suggesting sympathetic excitation (Appendix Figure 3). Similar effects indicating sympatho-excitation were not apparent while rats were at rest in their home cages in this pilot study or our previous studies (Carll et al., 2012; Carll et al., *in review*), indicating that the treadmill challenge could reveal latent autonomic effects of air pollutant exposure. Moreover, at 1 day post-exposure to DE, treadmill challenge provided the first evidence of sympathetic dominance within the research conducted toward this dissertation.

Acute Diesel Exhaust Inhalation Exposure Causes Autonomic-Mediated Cardiac Dysfunction in Heart Failure-Prone Rats.

Subsequently, treadmill challenge was used to determine if inhalation exposure of young adult SHHFs to DE at equivalent concentrations as the prior inhalation studies could provoke a similar pattern of autonomic imbalance as the instillation/treadmill study. An additional stress test was implemented involving the *i.v.* administration of a sympatho-mimetic (dobutamine) in order to mimic the effects of exercise on autonomic balance and cardiovascular function. This latter challenge also involved more direct determinations of cardiac function in anesthetized rats

(relative to conscious ECG or aortic blood pressure) through measures of left ventricular (LV) pressure. LV pressures before challenge indicated that DE impaired LV relaxation (lusitropy), decreased contractility, and increased LV filling pressures—key determinants of cardiac output. To the author's knowledge, this is the first study to demonstrate decrements in systolic and diastolic function after a single inhalation of DE. Several groups have demonstrated in rodents that longer or much higher exposures to DE, DE particles, or non-vehicular PM can impair contraction and relaxation of the heart (Huang, et al., 2010; Lord *et al.*, 2011; Tankersley, et al., 2008b; Wold *et al.*, 2012; Yan, et al., 2008). Much like the previous instillation study, treadmill challenge revealed that DE inhalation increased parasympathetic influence over the heart at 3-5 h post-exposure, whereas, 1 day later, it revealed that DE caused sympathetic dominance, impaired contractility, and decreased systolic blood pressure. Responses to dobutamine challenge complemented these findings, as heart rate at the final 10 sec of dobutamine infusion was elevated in the DE group relative to the Air group, suggesting impaired parasympathetic reflexes to sympathetic stimulation. Additionally, dobutamine had the same effect on lusitropy in the Air group as did DE exposure at pre-infusion, suggesting that the DE group's impairments in lusitropy were mediated by sympathetic dominance. Therefore, the treadmill and dobutamine stress tests were useful for revealing DE-induced cardiac dysfunction and imbalance of the autonomic nervous system.

The following experiments were conducted to more directly determine whether the autonomic effects of DE mediate cardiac dysfunction. Pharmacologic inhibitors of sympathetic (atenolol) or parasympathetic (atropine) influence over cardiac function were administered immediately before DE inhalation. HRV and HR measurements during treadmill stress tests at 1 day post-exposure revealed that sympathetic and parasympathetic inhibition both abolished DE-

induced sympathetic dominance. Because DE caused parasympathetic dominance at treadmill challenge 3-5 h post-exposure, and administration of a parasympathetic inhibitor prevented subsequent sympatho-excitation, the findings also suggested that parasympathetic blockade during DE exposure may prevent subsequent sympatho-excitation. Yet, only sympathetic inhibition prevented decrements in contractility and blood pressure, indicating that the effects of DE on cardiac function may be primarily mediated by a relative dominance of sympathetic regulation—either through diminished parasympathetic or increased sympathetic output to the heart. In addition, after the initial post-exposure dobutamine challenge, surgical vagotomy was performed and followed by a second infusion. Vagotomy revealed that the dobutamine-induced increases in lusitropy for the Air group were likely parasympathetic-mediated reflexes to sympathetic agonism, while the DE group's diminished lusitropic responses to infusion as well as to vagotomy were likely due to a pre-established deterioration in parasympathetic function. This was further supported by a lack of chronotropic inhibitory responses to dobutamine for the DE group before vagotomy and for the Air group after vagotomy. Thus, DE abolished the impact of vagotomy on lusitropic and chronotropic responses to dobutamine, whereas sympathetic inhibition prevented DE-induced changes in post-exercise measures of contractility, systolic blood pressure, and HR. Collectively, these findings correspond with demonstrations by others that air pollutant exposure can diminish parasympathetic output to the heart (Pham et al., 2009), and suggest that this effect results in sympathetic dominance, consequently, cardiac dysfunction.

While the investigations herein did not examine the role of NO in the cardiovascular effects of DE, it should be noted that both NO and the autonomic nervous system can modulate each other upstream of changes in cardiac or vascular function (Katz et al., 2006; Danson &

Paterson, 2006). Interestingly, observations of both DE-enhanced venoconstriction and PM-induced cardiac dilation appear to involve mediation, at least in part, by the uncoupling of nitric oxide (NO) synthase, which promotes superoxide production and oxidative stress (Tankersley et al., 2008; Knuckles et al., 2008). Others have noted that autonomic inhibition prevents PM exposure-induced oxidative stress, and conversely, administration of an anti-oxidant can prevent PM-induced autonomic imbalance and adverse cardiac effects (Rhoden et al., 2005). Of additional consideration with respect to the physiologic effects reported here, DE exposure appears to increase circulating nitrates through the inhalation of NO (Knuckles et al., 2011). Further studies are necessary to disentangle the interactions between oxidative stress, NO, and autonomic balance in air pollutant cardiotoxicity.

Conclusion

The multiple studies toward this dissertation consistently indicated that diesel exhaust exposure caused an early parasympathetic predominance over cardiac function. Such findings are frequently neglected in literary reviews of air pollutant exposure's effects on autonomic balance and cardiac function. Nevertheless, myriad human studies have reported air pollutant-induced increases in HRV (Peretz, et al., 2008a) (Mills, et al., 2011a; Pope *et al.*, 1999; Riediker, 2007; Riediker *et al.*, 2004; Routledge *et al.*, 2006; Yeatts *et al.*, 2007). While at least one of these studies noted an increase in supraventricular arrhythmias in association with increased HRV, the health implications of air pollutant-induced elevated HRV and parasympathetic dominance remain largely unacknowledged and underexplored. Interestingly, the findings of the present research also contrast with findings that DE exposure in humans increases blood pressure (Cosselman, et al., 2012). Discrepancies in blood pressure and HRV

responses between studies may stem from differences between rodents and humans in cardiovascular and thermoregulatory responses to toxins (Rowan, et al., 2007; Watkinson *et al.*, 2001; Watkinson & Gordon, 1993); differences in study design, underlying disease of subjects, conditions, and exposures (i.e., engine load modifies emission toxicity (McDonald *et al.*, 2011)); or a divergence in unidentifiable covariates.

The results herein suggest that the parasympathetic effects of air pollution exposure may precipitate sympathetic dominance over cardiac function. Increased sympathetic influence over cardiac function is closely associated with adverse cardiac outcomes. To this end, the effectiveness of β-adrenergic receptor blockers on the prevention of cardiac disease and related deaths provide a compelling case in point (Go *et al.*, 2008; Ram, 2010). The vascular effects that would likely accompany air pollutant-induced sympathetic activation (e.g., hypertension) are also of significant public health concern. Interestingly, the absence of hypertensive responses to DE indicates that central sympathetic excitation did not occur. This seems especially likely given that the parasympathetic branch has relatively minimal effects on vascular tone, which is disproportionately mediated by sympathetic input in humans (Chong & Michel, 2012) and Spontaneously Hypertensive rats (Friberg, et al., 1988). Of additional note, traditional β-blockers such as atenolol (a β_1-adrenergic receptor antagonist) do not cause vasodilation and have been deemed ineffective in the prevention of hypertension-associated cardiovascular disease progression (Ram, 2010). Thus, the DE-induced decrements in contractility and systolic BP which atenolol prevented were probably not the result of hypertensive reflexes to exposure. Ultimately, the present research indicates that DE-induced impairments in cardiac function may depend upon enhanced sympathetic stimulation of the β_1-adrenergic receptor, which is likely a result of impaired parasympathetic inhibition of sympathetic input to the heart. Direct

examinations of sympathetic and parasympathetic nerve activation and norepinephrine spillover rates may enable a more definitive understanding of the autonomic effects of air pollutant exposure.

Several questions persist about the basis for autonomic imbalance in air pollutant-induced cardiac toxicity. Firstly, the findings of more pronounced parasympathetic-associated effects of filtered DE on cardiovascular physiology indicate that the filtration of particles may enhance the autonomic and cardiac effects of DE. Additional studies are required to test this theory. Meanwhile, the involvement of oxidative stress and/or myocardial stretch in the activation of autonomic reflexes to air pollutant exposure remains largely unexplored. Although Rhoden and colleagues (2005) demonstrated a link between oxidative stress and autonomic reflexes, they did not assess cardiac function to determine whether the inhibition of either oxidative stress or autonomic reflexes prevented adverse physiologic effects. Likewise, myocardial stretch receptor inhibition has been shown to prevent cardiac arrhythmias (Hansen *et al.*, 1991) and stretch-mediated activation of the hypertrophic pathway (Scimia *et al.*, 2012) and thus could be readily incorporated into air pollution exposure studies. The influence of myocardial ischemia and systemic hypoxia on autonomic control of the heart also deserves study in the context of air pollutant exposure. For instance, hypoxia causes an immediate decrease in cardiac norepinephrine turnover and heart rate and a subsequent rebound increase in norepinephrine and heart rate upon return to normoxic conditions (Hirakawa & Hayashida, 2002; Johnson *et al.*, 1983). Myocardial ischemia also causes a cascade of compounds that activate cardiac nociceptors (pain receptors), including adenosine, bradkyinin, lactate, serotonin, prostaglandins, and other substances, some of which are known to affect autonomic balance (Horst, 2000). Finally, it is unclear to what extent autonomic imbalance may mediate the adverse cardiac effects

of long-term exposures to air pollutants. Wold and colleagues (2012) recently demonstrated that long-term inhalation exposure to particulate matter (PM) in mice causes myocardial remodeling and impairs contractile function. From the present findings of short-term effects of DE exposure, it is conceivable that repeated exposures could bear autonomic-mediated adverse effects on cardiac function.

Thus, the findings within this dissertation introduce many new questions about the etiology of autonomic-mediated cardiac effects of air pollutant exposure. As well, this research enhances understanding of the impact of diesel exhaust constituents, the role of disease in conferring susceptibility to diesel exhaust, and the mechanisms by which exposure to diesel exhaust causes adverse cardiovascular health effects. While decreasing exposure to air pollution remains the most assured means of harm prevention, there may be promise for β-adrenergic blockade in inhibiting the adverse cardiac effects of air pollutants,. Taken together, the findings in this dissertation may contribute to both therapeutic and air quality control strategies for mitigating the health effects of air pollution exposure.

APPENDIX

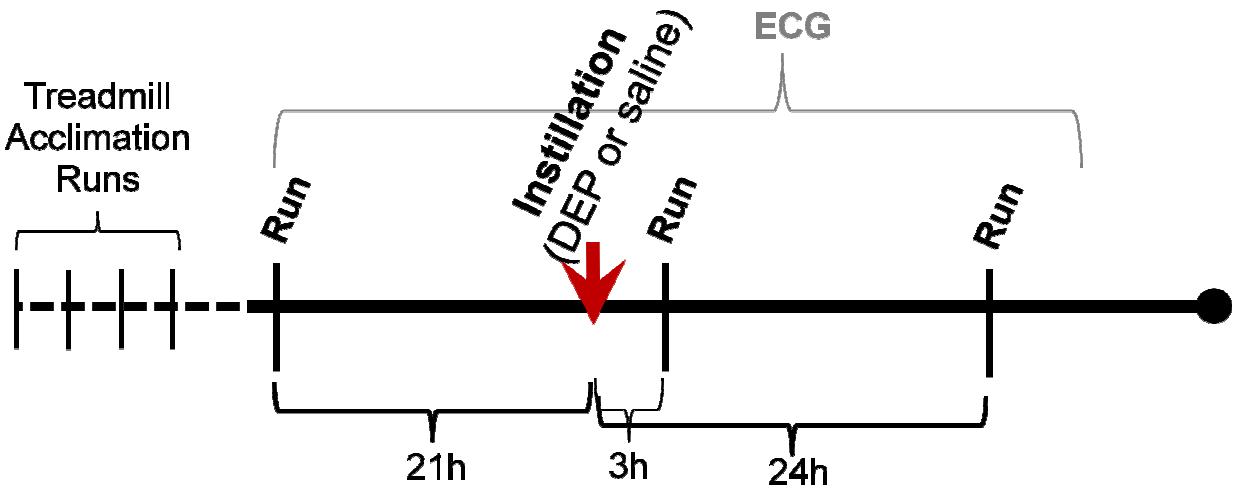


Figure A.1. Study Regimen: Treadmill stress test after intra-tracheal instillation (IT) of diesel exhaust particles (DEP; 500 μg/kg) or saline vehicle (1 ml/kg) in adult SHHF rats. Treadmill challenges occurred at 21 hours pre-IT, 3 hours post-IT, and 24 hours post-IT. Each challenge consisted of 2 consecutive runs separated by 25 min of rest. ECG was monitored by radiotelemetry during treadmill (continuous) or while in home cages (2 of every 15 min). Rats were trained 4 times before data collection on the treadmill.

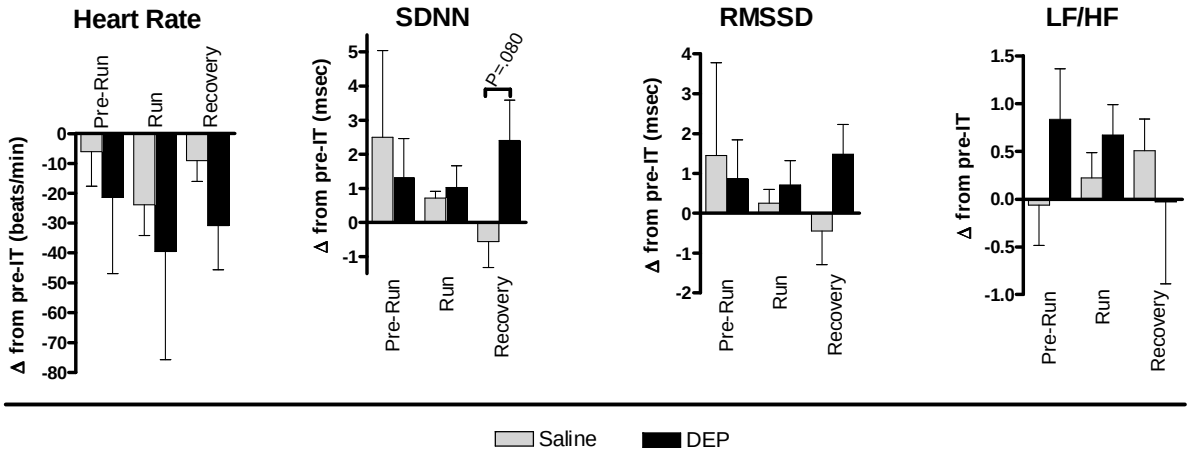


Figure A.2. Effects of DEP IT on autonomic regulation of the heart during treadmill challenge at 3 h post-IT. Trends in SDNN suggested parasympathetic activation at this time. All values are from 2nd of 2 consecutive treadmill runs and presented as change in means (± S.E) relative to pre-IT. n=4/group.

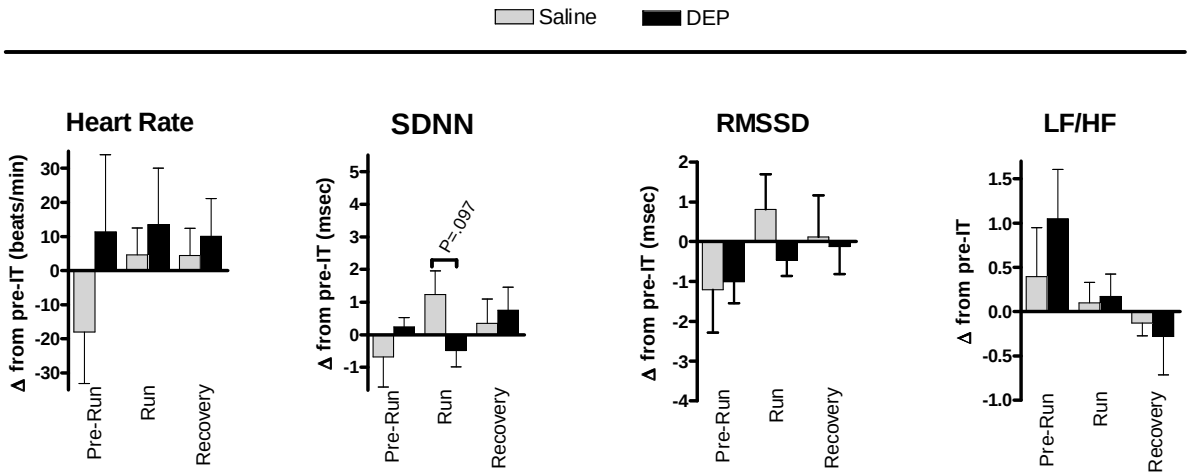


Figure A.3. Effects of DEP IT on autonomic regulation of the heart during treadmill challenge at 24 h post-IT. Trends in SDNN suggested potential sympathetic activation at this time. All values are from 2nd of 2 consecutive treadmill runs and presented as change in means (± S.E) relative to pre-IT. n=4/group.

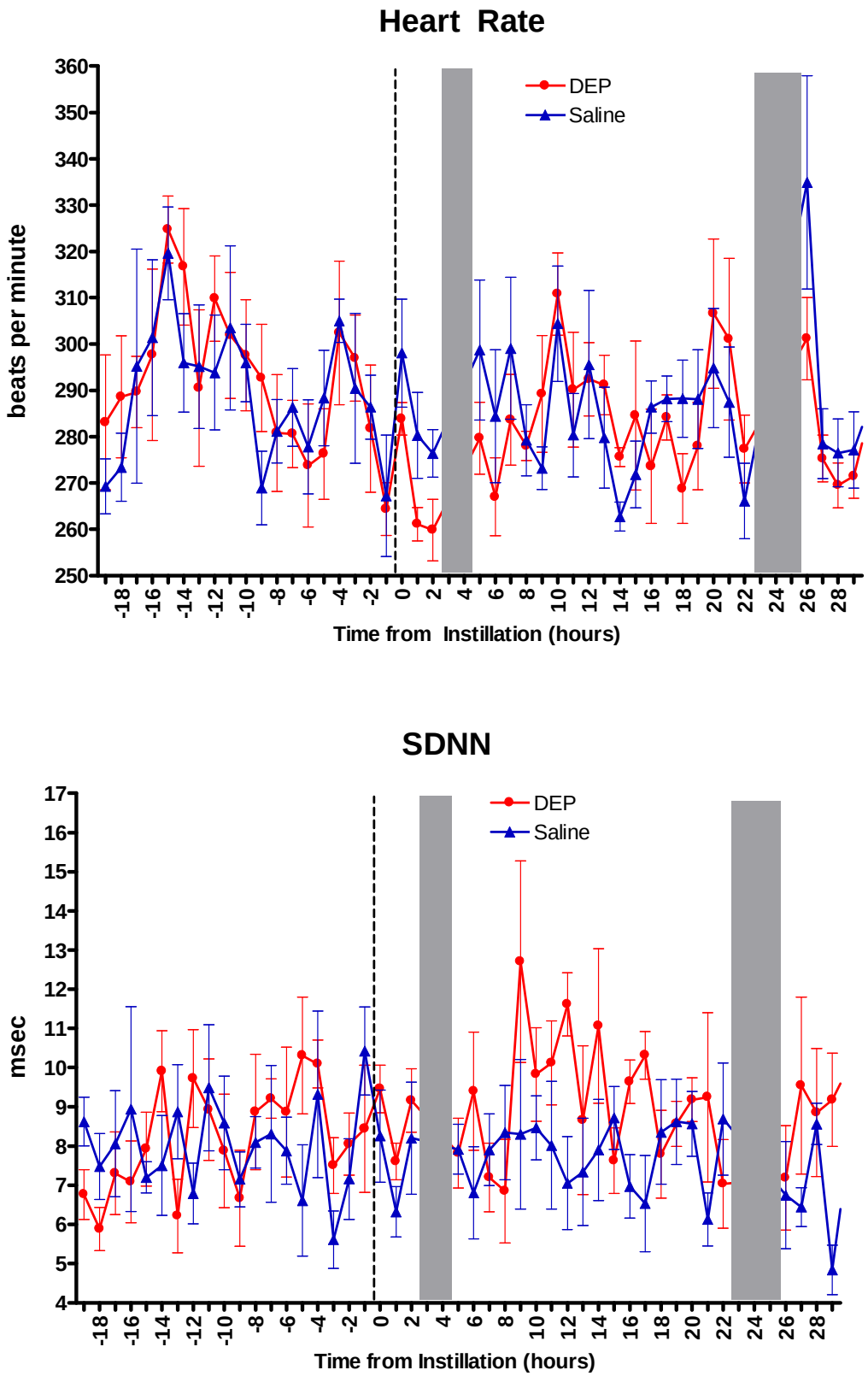
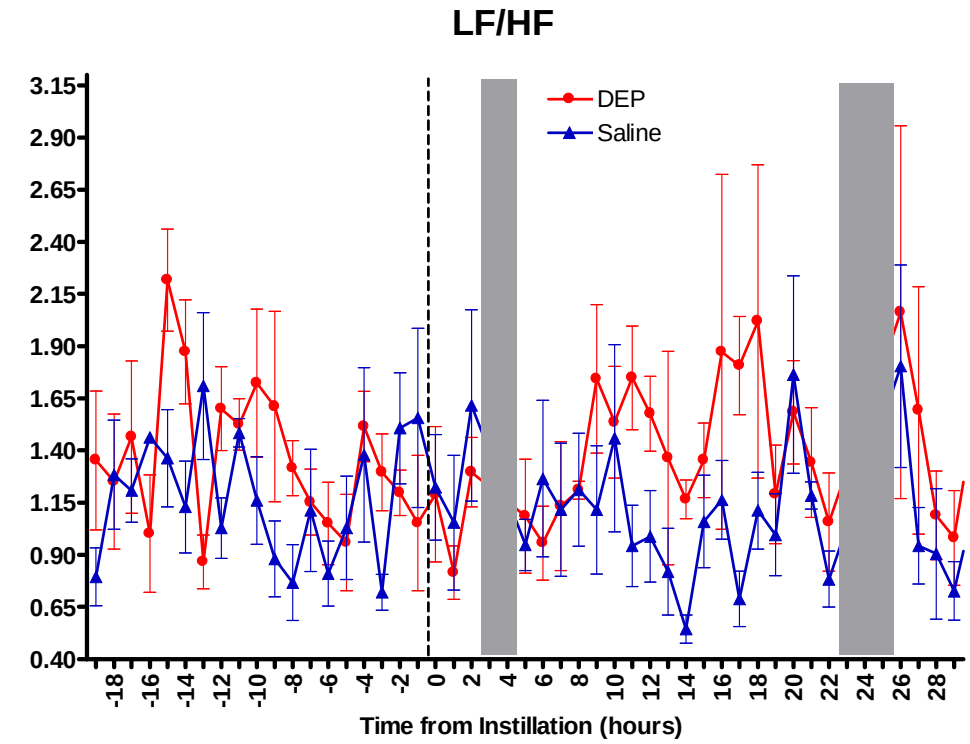


Figure A.4. Effects of DEP IT on autonomic regulation of the heart in home cages. Gray bars represent missing data for treadmill challenges. Group means (± S.E), n=4/group.



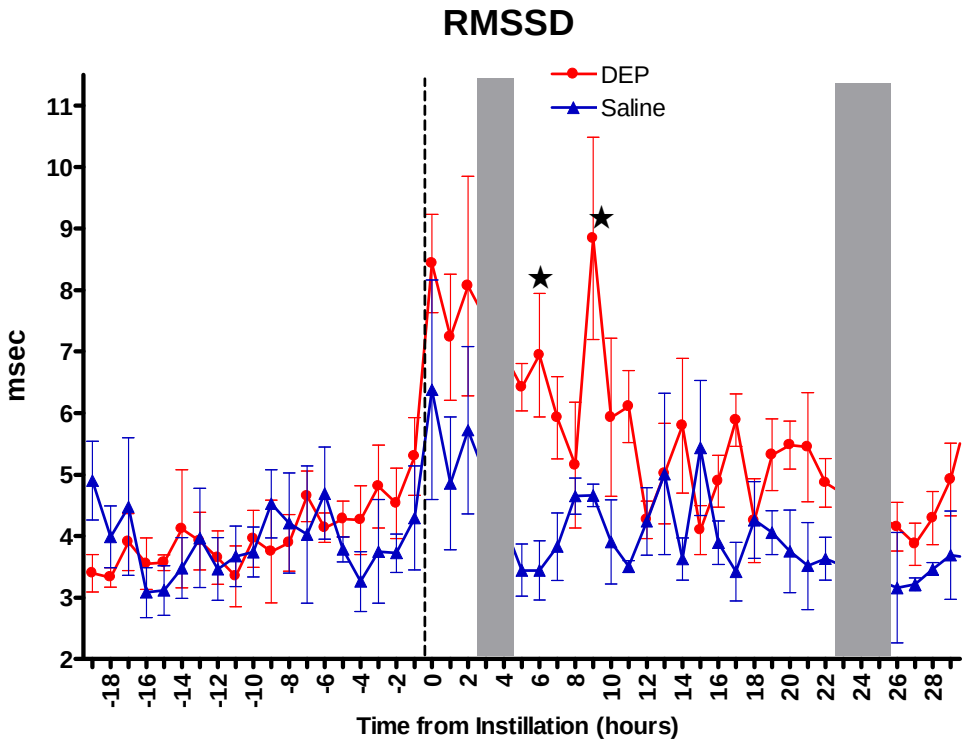


Figure A.5. Effects of DEP IT on autonomic regulation of the heart in home cages. Gray bars represent missing data for treadmill challenges. Group means (± S.E), n=4/group. Stars indicate significant differences between groups ($P < 0.05$). DEP significantly increased RMSSD at 6 h and 9 h post-IT, indicating parasympathetic excitation.

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