THE ROLE OF TRPA1 AND AUTONOMIC IMBALANCE IN THE CARDIAC RESPONSE TO AIR POLLUTION

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Toxicology.

Chapel Hill
2016

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ABSTRACT

Nicole Anne Kurhanewicz: The Role of TRPA1 and Autonomic Imbalance in the Cardiac Response to Air Pollution.
(Under the direction of Mehdi Hazari)

Exposure to air pollution has been shown to contribute to cardiovascular morbidity and mortality; this is especially true for certain susceptible subpopulations. One mechanism linking air pollution and cardiovascular dysfunction involves perturbation of autonomic nervous system balance initiated by air pollution activation of airway sensory receptors. One such "sensor" is transient receptor potential A1 cation channel (TRPA1), which is expressed on airway afferent nerves and is known to be activated by certain ubiquitous air pollutants. Although this mechanism has classically been known to mediate certain reflexive respiratory responses, research suggests that a subset of cardiovascular responses are similarly produced. Thus my global hypothesis for this dissertation project is that autonomic imbalance during an acute exposure to air pollution, as indicated by heart rate variability (HRV), is mediated by TRPA1 and contributes to cardiac dysfunction. To test this, we first characterized the cardiovascular response of mice to acute particulate matter and ozone exposure. We then examined the cardiovascular impacts of inhaled acrolein, which is a TRPA1 agonist, in both wild-type and TRPA1 knockout mice. Lastly we determined if inhibition of either or both arms of the autonomic nervous system affected the acrolein-induced changes HRV. Co-exposure to fine or ultrafine concentrated ambient particles and ozone produced ECG changes indicative of increased heterogeneity of repolarization, non-conducted p-wave arrhythmias, and decrements in cardiac mechanical function one day after exposure.

Exposure to acrolein caused increases in HRV and bradyarrhythmia during exposure, as well as changes in cardiac mechanical function one day after exposure. No exposure effects were observed in the TRPA1 knockout mouse. Pharmacological inhibition of either or both arms of the autonomic nervous system demonstrated that acrolein exposure caused a biphasic response in which early sympathetic activation was followed by prolonged vagal dominance. These data suggest that air pollution causes autonomic imbalance and cardiac dysfunction through TRPA1. This research fills important gaps in our understanding of mechanisms underlying air pollution-induced cardiovascular dysfunction and will aid in risk assessment.

PREFACE

The first manuscript presented in this dissertation (Chapter 2) is a pre-copy-editing, author produced version of an article accepted for publication in *Particle and Fibre Toxicology*. The definitive publisher-authorized version of this manuscript is available online at http://particleandfibretoxicology.biomedcentral.com/articles/10.1186/s12989-014-0054-4 and is cited as follows:

Kurhanewicz N, McIntosh-Kastrinsky R, Tong H, Walsh L, Farraj AK, Hazari MS. Ozone co-exposure modifies cardiac responses to fine and ultrafine ambient particulate matter in mice: concordance of electrocardiogram and mechanical responses. Part Fibre Toxicol. 2014 Oct 16;11:54.

ACKNOWLEDGEMENTS

Although it is my name that appears on the cover of this dissertation, a great many people have contributed to its creation. I owe my gratitude to all who have made this achievement possible. First and foremost I would like to express my sincere appreciation to my advisor Dr. Mehdi Hazari, who saw fit to take a chance on an untrained, unruly student. He has truly been a mentor, both as a superb researcher and as a well-balanced person. Through his patience, kindness, and immense knowledge he has guided me through turbulent years. A special thanks is due to Dr. Aimen Farraj as well for his welcome advice on everything from experimental design to data analysis, and friendly guidance through the graduate school process. I would also like to thank my committee members, Drs. David Peden, Dan Costa, Mark Zylka and Brian Jensen for their time, expertise, and thoughtful critique so generously provided over the course of my graduate career. This work could not have been completed without the support received from co-workers and colleagues. My deepest gratitude to all of the members of the laboratory and employees of the EPA that helped with the many facets of this project including Allen Ledbetter, Lenny Walsh, Patty Dillard, Najwa Haykal-Coates, Judy Richards, Rachel McIntosh-Kastrinsky, Kim Stratford, and Joey Stevens. I would like to thank Dr. Leslie Thompson in particular; his insightful comments and constructive criticisms at different stages of my research were thought-provoking and helped me focus my ideas. I am grateful to him for holding me to his own high research standard. Finally, I would especially like to acknowledge my parents and

family for their never ending love and support, my husband Everett for his unyielding encouragement and understanding over the years, and my son Abel for giving me perspective through trying times.

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LIST OF ABBREVIATIONS

ACE – angiotensin converting enzyme

ANOVA – Analysis of Variance

ANS – Autonomic Nervous System

BAL – Bronchoalveolar Lavage

CAPs – Concentrated Ambient Particles

CDC – Centers for Disease Control

CK – Creatine Kinase

COPD – Chronic Obstructive Pulmonary Disease

CRP – C-reactive protein

dP/dT (-) – Negative Derivative of left ventricular Pressure over Time

dP/dT (+) – Positive Derivative of left ventricular Pressure over Time

ECG – Electrocardiogram

EPA – United States Environmental Protection Agency

FA – Filtered Air

FCAPs – Fine Concentrated Ambient Particles (<2.5um)

GTR – glutathione-S-transferase

HAPs – Hazardous Air Pollutants

HBDH - α-hydroxybutyrate dehydrogenase

HF – High Frequency

HR – Heart Rate

HRV – Heart Rate Variability

KO – Knockout

LDH – Lactate dehydrogenase

LF – Low Frequency

LVDP – Left Ventricular Developed Pressure

MIA – Microalbumin

NAAQS – National Ambient Air Quality Standards

NAG - N-acetyl-b-d-glucosaminidase

NCPW – Non-Conducted P-Wave

NTS – Nucleus Tractus Solitarii

O3 – Ozone

PM – Particulate Matter

RAR – Rapidly Adapting Receptor

RAS – Renin-Angiotensin System

RMSSD – Root Mean Square of the Standard Deviation of subsequent NN intervals

RSA – Respiratory Sinus Arrhythmia

SA – Sinoatrial

SAR – Slowly Adapting Receptor

SDNN – Standard Deviation of subsequent NN intervals

SEM – Standard Error of the Mean

SOD – Superoxide dismutase

SP – Substance P

SSS – Sick Sinus Syndrome

TRP – Transient Receptor Potential Cation Channel

TRPA1 – TRP Subfamily A, Member 1

TRPV1 – TRP Subfamily V, Member 1

UFCAPs – Ultrafine Concentrated Ambient Particles (<100nm)

VOC – Volatile Organic Compound

WHO – World Health Organization

WT – Wildtype

CHAPTER I: INTRODUCTION

Air Pollution and Public Health

The health impacts of outdoor air pollution largely became apparent following the industrial revolution during the 19th and 20th centuries, when urban centers began to rapidly expand with dense concentrations of both homes and industrial areas, providing ideal conditions for a range of public health concerns including chronic diseases and daily exposure to a variety of environmental toxicants. During this time coal was widely used for the heating of homes and powering of factories and eventually resulted in some of the most deadly air pollution incidents in modern history. The most widely cited of these is the 1952 London Great Smog episode, where combustion-related emissions combined with a temperature inversion resulted in the excess deaths of an estimated 12,000 people (Bell & Davis 2001). Pollution levels between December 5th-9th in 1952 London were 50-300% increased compared with previous years (5-19 times current regulatory standards). The smog caused a sharp rise in hospital admission rates for both respiratory and cardiac diseases as well as pneumonia and influenza (Bell & Davis, 2001; Logan, 1956). Two years earlier in 1948 a similar episode had occurred in the U.S., in the small town of Donora, Pennsylvania where 20 of the town's 14,000 residents died and over 7000 were hospitalized for cardiopulmonary injury or exacerbation of disease (Boyd, 1960). Incidences of extreme air pollution like those observed in London and Donora were relatively uncommon, but were highly publicized. They highlighted the public health consequences of ambient air pollution exposure and the growing need for monitoring and regulation of air pollution emissions.

The first U.S. federal legislation addressing air pollution was the Air Pollution Control Act of 1955. This act declared that air pollution posed a risk to public health, and authorized the surgeon general to work with state and local air pollution control agencies to recommend research programs and develop methods to reduce or eliminate ambient air pollution. However it maintained that individual states had the responsibility to establish their own air pollution controls. The Air Pollution Control Act primarily provided funds for the federal government to research the sources and effects of air pollution (U.S. Congress 1955). In 1963 the Clean Air Act was passed, putting the onus of air pollution regulation on the federal government. This act required the U.S. Department of Health to create and enforce automobile emissions standards. The Clean Air Act of 1970 further expanded on the federal government's ability to develop and enforce air pollution standards and controls from both stationary and mobile sources (US EPA 2016). Also in 1970 the Environmental Protection Agency (EPA) was established for the purpose of consolidating federal research and monitoring programs pertaining to environmental and public heath, as well as developing and enforcing environmental pollution standards. A major component of the 1970 Clean Air Act was the establishment of the National Ambient Air Quality Standards (NAAQS) which today designates six criteria air pollutants that are routinely and broadly monitored by the EPA. These include ambient particulate matter (PM), ozone, carbon monoxide, sulfur dioxide, nitrogen dioxide, and lead (US EPA 2016). In addition to the six criteria air pollutants the EPA has identified and has regulatory standards for a list of 187 hazardous air pollutants (HAPs) which have the capacity to increase rates of cancer, produce other serious health impacts, or have significant environmental impacts (US EPA). Even with more stringent air quality regulations in place, and greatly improved ambient air quality, the impact

of air pollution is an estimated 200,000 premature deaths per year in the U.S (Caiazzo et al. 2013) and 3.7 million premature deaths per year worldwide (WHO, 2012), the vast majority arising from cardiopulmonary injury or disease.

Present day ambient air pollution comprises a diverse mixture of substances including particulate patter (PM), gases, organic compounds, and toxic metals (Brook et al. 2010; Verrier et al. 2002). PM exposure in particular has been linked with a variety of adverse cardiovascular outcomes including myocardial infarction, stroke, arrhythmia, and exacerbation of heart failure. However the 2010 American Heart Association scientific statement pointed out that, "Although PM2.5 mass has rightfully attracted attention as a target for regulation and epidemiological study, more than 98% of the air pollutant mass in the mixture we breathe in urban settings is from gases or vapor phase compounds such as CO, volatile organic carbons (OCs), NO2 , NO [nitric oxide], O3 , and SO2 ." (Brook et al. 2010). Each of these may have independent effects or combined effects with co-pollutants. Aldehydes, a class of volatile organic compounds, have attracted interest since the earliest days of air pollution research due to their ubiquitous and highly irritating nature (Quade R . Stahl 1969).

Acrolein

Acrolein is a common constituent of ambient air pollution and a prototypical airway irritant. It is a small, highly reactive, unsaturated aldehyde produced by the combustion of organic compounds. Because acrolein is so reactive it can bind nucleophiles such as glutathione as well as react with cysteine, histidine and lysine residues to form protein adducts (Kehrer & Biswal 2000). In this way acrolein exposure can produce structural

damage or functional changes which may result in cytotoxicity, oxidative stress and inflammation, as well as react with non-specific binding sites on dermal and airway nociceptive receptor channels. Humans are primarily exposed to acrolein via contact with cigarette smoke, automobile exhaust, structural and wildfire smoke, and some industrial processes including the burning of wood or coal (CDC-ASTDR 2007; Ghilarducci & Tjeerdema 1995). In 1999 estimated ambient acrolein concentrations ranged from a national average of 0.033 ppm to a high of 0.19 ppm (Woodruff et al. 2007; Walsh 2008). Cigarette smoke contains up to 90 ppm acrolein, and acrolein levels in side-stream tobacco smoke have been measured as high as 10 ppm (Esterbauer et al. 1991). The U.S. EPA currently classifies acrolein as a HAP and determined that it represents the leading non-cancer hazard in ambient air pollution causing adverse respiratory health effects (Ris 2007). Acrolein is known to primarily impact the upper airway, with nasal mucosa representing the most sensitive target of inhalation exposure (Shusterman 2012; CDC-ASTDR 2007), although the effects can extend deeper into the respiratory tract as exposure levels increase (CDC-ASTDR 2007). Inhalation of acrolein induces a variety of respiratory effects including nose and throat irritation (Beauchamp, Andjelkovich, Kligerman, Morgan, & Heck, 1985; Sim & Pattle, 1957), apnea (Lee et al. 1992), pulmonary edema (Hales et al. 1989; Kutzman et al. 1985), asthma like symptoms such as increased bronchial responsiveness and respiratory distress (Ben-Jebria et al. 1994; Bein & Leikauf 2011), and has been shown to exacerbate existing asthma (Leikauf et al. 1989). Acrolein exposure has also been shown to produce cardiovascular effects as well (DeJarnett et al. 2014) including cytotoxicity in pulmonary artery endothelial cells and cardiac fibroblasts *in vitro* (Kachel & Martin 1994; Toraason et al. 1989). *In vivo* animal studies have also demonstrated that acute exposure primarily

produces short-lived effects including bradycardia (Lee et al. 1992), while chronic exposure to inhaled acrolein can produce dilated cardiomyopathy (Ismahil et al. 2011) and contributes to the development of thromboses (Sithu et al. 2010; O'Toole et al. 2009).

Mechanisms Linking Pulmonary Exposure to Cardiovascular Effects

Historically, airway and pulmonary sites were believed to be the primary target of air pollution. However, now systemic effects including those on the central nervous system, metabolism and cardiovascular systems have been drawing increasing attention over the last 15 to 20 years (Calderón-Garcidueñas et al. 2007; Mills et al. 2009; Kodavanti 2015; Wagner et al. 2014). In fact, a growing body of evidence demonstrates that elevations in ambient air pollution, particularly PM, results in greater risks for cardiovascular related mortality than for any other causes including respiratory disease (Brook et al. 2010).

Biological mechanisms linking air pollution with adverse cardiovascular outcomes involve both the direct effects of pollutants on cardiovascular tissues and indirect effects mediated through more general pathways including oxidative stress and inflammation. Putative mechanism include 1.) Systemic inflammation and oxidative stress, 2.) The direct effect of agents which may readily pass through the pulmonary epithelium into the blood, and 3.) Perturbation of systemic autonomic nervous system balance by air-pollutant interactions with airway sensory receptors (Simkhovich et al. 2008; Brook et al. 2004; Brook et al. 2010).

Episodes of chronic air pollution exposure can induce sustained local oxidative stress and pulmonary release of pro-inflammatory mediators including cytokines, activated immune

cells, as well as vasoactive molecules (Grunig et al. 2014). Additionally, sustained airway inflammation sensitizes airway sensory nerves, provoking the further release of proinflammatory neurotransmitters and enhancing the proinflammatory state of the airways (Zholos 2015; Lee & Widdicombe 2001). Moreover prolonged periods of oxidative stress can deplete the airway antioxidant capacity, impairing a primary defense mechanism of the airway mucosa and producing further tissue damage (Domej et al. 2014). Over time profound airway inflammation may contribute to a state of systemic inflammation which can disrupt hemostatic pathways and impair vascular function. In fact, the primary adverse cardiovascular outcome linked with this mechanism is the development of atherosclerosis as well as its sequelae (Park et al. 2010; Gill et al.; Provost et al. 2015).

Constituents of air pollution capable of crossing the pulmonary epithelium include gases, soluble constituents of particulate matter such as organic compounds or metals, and possibly ultra-fine particles (Brook 2008). Since oxygenated blood leaving the lungs makes its first pass through the heart before entering systemic circulation the largest effect of translocated constituents of air pollution would be on the myocardium itself (Nemmar et al. 2002; Nemmar et al. 2004).

In addition, the airways are richly innervated with sensory nerves that react to irritating or noxious stimuli and initiate protective physiological responses (Widdicombe 2001; Lee & Pisarri 2001; Coleridge et al. 1983). These responses are mediated by reflexive changes in autonomic nervous system regulation of cardiopulmonary function which originate in the brainstem, as well as release of neurotransmitters which provoke local immune responses (Perez et al. 2015; Lee & Pisarri 2001; Bessac & Jordt 2010). In this way activation of airway sensory nerves by air pollutants can transiently alter autonomic nervous

system balance and cardiovascular function. Under normal physiological conditions, this system ensures that the body maintains its function (e.g. perfusion of tissues, oxygenation, anti-oxidant potential) during changing conditions or even certain extremes. Exposure to an environmental stressor can result in perturbation of internal homeostatic mechanisms including autonomic nervous system controls such that a subsequent trigger might significantly increase the risk of adverse cardiovascular events like arrhythmia (Brook et al. 2004). In individuals with underlying cardiovascular or pulmonary disease, the disruption of autonomic balance may actually result in cardiovascular morbidity or mortality given they already have impaired cardiopulmonary compensatory capacity (Goldberg et al. 2001; Routledge et al. 2003; Brook et al. 2004).

Airway Sensory Nerves

The challenge in understanding the cardiovascular risk attributable to short-term air pollution exposure lies in linking respiratory exposure and cardiovascular response by a mechanism capable of producing immediate and reversible effects, which are often latent. Historically adverse cardiovascular effects following acute exposure to air pollution have been readily observable in populations with underlying cardiovascular disease (Franchini & Mannucci 2007), however cohorts of healthy young individuals have also demonstrated a shift towards a higher risk for adverse cardiovascular events with acute exposure to air pollution (Shields et al. 2013; Allen et al. 2011; Brook et al. 2004), indicating a general mechanism shared by both populations. Activation of neural pathways through airway chemosensors and subsequent disruption of autonomic balance may explain not only the

worsening of existing disease but also sudden impairment in people who are seemingly healthy.

As such, chemosensory nerve endings in the airway mucosa are one of the first lines of defense against noxious chemicals and stimuli. Activation of these "sensors" trigger crucial protective reflexive responses such as coughing, sneezing and the sensation of irritation and pain (Widdicombe 2001; Bessac & Jordt 2008; Carr & Undem 2003). The respiratory system, from the nose into the deep lung, is innervated by multiple nerve types which have the ability to respond to a variety of stimuli including irritating molecules, cold, changes in osmolality, and changes in lung inflation (Perez et al. 2015; Bessac & Jordt 2008). The cell bodies of airway sensory nerve fibers reside in the jugular and nodose ganglia of the vagus nerve and project to the nucleus tractus solitarius (NTS) in the medulla. Afferent activity arising from airway sensory nerves is transmitted into the central nervous system (i.e. brainstem) where the signal is modulated and then eventually results in efferent signaling via autonomic nerves to the viscera (Carr & Undem 2003; Bonham et al. 2006). As mentioned above, this homeostatic system functions tonically on a breath-by-breath basis affecting respiratory and cardiovascular activity (e.g. respiratory sinus arrhythmia) but is also then immediately altered in the event of exposure.

Airway sensory nerve fibers are characterized by 3 primary receptor types including C-fibers, rapidly adapting pulmonary receptors (RARs), and slowly adapting pulmonary receptors (SARs) (Kubin et al. 2006; Perez et al. 2015; Widdicombe 2001). While both RARs and C-fibers can be broadly described as irritant sensors which mediate reflexive pulmonary responses, SARs are stretch receptors located in the airway smooth muscle of the lower airways which respond to inflation of the lungs (Widdicombe 2001). The homeostatic

role of these sensory fibers, as detailed above, is the modulation of HR and cardiac output in response to variation in breathing patterns that are both normal and abnormal (i.e. occurring due to irritation). This includes respiratory sinus arrhythmia (RSA), which is heart rate variability that occurs in synchrony with respiration and causes heart rate to increase during inspiration and decrease during expiration (Perez et al. 2015; Widdicombe 2001). Yet, in terms of air pollution health effects, the most important sensory fibers appear to be the C-fibers, which are characterized by their distinct sensitivity to both exogenous chemical stimuli including sulfur dioxide, acids, cigarette smoke, ozone, acrolein, ammonia and particulate matter, as well as endogenous inflammatory mediators (Kollarik et al. 2010) and which morphologic studies have demonstrated to be seventy-five percent of vagal afferent nerve fibers arising from the respiratory tract (Agostoni et al. 1957).

C-fibers were first identified by Coleridge and Coleridge in 1984 (Coleridge & Coleridge 1984) and since then they have become recognized as important regulators of cardiopulmonary function under both normal and abnormal physiologic conditions. General anatomical and functional features of C-fibers are well conserved across most species in which they have been studied including human, monkey, dog, cat, rabbit, guinea pig, rat, mouse and (Canning & Chou 2009). They are located throughout the respiratory tract including the nose, larynx, trachea, bronchi, and alveoli (Widdicombe 2001); they are usually found just below the airway epithelium but can occasionally be located deeper in the mucosa adjacent to arterioles (Widdicombe 2001). Responses to C-fiber activation are mediated through both central reflex pathways and via local release of neuropeptides (Lee & Pisarri 2001). Intense, rapid C-fiber activation produced by a bolus injection of established C-fiber activators such as capsaicin cause reflex apnea often followed by rapid shallow breathing

(Coleridge & Coleridge 1984), however more moderate levels of pulmonary fiber discharge, akin to the tonic level of pulmonary C-fiber activation physiologically present without robust exogenous stimulation, produces an increase in respiratory rate and a decrease in tidal volume (Lee & Pisarri 2001). C-fiber activation also causes reflex bronchoconstriction and airway mucus secretion (Lee & Pisarri 2001). Cardiovascular reflex responses to C-fiber activation include bradycardia along with systemic and pulmonary hypotension (Perez et al. 2015; Lee & Pisarri 2001) all of which are primarily mediated through enhanced vagal modulation through the brainstem (Lee et al. 1992). On the other hand, local effects in the airways are mediated via the release of neuropeptides including substance p, neurokinin A and calcitonin gene related protein neuropeptides (Saria et al. 1988); these include bronchoconstriction and the development of pulmonary edema, and induction of neuroinflammation (Lee & Pisarri 2001; Kollarik et al. 2010).

A common feature of all responses to C-fiber activation is that they act to prevent noxious stimuli, or at the very least limit its impact (Bessac & Jordt 2010; Lee et al. 1992; Coleridge & Coleridge 1984). Reflexive bronchoconstriction and decreased tidal volume reduce inhalation of the irritant into the lung while enhanced mucus secretion protects the airway mucosa. Increased breathing rate may aid in the removal of irritants from the deep lung, and decrease of heart rate reduces oxygen demands until normal physiological function can be resumed. Despite their robust response to noxious stimuli bronchopulmonary C-fibers are generally quiescent in healthy airways (Canning & Chou 2009; Kollarik et al. 2010). However under abnormal physiological conditions, particularly with persistent airway inflammation C-fibers become sensitized and produce enhanced responses (Lee & Pisarri

2001). Enhanced C-fiber excitability is believed to play a role in the pathophysiology of number of chronic airway diseases including asthma and COPD.

TRPA1

Transient receptor potential ion channels (TRP) are expressed throughout the upper and lower airways on trigeminal and vagal afferent C-fibers. In 1997 successful cloning of the TRP capsaicin, or vanilloid receptor, TRPV1 was reported (Caterina et al. 1997) and immunohistochemical studies confirmed the presence of TRPV1 on the airway sensory fibers in the nasal mucosa, trachea, bronchi and alveoli (Bessac & Jordt 2008). These discoveries started a new wave of chemosensory research with hundreds of studies dedicated to the identification of TRP family members, their pharmacology, structure, function, and physiological role in chemosensation and nociception in the airways (Eid & Cortright 2009). Initially identified in 1999 as ANKTM1, a theoretical protein expressed in cultured fibroblasts (Jaquemar et al. 1999), TRP ankyrin 1 (TRPA1) was rediscovered in 2003 as a cold-activated TRP channel found in a subset of nociceptive sensory nerve fibers where it is co-expressed with TRPV1 (Story et al. 2003).

TRPV1 and A1 are both cation-permeable channels that act as chemical sensors and react to a variety of exogenous and endogenous compounds, however activation of these channels produce sensations with divergent effects (Wetsel 2011). TRPV1 is known to be a key mediator of thermal and pain sensation. Activation by capsaicin, a well described agonist, produces a burning sensation. TRPV1 is also activated by painfully hot thermal stimuli (Story 2006; Caterina et al. 1997). Conversely, TRPA1 is activated by painfully cold

stimuli, but also displays activation by a variety of noxious compounds, many of which evoke cold and pain sensations (Story et al. 2003).

TRPA1 is the only member of the TRPA subset expressed in mammals. It is expressed in 20-35% of sensory neurons (Bessac & Jordt 2008). Structurally it is characterized by a large number of ankyrin repeats on the long cytoplasmic N-terminal domain. Four identical TRPA1 subunits combine to form the functional channel (Caterina 2007), however recently there has been evidence to suggest functional TRPA1:TRPV1 heteromers (Fischer et al. 2014). Whether or not TRPA1 regularly combines with TRPV1 to form heteromers, it has been repeatedly demonstrated that TRPA1 and TRPV1 channels interact both under normal physiological conditions and in disease states (Bessac & Jordt 2008; Staruschenko et al. 2010; Salas et al. 2009). Activation of TRPA1 is thought to occur primarily via covalent modification of cystine residues on the N-terminal domain (Hinman et al. 2006; Macpherson et al. 2007; Caterina 2007). Activation via a non-specific mechanism such as cysteine modification allows for the wide variety of known TRPA1 activators including structurally diverse substances found in industrial chemicals, environmental exposures, natural products, pharmaceuticals and endogenous molecules, particularly oxidizing agents and electrophiles (Bessac et al. 2008). As many TRPA1 agonists can react with thiols, antioxidant levels in the airways, particularly glutathione, can modulate the efficacy of TRPA1 activating airway irritants (Bessac et al. 2008; Lee & Widdicombe 2001). Poor antioxidant capacity or complete depletion allows for increasing numbers of covalent cysteine modifications with every breath of inhaled agonist. In this way irritant-induced TRPA1 activity may be heightened, even in low-level, sub-chronic exposures scenarios.

In 2006 a TRPA1 knockout mouse was developed (Kwan et al. 2006). These mice were generated by deleting exons that code for the pore domain of the ion channel, rendering the channels non-functional. Constitutive knockout animals were backcrossed to the C57BL/6J line for over 10 generations to produce a TRPA1 knockout animal that is essentially in the C57BL/6K genetic background. These mice demonstrate no behavioral responses to topical application of mustard oil, cold (0 C), or punctate stimuli. They do however demonstrate normal startle reflex to loud noise, a normal sense of balance, and normal auditory brainstem response (Kwan et al. 2006). It has also been reported that TRPA1 knockout mice not respond to inhaled oxidants but do respond predictably to the non-TRPA1 specific C-fiber irritant acetic acid (Bessac et al. 2008). Implying that the model's response to non-TRPA1-activating exposure types is not impaired.

TRPA1 has been investigated thoroughly in its role as a nociceptor in dermal pain pathways, particularly with regard to cold or noxious chemical sensation, and neuroinflammation (Bautista et al. 2006; Bodkin & Brain 2011; Guimaraes & Jordt 2007). Because of this TRPA1 presents a ready target in the development of anti-inflammatory drugs and analgesics (Brederson et al. 2013; Chen et al. 2011). It is also fairly well understood as an airway sensor (Bautista et al. 2006) and has been suggested previously as a mediator of cardiovascular effects via reflex modulation of autonomic reflex function (Hooper et al. 2015; Pozsgai et al. 2010). More recently a direct role for TRPA1 in cardiovascular function has been investigated and found that TRPA1 expressed in the vasculature may act as a cold and/or oxidant sensor there as well as contribute to autonomically mediated vasodilatory responses (Aubdool et al. 2014; Pozsgai et al. 2010; Bodkin et al. 2014; Earley 2012; Graepel et al. 2011).

Due to its presence on pulmonary C-fibers known to initiate autonomic reflex arcs upon activation, its propensity toward activation by a large variety of substances commonly found in ambient air pollution including acrolein, and the availability of a knockout mouse model, TRPA1 represents a prime target for research as a mediator of airway irritant induced cardiovascular dysfunction via the perturbation of autonomic balance and homeostasis.

Autonomic Control of the Heart

The autonomic nervous system is the division of the peripheral nervous system responsible for control of bodily functions not consciously directed, such as breathing, the heartbeat, and digestive processes. It is split into two branches: sympathetic and parasympathetic. In situations where an organism encounters a stressor the sympathetic nervous system dominates; heart rate and respiration increase, blood pressure increases, and blood is redirected to muscles and organs important to the "fight or flight" response and away from non-essential areas such as the gut. When the stressor is removed and the organism is returning to a resting state the parasympathetic nervous system dominates; heart rate and respiration slow, and activity in the gut recommences, hence the phrase "rest and digest". While the actions of the sympathetic and parasympathetic branches of the autonomic nervous system do oppose each other to some extent, it is not a reciprocal system: when one rises the other does not automatically fall. There can be co-activation or co-depression or any degree of activation of either branch simultaneously. Furthermore the actions of either branch of the autonomic nervous system are not systemically uniform; even in a resting state different tissues and organ systems are variably affected by either sympathetic or parasympathetic modulation. For example, in humans, control of heart rate is

primarily parasympathetically dominated while the vasculature is controlled by the sympathetic branch. Overall the important aspect of the relationship between the actions of the sympathetic and parasympathetic nervous systems is that they are coordinated such that cardiovascular homeostasis is maintained.

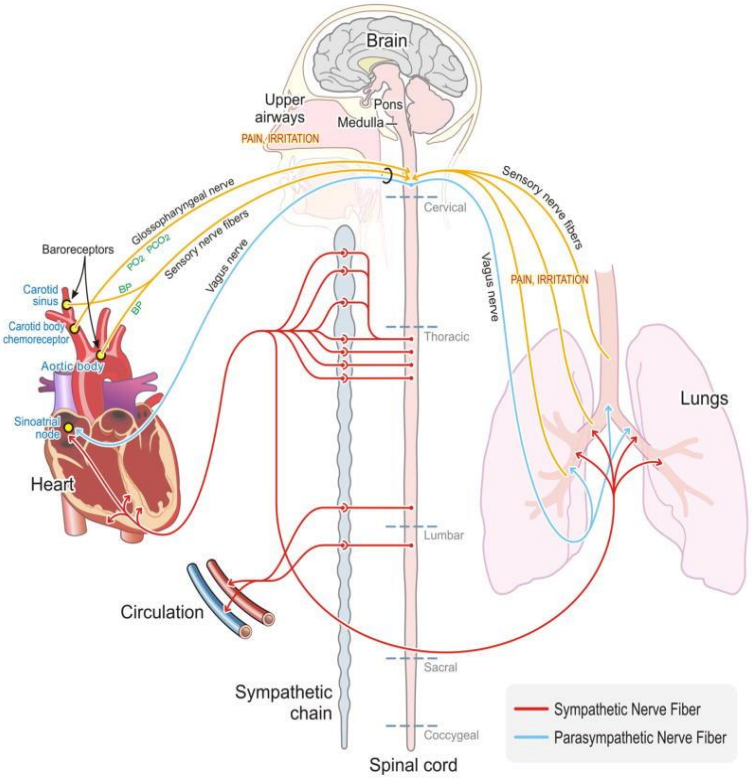
Like all vital bodily systems the cardiovascular system is dynamic, reacting moment to moment to both internal and environmental stimuli and adjusting physiological parameters such as heart rate, contractility, and blood pressure to ensure sufficient systemic perfusion and meet the ever changing metabolic demands of the body. The regulation of this complex balancing act is traditionally described in terms of homeostasis. This word comes from the Greek *homeo* (similar) and *stasis* (steady) and refers to the body's ability to return toward a relatively stable internal set point, even while continually subjected to external changes. An important aspect of cardiovascular homeostasis is heart rate. Heart rate is normally determined by periodic depolarization of the sinoatrial (SA) node, the frequency of which is primarily modulated by both the sympathetic and parasympathetic branches of the autonomic nervous system. Additionally, physiological feedback from the intrinsic cardiac nervous system, autonomic reflexes including baroreflex, chemoreflex and even irritant reflexes originating in the airways, thermal regulation and respiration can all contribute to beat by beat adjustments to heart rate (Figure 1.) (Perez et al. 2015; Perini & Veicsteinas 2003; Purves et al. 2001). This dynamic nature of the cardiovascular system directs our attention not only to average or baseline heart rate values, which can be regarded as the reference set point, but also to perturbations of this average as various studies have demonstrated these fluctuations are indeed much more than noise. As such, the study of heart rate variability

(HRV) represents a valuable source of information that can provide insight into the mechanisms of cardiovascular control (Appel et al. 1989).

Heart Rate Variability

HRV has become an increasingly popular endpoint for analysis of autonomic function, because of its non-invasive nature and because of its applicability in both humans and animal models including rodents (Farraj, Hazari & Cascio 2011; Rowan et al. 2007; Thireau et al. 2008). Since a simple ECG readout and HR analysis is all that is required for HRV calculation it is now used commonly as a prognostic indicator in clinical settings (Anon 1996) and in commercially available software used in the fields of psychology and sports medicine. Two broad approaches to calculating HRV are time and frequency domain analysis. Time domain analysis are basic statistical analyses of normalized RR intervals: the standard deviation of subsequent normalized RR intervals (SDNN) and the root mean square of the standard deviation of subsequent normalized RR

Figure 1. Multiple autonomic and neural pathways contribute to the body's response to stimuli



Perez et al., 2015. Cardiovasc Toxicol. 2015

intervals (RMSSD). SDNN represents all cyclic components contributing to variability, therefore representing an overall measure of HRV while RMSSD more specifically correlates with parasympathetic modulation (Rowan et al. 2007). HRV can also be calculated via frequency domain analysis whereby high or low frequency bands are assigned and power spectral density analysis is performed using a fast Fourier transformation. This provides basic information regarding the power across a low frequency (LF) vs high frequency (HF) bands. LF power is believed to represent a mixture of sympathetic and parasympathetic components as well as inputs from mechanisms governing blood pressure regulation (Reyes del Paso et al. 2013; Goldstein et al. 2011; Rahman et al. 2011; Moak et al. 2007) while HF power again correlates with parasympathetic modulation but likely also represents inputs from changes in cyclic ventilatory patterns (Billman 2013; Reyes del Paso et al. 2013; Chess et al. 1975; Piccirillo et al. 2009; Heathers 2014).

Changes in HRV have been observed in various stressful circumstances including during exercise (Perini & Veicsteinas 2003; Baumert et al. 2006), in response to fearful stimuli (Liu et al. 2014) and in response to perceived stress (Dishman et al. 2000). Both acute and chronic stress have been shown to induce an overall decrease in HRV whereby some combination of increased sympathetic modulation and vagal withdrawal occurs (Liu et al. 2014; Schubert et al. 2009). With repeated stressful stimulus however habituation is observed, attenuating the intensity and duration of the HRV response (Liu et al. 2014).

Under normal conditions heart rate, which is governed by a variety of regulatory mechanisms (e.g. ANS, pre-load, after-load, circulating peptides), displays robust R-R interval variability which allows the heart to effectively adapt to external stressors and maintain homeostasis. Thus, a shift towards stronger HR regularity may indicate decreased

responsiveness to both external stimuli and internal control mechanisms (Rahman et al. 2002; Lipsitz & Goldberger 1992; Pincus 1994; Schubert et al. 2009), representing a risk for adverse cardiovascular events due to reduced compensatory capacity. In fact it has been demonstrated that decreased HRV occurs over a broad range of cardiovascular illnesses and injury (Gang & Malik 2003; Thayer et al. 2010; Tsuji et al. 1996). Reduced HRV has been associated with increased risk of fatal arrhythmias, heart attack and stroke, particularly in populations with existing cardiovascular disease (Ponikowski et al. 1997; Valkama et al. 1995; Kop et al. 2001). Significantly, low HRV has also been associated with adverse cardiovascular events in apparently healthy populations as well (Hillebrand et al. 2013). Although classically low HRV has been accepted as an indicator of heightened cardiovascular risk, elevated HRV has also been linked to adverse cardiovascular outcomes (Stein et al. 2005; Amar et al. 2003; Schwartz et al. 2011; Eguchi et al. 2010). Specifically, increased HRV, indicating enhanced vagal modulation, has been implicated in a group of heart rhythm disorders that involve impaired SA node function termed sick sinus syndrome (SSS) which is characterized by severe sinus bradycardia, often with alternating tachycardia, SA node arrest or block (Keller & Lemberg 2006; Brignole 2002; Adán & Crown 2003; Gladuli et al. 2011; Semelka et al. 2013). Symptoms can occur in intermittent periods or chronically, and depending on severity of disease may produce dizziness and syncope. There are a number of pathophysiologies which could produce SSS. It is often idiopathic and the result of degenerative diseases affecting nodal tissue (Keller & Lemberg 2006). Conversely, sinus node dysfunction is also associated with diseases affecting the autonomic nervous system (Brignole 2002; Semelka et al. 2013), thus abnormal parasympathetic modulation and

resulting ECG features may be originating in the central nervous system (rather than degeneration of SA node itself).

Ambient air pollution, depending on its physical and chemical composition, has the capacity to act as a potent chemical stressor and has been shown to elicit cardiac autonomic responses (Arjomandi et al. 2015; Gold et al. 2000; Pope et al. 1999; Godleski et al. 2000). While decreased HRV is a commonly accepted indicator of heightened cardiovascular risk following air pollution exposure in humans (Gold et al. 2000), increases HRV have also been demonstrated (Chen & Hwang 2005; Bortkiewicz et al. 2014). For example, increased RMSSD has been shown to be associated with elevated risk of air pollution-induced arrhythmia (Davoodi et al. 2010) In fact our lab has consistently shown that exposure to different types of air pollutants such as residual oil fly ash (Farraj, Hazari, Haykal-Coates, et al. 2011; Carll et al. 2015) diesel exhaust (Hazari et al. 2011; Carll et al. 2013), ozone (Farraj et al. 2012) and acrolein (Hazari et al. 2014) all lead to an increase in HRV in rodent models. Considering that parasympathetic modulation is a well-characterized reflex response to airway sensory activation (Lee & Pisarri 2001; Alarie 1973; Bessac & Jordt 2010) these results are not surprising.

Interestingly there is also evidence that abnormal variation in heart rate and heart rate variability as seen with enhanced vagal tone (and sick sinus syndrome) has a destabilizing effect on the atrial tissue (Shetty & Scott 2015) which can contribute to the pathophysiological progression of cardiac disease or injury. So, when we are considering sensitive subpopulations, such as those with underlying cardiac disease, the capability of air pollution to induce these types of changes not only increases their risk for adverse cardiac

events but through this mechanism can exacerbate and may also physically worsen their condition.

Taken together it is apparent that any perturbation of homeostatic balance has the potential to increase risk of adverse cardiovascular events if the shift is drastic enough or combined with other perturbation causing stimuli. In that sense, HRV changes in either direction might indicate that sympathetic and parasympathetic nervous systems are not properly coordinating to provide an appropriate heart rate response to environmental stressors or internal cues.

TRPA1: Connecting the Airways and Heart through Central Neural Circuits

Linking all these processes, the activation of TRPA1, neuro-modulation in the brainstem and autonomic effects, including those on the heart, is substance P, a tachykinin neuropeptide involved in numerous neuro-modulatory processes. Activation of TRPA1 causes a release of Substance P in the airways (Saria et al. 1988), which produces its own set of local effects, while transmission of the signal via afferent airway sensory C-fibers to the NTS releases substance P there as well (Morilak et al. 1988). This secondary release of substance P in the NTS upon activation of airway C-fibers has been shown to modulate efferent signaling and contribute to changes in autonomic nervous system function. This connection was previously described with respect to respiratory responses to airway irritants whereby activation of airway sensory nerves by side-stream cigarette smoke produced excitability of lung sensory afferent fibers, this in turn caused substance P release and increased excitability at the NTS synapses and finally enhanced respiratory reflex responses (Mutoh, Joad, et al. 2000; Bonham et al. 2001). Furthermore it has been demonstrated that

the NTS plays a critical role in the integration of a wide range of peripherally initiated sensory information in addition to airway receptor activation, including baroreflex and chemoreflex receptors activity, as well as the central control of cardiovascular function (Lawrence & Jarrott 1996; Potts). Thus it is reasonable to assert that cardiovascular and respiratory reflex responses to C-fiber activation are similarly mediated via substance P release in the NTS. It is this arc of neural activity (Figure 2.) that may determine the final cardiovascular outcome. The key question as research moves forward in this field is how long do these effects persist and how can the key mediators be identified in those most susceptible? With regards to the latter, I hypothesize that alteration of homeostatic mechanisms like autonomic control by air pollution exposure, however short-lived, will alter those regulatory processes that employ autonomic efferent signals to maintain a given function. For example, we have already demonstrated that acrolein exposure desensitizes the baroreflex response by blunting the sympathetic outflow to the heart (Hazari et al. 2014). Thus, it may not necessarily be the exposure that directly causes the adverse response but rather some other subsequent event that further "pushes" the body out of balance.

Summary

Although air quality has consistently improved over the last few decades, ~40% of the US population still lives in areas that meet or exceed National Ambient Air Quality Standards (NAAQS) for at least one primary air pollutant as established by the U.S. Environmental Protection Agency (EPA)(Anon n.d.). Center for Disease Control (CDC) reports additionally state that ~11.5% of the adult, non-institutionalized population in the U.S. has a diagnosed cardiovascular disease (CDC 2016). Recent studies demonstrating the

relationship between high air-pollution days and a significant increase in hospital visits for serious cardiovascular events, particularly in populations with existing cardiovascular disease, highlight the need for a more complete understanding of the mechanisms underlying this relationship (Colais et al. 2012; Dockery et al. 1993; Pope et al. 2004). On the other hand, when air pollution levels are not exceedingly high, or in the case of acute exposure, it is assumed that the effects are minimal or even non-existent, especially when clinically observable symptoms are lacking. As such, subtle effects may go unnoticed but nonetheless represent a serious risk (Hazari et al. 2011). At these lower and seemingly benign air pollution concentrations or durations, and in the absence of host perception of an effect, it would be suitable to examine the role of airway sensory nerves which not only "sense" the toxicant but also cause internal homeostatic changes that may predispose to cardiopulmonary dysfunction. Airway sensors, particularly TRPA1, located on airway C-fibers have been demonstrated to play a major role in airway reflex responses, and have been suggested as possible mediators of some cardiovascular responses as well (Hazari et al. 2011; Jones et al. 1995; Lee 2010; Wang et al. 2000)(Lee 2010; Wang et al. 2000; Jones et al. 1995; Hazari et al. 2011). The studies presented herein explore TRPA1 as a major sensor of airway irritant pollutants and address the mechanisms through which it produces autonomic imbalance and adverse cardiovascular effects following exposure to air pollution.

Purpose of Research and Global Hypothesis

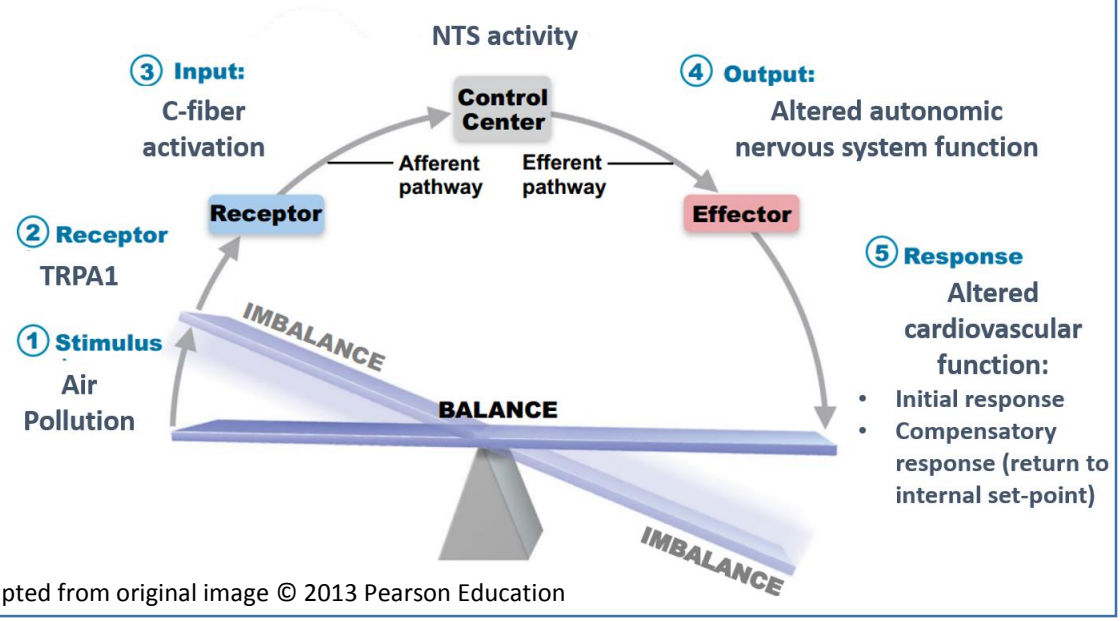
Although acute exposure to air pollution has been firmly associated with an increased risk of adverse cardiovascular events, the precise mechanisms of toxicity may not be easy to identify given they are quite numerous and often co-exist. Time-series studies indicate that

the risk of cardiac events and arrhythmias increase significantly in the hours and days immediately following air pollution exposure. Yet, epidemiological studies by their nature cannot quantify the physiological response in humans during exposure; this is crucial information in air pollution toxicology because it can shed light on the early initiating events resulting from exposure which influence and direct subsequent pathways in the hours and days following exposure. Although numerous biological pathways have been proposed by which air pollution may cause cardiovascular events, airway sensory activation may be the most relevant when considering the short-term, reversible, and often latent effects of an acute exposure. Toxic air pollutants, including particulate matter, acrolein, and ozone stimulate sensory nerves in the airways causing several well-described ventilatory and pulmonary effects, however the cardiovascular responses have not been sufficiently characterized.

A subset of these responses are known to be mediated by the transient receptor potential cation A1 (TRPA1) channels present on chemosensitive C-fibers. Thus, TRPA1 represents a likely candidate as an initiator of air-pollution induced cardiovascular dysfunction. The mechanism through which airway sensory nerve activation perturbs cardiopulmonary function has been broadly described as occurring through modulation of autonomic nervous system function, however specific changes in measures of autonomic modulation have not yet been clearly characterized or linked with changes in cardiopulmonary function. Thus my global hypothesis for this dissertation is that cardiac dysfunction following a single acute exposure to air pollution is in part driven by airway TRPA1 activation and that autonomic imbalance initiated during exposure may contribute to the changes observed up to one day after exposure (Figure 2.). The primary purpose of this research is to better understand the specific autonomic mechanisms underlying air pollution

induced cardiotoxicity, which will contribute relevant data to improve assessments of risk, as well as identify potentially sensitive subpopulations and define windows of heightened susceptibility following exposure.

Figure 2. Acute air pollution exposure produces cardiovascular dysfunction via autonomic imbalance mediated by TRPA1



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In order to address this hypothesis the following specific aims were established:

Aim 1) Characterize decrements in cardiac mechanical and electrical function observed in the mouse with exposure to air pollution. The aim of this study was to determine the independent effects of fine (FCAPs) and ultrafine (UFCAPs) concentrated ambient particles on cardiac function in C57BL/6 mice, and explore whether ozone (O_3) co-exposure would alter the response. These experiments examined a wide range of cardiac endpoints including electrical function and rhythm, which were assessed via electrocardiogram (ECG) analysis, mechanical properties and post-ischemia recovery, which were measured using a Langendorff technique, as well as biochemical indicators of cardiac

toxicity. This work fills a unique gap in the existing literature as few studies, if any, have examined the effects of simultaneous particulate matter (PM) and O_3 exposure on both ECG and mechanical function (e.g. contractility) of the heart. Risk assessments of air pollution health effects have become increasingly challenging given the complexity of present-day air pollution mixtures. Previous data suggests that PM size determines the physiological impact with fine PM causing primarily pulmonary effects and ultrafine PM altering cardiac function. However, air pollution is a mixture of not only PM, but also gaseous irritants, vapors, and biological substances. Thus when examining the effects of a given pollutant, the influence of other components must be considered, and so studies are needed to determine whether the physiological and biochemical responses to multipollutant exposures represent the simple additive effects of the pollutants, their synergism or antagonism. One particularly relevant interaction is that of PM and the ubiquitous gaseous co-pollutant O_3. Thus, we hypothesized (1) that inhalation of either fine (FCAPs) or ultrafine CAPs (UFCAPs) would cause cardiac electrical dysfunction, mechanical decrements and arrhythmogenesis in mice; but (2) that UFCAPs, due to its size, would have a greater effect on the heart than FCAPs; and (3) that O_3 co-exposure would potentiate the response elicited by both particle sizes.

Aim 2) Determine the role of TRPA1 in acute cardiovascular dysfunction during exposure to air pollution. Wild-type C57BL/6 (WT) and TRPA1 knockout (KO) mice were exposed to prototypical airway irritants acrolein and ozone to determine the role of TRPA1 in air pollution-induced cardiovascular dysfunction. The cardiovascular response during exposure was characterized using radiotelemetry to record continuous electrocardiogram (ECG) in conscious, unrestrained animals, using whole body exposure chambers. EGC

interval analysis, heart rate variability (HRV) analysis, and identification and quantification of arrhythmia were the primary endpoints. Potential pollutant-driven alterations in cardiac mechanical function were evaluated post-exposure using a Langendorff cardiac perfusion preparation. The majority of research on TRP channels done thus far has utilized in-vitro methods or isolated organ perfusion systems. While these techniques provided a great deal of insight regarding channel activation and elucidation of the neural pathways potentially involved in the adverse cardiopulmonary response to airway nerve activation, physiological endpoints in the whole organism could only be inferred. Furthermore, the few studies identified which used whole organism, including the TRPA1 knockout mouse, generally anesthetized the animal prior to exposure, potentially confounding a reflex response. Previous studies from this lab have demonstrated that pharmacological inhibition of TRPA1 attenuated the development of diesel exhaust-induced arrhythmia. Thus, this study sought to further establish a role for TRPA1 in air pollution induced cardiac dysfunction and we hypothesized that adverse cardiac electrical and mechanical responses due to acrolein or ozone were mediated by TRPA1.

Aim 3) Characterize baseline autonomic tone and the effects of pharmacological inhibition of sympathetic or parasympathetic neurotransmission in wild-type and TRPA1 knockout mice with exposure to airway irritants.

HRV is an indirect measure of autonomic influence on the heart which represents input from homeostatic control mechanisms that dynamically regulate cardiovascular and respiratory function. My previous studies demonstrate that air pollution exposure modulates cardiac function and increases arrhythmia in mice through altered autonomic balance. To

fully determine the degree of autonomic modulation which results from TRPA1-mediated sensory activation in the airways we need to better understand baseline cardiac autonomic properties in our model, as well as how these measures change with exposure. Hence, I explored how pharmacological blockade of each autonomic branch affects measures of HRV as well as the cardiac response to air pollution in mice. Wild-type C57BL/6 (WT) and TRPA1 (KO) mice were treated with one of three pharmacological agents producing either sympathetic blockade, parasympathetic blockade, or blockade of both branches of the autonomic nervous system, before being exposed to filtered air or acrolein. We hypothesized that pharmacological disruption of normal autonomic balance in the mouse would disrupt basic cardiovascular function as shown by heart rate and heart rate variability as well as blunt the cardiac response to acrolein.

CHAPTER II: OZONE CO-EXPOSURE MODIFIES CARDIAC RESPONSES TO FINE AND ULTRAFINE AMBIENT PARTICULATE MATTER IN MICE: CONCORDANCE OF ELECTROGRAM AND MECHANICAL RESPONSES

Studies have shown a relationship between air pollution and increased risk of cardiovascular morbidity and mortality. Due to the complexity of ambient air pollution composition, recent studies have examined the effects of co-exposure, particularly particulate matter (PM) and gas, to determine whether pollutant interactions alter (e.g. synergistically, antagonistically) the health response. This study examines the independent effects of fine (FCAPs) and ultrafine (UFCAPs) concentrated ambient particles on cardiac function, and determine the impact of ozone (O_3) co-exposure on the response. We hypothesized that UFCAPs would cause greater decrement in mechanical function and electrical dysfunction than FCAPs, and that O_3 co-exposure would enhance the effects of both particle-types. Conscious/unrestrained radiotelemetered mice were exposed once whole-body to either 190 $\mu g/m^3$ FCAPs or 140 $\mu g/m^3$ UFCAPs with/without 0.3 ppm O_3; separate groups were exposed to either filtered air (FA) or O_3 alone. Heart rate (HR) and electrocardiogram (ECG) were recorded continuously before, during and after exposure, and cardiac mechanical function was assessed using a Langendorff perfusion preparation 24 hrs post-exposure. FCAPs alone caused a significant decrease in baseline left ventricular developed pressure (LVDP) and contractility, whereas UFCAPs did not; neither FCAPs nor UFCAPs alone caused any ECG changes. O_3 co-exposure with FCAPs caused a significant decrease in heart

rate variability when compared to FA but also blocked the decrement in cardiac function. On the other hand, O_3 co-exposure with UFCAPs significantly increased QRS-interval, QTc and non-conducted P-wave arrhythmias, and decreased LVDP, rate of contractility and relaxation when compared to controls. These data suggest that particle size and gaseous interactions may play a role in cardiac function decrements one day after exposure. Although FCAPs + O_3 only altered autonomic balance, UFCAPs + O_3 appeared to be more serious by increasing cardiac arrhythmias and causing mechanical decrements. As such, O_3 appears to interact differently with FCAPs and UFCAPs, resulting in varied cardiac changes, which suggests that the cardiovascular effects of particle-gas co-exposures are not simply additive or even generalizable. Additionally, the mode of toxicity underlying this effect may be subtle given none of the exposures described here impaired post-ischemia recovery.

Introduction

Risk assessments of air pollution health effects have become increasingly challenging given the complexity of present-day air pollution mixtures. Epidemiological studies indicate that fine ($PM_{2.5}$) and ultrafine ($PM_{0.1}$) particulate matter (PM) are the principal instigators of adverse clinical events, particularly those involving the cardiovascular system (Brook et al. 2010). However, air pollution is a mixture of not only PM, but also gaseous irritants, vapors, and biological substances; thus when examining the effects of any given pollutant, the influence of other components must be considered. As such, studies need to determine whether the resultant physiological and biochemical effects of multipollutant exposures represent the simple additive effects of the pollutants, their synergism or antagonism. One particularly relevant interaction is that of PM and the ubiquitous gaseous co-pollutant O_3.

Although studies have examined the effects of sequential exposures, for example, ozone (O_3) and then $PM_{2.5}$ causes decreased HRV, systolic blood pressure and heart rate (HR) in rat (Wang et al. 2013), only a few studies have addressed the health effects of simultaneous exposures with distinct pollutants and the effects are still not fully clear. For instance, Brook et al. demonstrated acute arterial vasoconstriction in healthy subjects co-exposed to $PM_{2.5}$ and O_3 (Brook et al. 2002), whereas Urch et al. (Urch et al. 2005) found no significant changes in mean arterial pressure, systolic blood pressure or HR in a similar study population; although constriction was observed with $PM_{2.5}$ alone. Animal studies also indicate that the effect of combining pollutants does not necessarily yield the expected synergistic response, especially in the case of susceptible models. Wagner et al. recently showed that depression of heart rate and blood pressure during $PM_{2.5}$ and O_3 co-exposure was not as great as either pollutant alone in rats fed a high-fructose diet (Wagner et al. 2013). The respiratory effects of O_3 and PM co-exposure are equally conflicting. For example, rats instilled with ozonized DEP had increased inflammatory cells and protein in the lungs (Madden et al. 2000), whereas mice co-exposed to O_3 and DEP did not have increased cytotoxicity or inflammation (Farraj et al. 2010). Instead, in this latter study, co-exposed mice had increased bronchoconstriction, which is a measure of lung function. Similar investigations into the effects of simultaneous exposure on cardiac function have not been widely conducted.

Rodent electrocardiograms (ECG) can provide valuable insight into cardiovascular function in air pollution studies, particularly when pollutant concentrations are low and overt inflammation or toxicity are not observed. ECG is now routinely used in rodents for the detection of disturbances in myocardial impulse formation and conduction, as well as abnormal cardiac rhythm and altered autonomic regulation of the heart. As such, a wide range of cardiac

responses demonstrated by controlled human and animal PM exposure studies have provided biological plausibility to the health effects of air pollution (Brook et al. 2010; Dockery et al. 1993; Kodavanti et al. 2011; Peters 2005). Some of these are responses observed using ECG and have been shown to be similar in humans and animals (Hazari, Callaway, Winsett, Lamb, Haykal-Coates, et al. 2012; Mills et al. 2007). For instance, some human subjects exposed to PM have decreased heart rate variability (HRV), which is a predictor of increased risk (Tsuji et al. 1996; Tsuji et al. 1994; Gold et al. 2000; Gong et al. 2004), and enhanced arrhythmogenesis (Zareba et al. 2009). Experiments in animals not only show a similar PM-induced decrease in HRV and increased incidence of arrhythmia (Brook et al. 2010), but also functional decrements in the heart such as diminished left ventricular developed pressure (LVDP) and decreased contractility (Chen & Hwang 2005; Gurgueira et al. 2002; Hwang et al. 2008). On the other hand, few studies, if any, have examined the effects of simultaneous PM and O_3 exposure on both ECG and mechanical function (e.g. contractility) of the heart.

Thus, the purpose of this study was to determine the effects of concentrated ambient particles (CAPs), with and without O_3 co-exposure, on cardiac electrical and mechanical function in mice. Previous data suggests that PM size determines the physiological impact with fine PM causing primarily pulmonary effects and ultrafine PM altering cardiac function (Tong et al. 2010; Amatullah et al. 2012). We hypothesized (1) that inhalation of either fine (FCAPs) or ultrafine CAPs (UFCAPs) would cause cardiac electrical dysfunction, mechanical decrements and arrhythmogenesis in mice; but (2) that UFCAPs, due to its size, would have a greater effect on the heart than FCAPs; and (3) that O_3 co-exposure would potentiate the response elicited by both particle sizes, respectively.

Materials and Methods

Animals - Ten to twelve-week old female C57BL/6 mice (body weight = 21.6 ± 0.1 g) were used in this study (Jackson Laboratory - Bar Harbor, ME). Mice were initially housed five per cage and maintained on a 12-hr light/dark cycle at approximately 22°C and 50% relative humidity in an AAALAC–approved facility. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum. Each mouse implanted with a radiotelemeter was singly housed after surgery. All protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency and are in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The animals were treated humanely and with regard for alleviation of suffering.

Experimental Groups - Mice were randomly assigned to one of six exposure groups: (1) fine concentrated ambient particles (FCAPs); (2) ultrafine CAPs (UFCAPs); (3) ozone (O_3); (4) FCAPs and O_3 co-exposure (FCAPs + O_3); (5) UFCAPs and O_3 co-exposure (UFCAPs + O_3); and (6) filtered air (FA). Each group had n = 6. Separate groups (same as above) of mice were used for Langendorff cardiac perfusion experiments (n = 5-8).

Surgical implantation of radiotelemeters - Animals were weighed and then anesthetized using inhaled isoflurane (Isothesia, Butler Animal Health Supply, Dublin OH). Anesthesia was induced by spontaneous breathing of 2.5% isoflurane in pure oxygen at a flow rate of 1 L/min and then maintained by 1.5% isoflurane in pure oxygen at a flow rate of 0.5 L/min; all animals received the analgesic Buprenorphrine (0.03 mg/kg, i.p. manufacturer). Briefly, using aseptic technique, each animal was implanted subcutaneously with a radiotelemeter

(ETA-F10, Data Sciences International, St Paul, MN); the transmitter was placed under the skin to the right of the midline on the dorsal side. The two electrode leads were then tunneled subcutaneously across the lateral dorsal sides; the distal portions were fixed in positions that approximated those of the lead II of a standard electrocardiogram (ECG). Body heat was maintained both during and immediately after the surgery. Animals were given food and water post-surgery and were housed individually. All animals were allowed 7-10 days to recover from the surgery and reestablish circadian rhythms.

Radiotelemetry data acquisition - Radiotelemetry methodology (Data Sciences International, Inc., St. Paul, MN) was used to track changes in cardiovascular function by monitoring heart rate (HR), and ECG waveforms immediately following telemeter implantation, through exposure until 24 hours post-exposure. This methodology provided continuous monitoring and collection of physiologic data from individual mice to a remote receiver. Sixty-second ECG segments were recorded every 5 minutes during the pre- and post-exposure periods and continuously during exposure (baseline and hours 1-4); HR was automatically obtained from the waveforms (Dataquest ART Software, version 3.01, Data Sciences International, St. Paul, MN, USA).

Electrocardiogram analysis - ECGAuto software (EMKA Technologies USA, Falls Church VA) was used to visualize individual ECG waveforms, analyze and quantify ECG segment durations and areas, as well as identify cardiac arrhythmias as previously described (Hazari et al. 2014). Briefly, using ECGAuto, P-wave, QRS complex, and T-wave were identified for individual ECG waveforms and compiled into a library. Analysis of all experimental ECG waveforms was then based on established libraries. The following parameters were

determined for each ECG waveform: PR interval (P_{start}-R), QRS complex duration (Q_{start}-S), ST segment interval (S-T_{end}) and QT interval (Q_{start}-T_{end}). QT interval was corrected for HR using the correction formula for mice $QTc = QT/(RR/100)^{1/2}$(Mitchell et al. 1998). Figure 3A and B show a typical ECG trace as well as a typical non-conducted p-wave (NCPW) arrhythmia, which indicates an intermittent atrioventricular block, as observed in mice, respectively.

HRV Analysis - Heart rate variability (HRV) was calculated as the mean of the differences between sequential RRs for the complete set of ECG waveforms using ECGAuto. For each 1-min stream of ECG waveforms, mean time between successive QRS complex peaks (RR interval), mean HR, and mean HRV-analysis–generated time-domain measures were acquired. The time-domain measures included standard deviation of the time between normal-to-normal beats (SDNN), and root mean squared of successive differences (RMSSD). HRV analysis was also conducted in the frequency domain using a fast-Fourier transform. The spectral power obtained from this transformation represents the total harmonic variability for the frequency range being analyzed. In this study, the spectrum was divided into low-frequency (LF) and high-frequency (HF) regions. The ratio of these two frequency domains (LF/HF) provides an estimate of the relative balance between sympathetic (LF) and vagal (HF) activity.

Concentrated ambient particle and ozone exposure - Concentrated ambient particles (CAPs) and ozone (O_3) were generated in the U.S. EPA's Concentrated Air Particles Laboratory, Research Triangle Park, NC. All exposures were carried out in the summer months of June and July and under sunny and warm climate conditions. Ambient air

containing PM from outside the facility entered the systems and passed through a size selective inlet removing PM > 2.5 μm so that remaining particles were in the size fractions of interest. The largest source of PM was from mobile sources (≈20%), wood combustion (≈21%), road dust (≈4%) and other minor sources such as brake wear and marine salt; the remaining PM was from secondary sulfates (≈50-55%). Incoming air was then split into two streams and particles were selectively concentrated into either the fine (0.1 to 2.5 μm) or ultrafine mode (<300 nm) and then delivered into two separate chambers. Real time measurements of number concentration and particle size distribution were performed using a scanning mobility particle sizer (SMPS) and an Aerodynamic Particle Sizer *(*APS). A generator was used to produce O_3 (0.3 ppm), which was then delivered to a third chamber. Chamber plumbing was altered to allow different configurations of concentrated PM and/or O_3 including: FCAPs alone, UFCAPs alone, FCAPs + O_3, UFCAPs + O_3, O_3 alone, or filtered air (FA). Exposure to FCAPs/UFCAPs alone had to be done on separate days from FCAPs/UFCAPs co-exposures with O_3 due to limitations in the exposure system (i.e. exposure to CAPs alone and CAPs + O_3 could not be done on the same day); day-to-day variations in particle concentrations and composition were expected due to this. The study protocol included two days of animal-to-chamber acclimatization prior to exposure. A normal four-hour exposure (Exp1 (exposure hour 1), Exp2, Exp3, and Exp4) started with one hour of additional chamber acclimatization (Baseline). All mice were moved back to their home-cages after the exposure (Recovery). The Multiple Pathway Particle Dosimetry (MPPD; Version 3.0) model was used to estimate particle doses (Anjilvel & Asgharian 1995;

Cassee et al. 2002) for mice and humans; ventilatory parameters were estimated using typical values (Méndez et al. 2010).

Cardiac Perfusion - The procedure for cardiac perfusion has been previously described (Tong et al. 2010). Briefly, 24 hours after exposure, mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.). Heparin (100 units) was injected intravenously before removal of heart. The hearts were rapidly removed and placed in ice-cold Krebs-Henseleit buffer, after which the aortas were cannulated. Retrograde perfusion via the aorta was performed under constant pressure (100 cmH_2O) above the heart. The non-recirculating perfusate was a Krebs-Henseleit buffer containing (in mmol/L) 120 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.75 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose. The buffer was aerated with 95% O_2—5% CO_2 and maintained at pH 7.4 and a temperature of 37°C. For assessment of contractile function, a latex balloon on the tip of a polyethylene catheter was inserted through the left atrium into the left ventricle. The catheter was connected to a pressure transducer (Argon Medical Devices, Athens, TX) at the same height as the heart. The pressure of the left ventricular balloon was inflated to 0-5 cmH_2O. A PowerLab system was used to collect and process the heart rate, left ventricular developed pressure (LVDP = LV peak minus end-diastolic pressure (LVEDP)), and contractility (dP/dt) data (AD Instruments, Milford, MA). All hearts were perfused for 25 min; we then initiated 20 min of global no-flow ischemia by stopping the flow of oxygenated perfusion buffer, followed by 1 h of reperfusion. Onset of ischemic contracture was measured as the time from the start of ischemia until initial contracture (at least 5 cmH_2O increase in left ventricular pressure). Recovery of LVDP, expressed as a

percentage of the initial pre-ischemic LVDP, was measured at 20, 40 and 60 min of reperfusion after 20 min of ischemia.

Tissue collection and analysis - See Additional file 1 for full details, procedures were performed as previously described (Farraj et al. 2010). Briefly, 24 hrs after exposure, mice were euthanized and blood and lung lavage fluid (BAL) were collected, processed and analyzed. Multiple biochemical markers (e.g. lactate dehydrogenase, protein, etc) were assessed in the BAL, and serum or plasma supernatants were analyzed for creatine kinase, C-reactive protein (CRP), and other markers to assess cardiopulmonary inflammation, injury and oxidative stress.

Statistics - All data are expressed as means ± SEM. Statistical analyses of the data were performed with GraphPad Prism 5 (GraphPad software, San Diego CA). For HR, ECG intervals and HRV, two-way analysis of variance (ANOVA) for repeated-measures and Bonferroni *post hoc* tests were used to determine statistical differences. A one-way ANOVA was used to analyze arrhythmia counts. For Langendorff cardiac perfusion data, comparisons between groups were performed by one-way ANOVA followed by Bonferroni post hoc test for multiple comparisons. Comparisons were made across all groups taking into account the multiple endpoints, exposure groups and time points as well as any interactions. An oblique principal component cluster analysis and multivariate analysis of variance (MANOVA – GLM procedure and least squares means post hoc test) were performed using SAS version 9.3 software, (SAS Institute Inc, Cary, NC) to determine whether the elements found in the CAPs on their own or in combination with one another had an effect on the cardiac responses. The objective of this approach was to reduce the large number of variables (i.e.

elements) to a smaller set that still retain the information in the original data set and then examine for effects. Five clusters were revealed and elements belonging to the same cluster had strong correlations. A p-value < 0.05 was considered statistically significant.

Results

Chamber and exposure characteristics - Table 1 shows the concentration and particle size of CAPs and O_3, and chamber characteristics for each exposure group. Table 2 indicates the elemental composition of the particulate matter from each of the exposure groups. Other than iron (Fe), FCAPs and UFCAPs particulate matter were of similar composition with the majority of the elemental fraction composed of SO_4.

Estimated particle doses - The following particle doses were calculated for the mice in each of the PM-exposed group: (1) UFCAPs - 0.418 μg (2) FCAPs - 0.426 μg (3) UFCAPs + O_3 - 0.264 μg and (4) FCAPs + O_3 - 0.446 μg. Using the same model and exposure characteristics the estimated human doses were determined to be: (1) UFCAPs - 103.4 μg (2) FCAPs - 81.3 μg (3) UFCAPs + O_3 - 65.8 μg and (4) FCAPs + O_3 - 85.0 μg.

Heart Rate - Although all animals experienced an increase in HR while in the exposure chamber before the start of the exposure (Baseline) and a progressive decrease during the 4-hour exposure (Exp1, Exp2, Exp3 and Exp4), there were no significant differences in HR among any of the exposure groups during any time period (Figure 1).

Heart rate variability (HRV) - Exposure to FCAPs + O_3 caused a significant decrease in the SDNN (4.8 ± 0.4 ms) compared with FA controls (7.7 ± 0.5 ms) (Figure 1). No other significant differences in time-domain HRV measurements were found among any of the

exposure groups pre-, during or post-exposure. There were also no significant differences in the LF/HF between any exposure groups.

Electrocardiogram - Figure 2 shows the electrocardiogram data before, during and after exposure. There were no significant differences in ECG between any of the groups during pre-exposure or recovery. All animals experienced a decrease in PR interval, QRS, ST interval, and QTc during the baseline, which was likely related to the increase in HR. Thereafter, PR interval and ST interval increased in all animals during the exposure; though there were no significant differences. In contrast, QRS and QTc were significantly increased in mice exposed to UFCAPs + O_3 when compared to FA. Exposure to O_3 alone demonstrated a trend towards decreased QTc when compared with FA.

Cardiac arrhythmia - There was a significant increase in the number of non-conducted P-wave arrhythmias during the 4-hour exposure period to UFCAPs + O_3 when compared with FA (Figure 3C). No other significant differences in arrhythmias were observed among any of the exposure groups. Although other types of arrhythmias were noted, they were few in number and not statistically different between any of the groups**.**

Cardiac effects before ischemia - Post-exposure (baseline) hemodynamics and the onset time to ischemic contracture for each of the exposure groups are listed in Table 3. As shown in Figure 4, there was a significant decrease in LVDP in the FCAPs (31.9 ± 6.7 cmH_2O), O_3 (54.7 ± 12.6 cmH_2O) and UFCAPs + O_3 (45.0 ± 9.2 cmH_2O) groups compared to FA (96.7 ± 9.6 cmH_2O) 24 hours after exposure and before ischemia. Left ventricular contractility was also significantly depressed in the UFCAPs, FCAPs, O_3 and UFCAPs + O_3 groups compared to the FA control group. The maximum d*P*/d*t* was significantly lower in FCAPs (1397 ± 296

cmH_2O/sec), O_3 (2483 ± 480 cmH_2O/sec) and UFCAPs + O_3 (1975 ± 306 cmH_2O/sec) when compared to FA (3880 ± 208 cmH_2O/sec) and the minimum d*P*/d*t* before ischemia was also significantly lower in the UFCAPs (-1452 ± 395 cmH_2O/sec), FCAPs (-982 ± 259 cmH_2O/sec), O_3 (-1520 ± 318 cmH_2O/sec) and UFCAPs + O_3 (-1323 ± 286 cmH_2O/sec) groups when compared to FA (-2744 ± 317 cmH_2O/sec) (Figure 5; Table 3). There was no difference in HR, coronary flow rate or ischemic contracture between any exposure groups before ischemia (Table 3).

Multivariate analysis of variance demonstrated that differences in LVDP, maximum d*P*/d*t* and minimum d*P*/d*t* between the FCAPs alone and FCAPs + O_3 groups could be accounted for by the decrease in aluminum (Al), barium (Ba), copper (Cu), iron (Fe) or silicon dioxide (SiO_2) compositions (Table 2); these elements clustered together however the analysis could not determine which element specifically was responsible. There were no apparent differences in elemental composition between UFCAPs alone and UFCAP + O_3, except nickel (Ni), which were linked to any cardiac response changes, nor were there any other significant linkages with any other cardiac endpoints.

Cardiac Effects Post-Ischemia - After ischemia there were minimal differences among the groups. There was a significant decrease in HR 20 min after reperfusion in the O_3 group (213.8 ± 14.2 bpm) compared to FA (285.3 ± 17.5 bpm) (Figure 6). There were no differences in the post-ischemia coronary flow rates of any of the groups. Although all groups experienced a significant decrease in LVDP recovery when compared to pre-

ischemia, there was no significant difference in post-ischemia recovery of LVDP (Figure 7), dP/dt_{max}, and dP/dt_{min} between any exposure groups.

Biochemical markers and inflammatory cells in BAL and blood **-** Exposure to O_3 alone or UFCAPs + O_3 caused a significant decrease in glutathione S-transferase (GTR) when compared to controls. There were no other significant differences in any other BAL cells or markers, or any of the serum or plasma markers (Table 4).

Discussion

This study demonstrates that a single inhalation exposure to either FCAPs or UFCAPs differentially affects cardiac mechanical and electrical responses in mice, and that the effect of O_3 co-exposure on the response varies for each particle size. FCAPs alone caused decreased ventricular contractility but contrary to our original hypothesis UFCAPs alone had no effect. However, introduction of O_3 as a co-pollutant with UFCAPs caused a significant decrease in cardiac contractility 24 hours after exposure and blunted the effects of FCAPs. In contrast, although exposure to either FCAPs or UFCAPs alone did not cause any significant electrocardiogram effects, co-exposure to each with O_3 caused electrical and HRV changes that might indicate increased cardiac risk. Overall, our results demonstrate that UFCAPs + O_3 produces the most significant effects across both mechanical and electrical cardiac function (Table 5.). Thus, these data suggest there is a differential effect of particle size, which holds true in the presence or absence of O_3, confirming the health effects resulting from a PM-gas co-exposure are not simply the sum of both pollutants. Instead, it appears each interaction (FCAPs + O_3 vs. UFCAPs + O_3) is complex and needs to be examined separately, particularly when exposure concentrations are low and the responses are subtle.

Our previous findings (Tong et al. 2010) suggested that UFCAPs would cause greater cardiac effects than FCAPs. Ultrafine black carbon particles have been shown to translocate into the blood circulation and have the potential to cause direct effects on the cardiovascular system (Takenaka et al. 2001; Furuyama et al. 2009). UF particles cause heterogeneity of repolarization and decreased HRV in humans (Samet et al. 2009), whereas mechanical assessments in animals reveal decreased LVDP, contractility and coronary flow (Hwang et al. 2008; Tong et al. 2010; Bagate et al. 2006). In this study, animals were exposed via whole-body inhalation as opposed to instillation (Tong et al. 2010; Bagate et al. 2006), direct perfusion (Hwang et al. 2008), or nasal inhalation (Amatullah et al. 2012), which could have resulted in a comparatively lower effective dose and milder response (Costa et al. 2006; Osier & Oberdörster 1997). However, among our animals, calculations of estimated total dose indicated that there was no difference between UFCAPs and FCAPs suggesting that in the absence of O_3 more than just particle burden was responsible for the cardiac decrements. Instead, the site of deposition (i.e. pulmonary vs. extra-thoracic) may have played a more important role. FCAPs, which we estimated had a higher extra-thoracic deposition when compared to UFCAPs (0.232 μg vs. 0.142 μg, respectively), may have caused its effects through the activation of upper airway sensory mechanisms. Previous studies have shown that $PM_{2.5}$ can cause irritation and subsequent activation of autonomic reflex arcs, particularly due to the presence of acidic components; UF particles did not produce the same response (Nadziejko et al. 2002). Thus, the higher relative exposure concentrations and differential deposition of FCAPs may have resulted in variable epithelial injury, inflammation, clearance and thus toxicological presentation (Johnson 2004).

On the other hand, it is not entirely surprising that on their own FCAPs and UFCAPs did not cause any significant changes in ECG given our previous negative results with a more toxic pollutant (Farraj et al. 2009). Similarly, Campen et al. (Campen et al. 2006) found that Apolipoprotein E (ApoE) -/- mice on a high fat diet, which are assumed to be susceptible to the cardiotoxic effects of inhaled pollutants, did not have any ECG changes when exposed to high concentrations of road dust PM or the vapor phase of gasoline engine exhaust. As far as arrhythmias are concerned, spatial dispersion of cardiac repolarization, which contributes to arrhythmogenesis, is increased in people after co-exposure to CAPs and O_3 with each pollutant causing minimal effects on their own (Sivagangabalan et al. 2011). Even in the presence of O_3, it is clear from not only our results, but the previously mentioned human data and other humans studies (Fakhri et al. 2009), that relatively low CAPs exposures will likely only cause mild electrical and HRV changes in healthy populations. Thus, a significant ECG effect due to acute exposure may not necessarily be direct evidence of serious cardiovascular morbidity or premature mortality; rather, it may reflect a transient instability that can worsen if exposure continues over a longer period.

Co-exposure to UFCAPs and O_3 produced electrophysiological changes indicative of increased heterogeneity of repolarization, as well as an increased incidence of non-conducted p-wave arrhythmias, which suggest atrioventricular block. In humans, this form of arrhythmia is usually seen with a wide QRS complex (Pelter & Adams 2003), which was also observed in our mice exposed to UFCAPs + O_3; this sometimes indicates that conduction is impaired in the ventricles, particularly when observed with a block. These results corroborate findings from human studies of PM exposure (Baja et al. 2010; Tong et al. 2012) as well as human studies of PM and O_3 co-exposure (Sivagangabalan et al. 2011). Similarly, a long QTc due to

prolonged repolarization suggests increased risk of early after-depolarization, which can trigger arrhythmias and potentially myocardial infarction when propagated. Indeed it is not unusual that electrical and mechanical dysfunction were both observed in mice exposed to UFCAPs + O_3 given increased arrhythmogenesis has been shown to be associated with changes in myocardial stretch(Milan & MacRae 2005).

Consequently, clarifying the role of each pollutant in the health response is challenging. It has been suggested that PM may be the main driver of the cardiovascular response in some instances with O_3 acting as a modifier. Brook et al. previously showed that PM and O_3 together cause acute arterial vasoconstriction in healthy humans subjects, but so does PM alone (Brook et al. 2002; Brook et al. 2009). However, we observed depression of mechanical function with O_3 alone; although much of the current research is focused on PM as a cardiotoxicant, several studies have also noted the adverse cardiovascular effects of O_3 inhalation; which include decreased HR, alteration of cardiac repolarization, and increased inflammation (Kodavanti et al. 2011; Whitsel et al. 2009; Farraj et al. 2012; McIntosh-Kastrinsky et al.). As such, the type of cardiac responses following air pollution may be dependent on the type of pollutant, or combination of pollutants, with some degree of overlapping effects. Tankersley et al. (Tankersley et al. 2010) showed that both carbon black particles and O_3 caused reduced cardiac output in mice but due to two different mechanisms. Thus, we speculate that although both particles and gases produce similar cardiac decrements, the mechanisms mediating the response may not be the same (e.g. translocation vs. airway sensory irritation). Combinations of pollutants only complicates the assessment due the involvement of various separate or overlapping mechanisms. Regardless, the responses appear to be independent of total particle

dose or even pulmonary deposition given UFCAPs was estimated to be less than FCAPs (pulmonary dose - 0.104 μg vs. 0.135 μg, respectively).

The role of the autonomic nervous system cannot be entirely discounted either; as demonstrated through HRV, the responses observed here and in other studies with respect to PM exposure appear to be dependent on the size of the particles. The interpretation and importance of HRV in air pollution studies is still not entirely agreed upon, particularly when examining populations with underlying cardiovascular disease. Mills et al. (Mills et al. 2011) and Perez et al. (Peretz et al. 2008) did not observe any HRV changes in humans exposed to diesel exhaust, however this lack of effect does not necessarily imply that there are no autonomic changes, instead a trigger (e.g. stress, exercise, etc) may be necessary to reveal any HRV differences. On the other hand, some studies show that particles, especially fine, cause HRV effects in humans. Several studies have demonstrated that exposure to FCAPs causes decreased HRV in young healthy or elderly adults (Devlin et al. 2003; Pope et al. 2004; Gold et al. 2000) with O_3 co-exposure only potentiating the response (Gold et al. 2000). In healthy young adults, there was no dose-response relationship between FCAPs mass and HRV, however when combined with O_3, increases in CAPs mass decreased HRV in a dose-dependent manner (Fakhri et al. 2009). On the other hand, UFCAPs either have no effect (Schneider et al. 2010) or increase HRV (Zareba et al. 2009; Samet et al. 2009) or the results are less conclusive across all studies. Long-term exposure to UFCAPs, or a higher concentration, may have caused a significant change in HRV given these particles have the ability to penetrate deep into the lung, cause inflammation and activate autonomic reflex pathways (Carll et al. 2010).

Some of these pathways may lead to subsequent ischemic damage, which has been shown to be increased by PM. Cozzi et al. showed that in mice intra-tracheally instilled with ultrafine PM, infarct size and oxidative stress in the myocardium were significantly increased (Cozzi et al. 2006). This corroborates our previous PM instillation studies which also demonstrated an increase in post-ischemia infarct size and decreased recovery of LVDP (Tong et al. 2010). It appears that the method of exposure significantly impacts the post-ischemia response because even though exposure to FCAPs or UFCAPs + O_3 caused significant pre-ischemia functional decrements, there was no change in coronary flow post-ischemia and there appeared to be an improvement of LVDP recovery (Figure 7). These findings are similar to what we observed with inhalation of multipollutant mixtures (McIntosh-Kastrinsky et al. 2013) and may represent activation of some compensatory mechanism post-exposure that actually protects the heart during ischemic injury. Lastly, although infarct size was not measured in our animals, we theorize that there was probably minimum to no increase particularly given we previously observed a decrease in infarct size with multipollutant mixture inhalation (McIntosh-Kastrinsky et al. 2013). Thus, acute inhalation of fine or ultrafine PM alone or in combination with O_3 may not be potent enough to cause serious ischemia-related damage and that a higher concentration is necessary to overcome this apparent response threshold.

Other than the mode of exposure, the chemical and physical characteristics of the PM might also account for some of the differences in response observed in this study. Indeed it is a limitation that exposure to CAPs alone could not be done on the same days as CAPs + O_3; this accounts for the variation in not only particle numbers but composition as well. However, it is our assertion that the responses to these "real-world" particle concentrations are important, especially given the daily fluctuation of particulate air pollution and the ubiquitousness of O_3.

It is also important to note that although we compare these results to our previous study (Tong et al. 2010), the composition of the current FCAPs and UFCAPs is different. Our CAPs, particularly the UFCAPs, had a higher organic (OC) and total carbon (TC) content; thus possibly explaining the differences in response.

As such, there was a 1/3rd of Cu and ½ the Fe in the FCAPs + O_3 exposure when compared to FCAPs alone which may have contributed to the lack of effect in the former. There was also an increase in Ni and Cu, which have been shown to be two of the most toxic metals found in PM (Riley et al. 2003), in the UFCAPs + O_3 exposure when compared to UFCAPs alone. In contrast, even though it appears that mass was not a factor in the observed decrements because there was less PM in the UFCAPs + O_3 exposure than UFCAPs alone and the opposite for the FCAPs and O_3 co-exposures, there was a significantly higher sulfate and OC/TC content in the UFCAPs, especially the UFCAPs + O_3, when compared to FCAPs, which may explain the larger cardiac effect (Chuang et al. 2007). On the other hand, responses to FCAPs and UFCAPs combined with O_3 might also be partially explained by the chemical changes occurring in PM upon ozonization. Ozone is highly reactive and therefore it has the potential to react with certain components of PM such as the aromatic compounds (Madden et al. 2000). It has been documented that ozonization of aromatic substances can result in the formation of carbonyls, carboxylic acids, quinones, and epoxides, which can be more toxic than the parent compound (Kozumbo & Agarwal 1990; Pitts et al. 1980), but also less potent due to "over-ozonization" (Madden et al. 1993). It is yet unclear which mechanism is at play here.

Additionally, O_3 may cause epithelial injury and oxidative stress, which facilitate the PM effects (Elder et al. 2000). Adamson et al. (Adamson et al. 1999) showed that O_3 and urban particulate co-exposure resulted in greater epithelial injury and interstitial inflammation than for either component alone; not to mention UFP did not have a large biological effect without O_3. As such, co-exposures may produce differential responses due to toxicological interactions within the host. Thomson et al. (Thomson et al. 2005) showed that on their own, PM and O_3 increased expression of the potent vasoconstrictor endothelin-1 (ET-1) in the lungs and its circulating levels in the plasma, however, together they only caused an upregulation (i.e. without plasma "spill-over"). Although there were no significant changes in inflammatory cells or markers in the blood or lavage, we found that O_3 alone and UFCAPs + O_3, but not FCAPs or UFCAPs alone, caused significantly decreased serum glutathione S-transferase (GTR) levels, which is indicative of increased oxidative stress; direct measurement of oxidative stress in the myocardium may have revealed a greater involvement as was shown by Cozzi et al. (2006) (Cozzi et al. 2006). Wang et al. previously showed that $PM_{2.5}$ and O_3 increased several markers of inflammation and oxidative stress in rats however their exposure concentrations were significantly higher than those used here (Watkinson et al. 2001). Regardless, synergistic interactions between inhalable PM and O_3 can increase the generation of reactive oxygen species due to the porous surface of particles which provides ample surface area for reactivity, but that the potency still depends on particle concentration, size and other factors (Valavanidis et al. 2009; Park et al. 2006).

The results of this study demonstrate that fine and ultrafine CAPs differentially alter cardiac responses, which include both mechanical and electrical effects. More importantly, these data clearly show that the effects of co-exposure may not be simply additive or

synergistic, nor even generalizable. Although only fine CAPs had significant effects on its own, O_3 co-exposure with FCAPs caused decreased HRV whereas with UFCAPs caused electrical changes and arrhythmia. Interestingly, O_3 co-exposure only caused mechanical decrements with UFCAPs and to our surprise blunted the effects of FCAPs. This indicates that the size, and potentially the chemical composition, of the particle is an important determinant of the type of cardiac response, particularly when gaseous co-pollutants are present. Although the responses were subtle, the important message may be that latent underlying changes are occurring post-exposure and that the deleterious effects of even a single exposure to air pollution needs to be considered. Some of these might not manifest as overt symptoms, however the latent effect might not be any less serious, instead increasing the susceptibility to subsequent triggered adverse responses (i.e. due to loss of compensatory capacity), particularly in people with existing cardiovascular disease.

Tables and Figures

Table 1. ***Chamber and exposure characteristics***

	GROUPS					
	FA	**UFCAPs**	**UFCAPs + O_3**	**FCAPs**	**FCAPs + O_3**	**O_3**
Temperature (°C)	22.3 ± 0.1	23.0 ± 0.1	22.6 ± 0.1	22.0 ± 0.1	22.2 ± 0.1	22.5 ± 0.2
Rel. humidity (%)	50.2 ± 0.7	70.5 ± 4.6	56.0 ± 3.6	59.8 ± 3.4	59.0 ± 5.8	52.4 ± 2.2
O_3 (ppb)	4.0 ± 0.0	25.7 ± 4.7	298.3 ± 0.7	33.1 ± 2.0	300.0 ± 0.4	299.0 ± 1.1
PM Mass (ug/m^3)	4.9 ± 2.2	138.8 ± 33.1	85.7 ± 6.5	190.9 ± 32.8	211.5 ± 37.3	3.4 ± 1.3
PM Total # (particles/cc)	24.2 ± 1.4	$2.1E^5 \pm 5.6E^3$	$1.6E^5 \pm 2.0E^3$	$1.0E^4 \pm 5.2E^1$	$1.1E^4 \pm 1.6E^2$	20.2 ± 4.2
Particle size (um)	-	0.076	0.072	0.246	0.235	-
Geo. Std. Dev.	-	1.67	1.66	1.96	1.67	-
	PM CARBON					
TC ($\mu g/m^3$)	3.4 ± 0.2	67.6 ± 6.5 (48.7%)	46.4 ± 3.4 (54.1%)	53.8 ± 4.4 (28.2%)	47.8 ± 7.3 (22.6%)	4.3 ± 0.4
OC ($\mu g/m^3$)	3.7 ± 0.2	64.5 ± 5.9 (46.5%)	44.6 ± 3.1 (52.0%)	50.3 ± 4.0 (26.3%)	45.7 ± 6.6 (21.6%)	4.6 ± 0.4
EC ($\mu g/m^3$)	**	3.1 ± 0.6(2.2%)	1.8 ± 0.3 (2.1%)	3.4 ± 0.5 (1.8%)	2.2 ± 0.7 (1.0%)	**

Reported values are mean ± SEM for each group over all exposure days

** = below detection limit

Carbon percentages are by mass

Table 2. ***Elemental Composition of Particulate Matter in Exposure Groups***

Element(μg/m^3)	**GROUPS**					
	FA	**UFCAPs**	**UFCAPs + O_3**	**FCAPs**	**FCAPs + O_3**	**O_3**
Al	---	BDL	BDL	1.8364	0.8428◊	---
As	---	0.0020	0.0040	0.0044	0.0077	---
Ba	---	0.0070	0.0047	0.0865	0.0480◊	---
Ca	---	BDL	BDL	1.1741	0.6475	---
Cd	---	0.0005	0.0005	0.0015	0.0010	---
Co	---	0.0002	0.0002	0.0008	0.0006	---
Cr	---	0.0088	0.0198	0.0129	0.0134	---
Cu	---	0.0119	0.0727Δ	0.1550	0.0537◊	---
Fe	---	0.0723	0.0848	2.1031	1.0555◊	---
K	---	0.7448	0.4325	2.6025	1.3867	---
Li	---	BDL	BDL	0.0017	0.0013	---
Mg	---	0.0282	0.0254	0.5585	0.6145	---
Mn	---	0.0048	0.0058	0.0604	0.0348	---
Mo	---	0.0014	0.0032	0.0033	0.0036	---
Na	---	0.1491	0.2550	0.8990	3.7144	---
Ni	---	0.0053	0.0348Δ	0.0079	0.0084	---
P	---	BDL	BDL	0.1278	0.1565	---
Pb	---	0.0128	0.0124	0.0373	0.0271	---
SO_4	---	56.4662	39.8730	39.4038	49.3026	---
Sb	---	0.0035	0.0040	0.0146	0.0108	---
Se	---	BDL	BDL	0.0246	0.0248	---
SiO_2	---	BDL	BDL	4.9640	2.8436◊	---
Sn	---	0.0182	0.0239	0.0151	0.0121	---
Sr	---	0.0016	0.0006	0.0179	0.0101	---
Ti	---	BDL	BDL	0.0691	0.0429	---
V	---	0.0023	0.0052	0.0092	0.0122	---
Zn	---	0.1527	0.0989	0.2171	0.2057	---

BDL - Below Detection Level

--- Very low PM concentrations, insufficient sample mass for elemental analysis

Δ Significantly different from UFCAPs

◊ Significantly different from FCAPs

Table 3. ***Baseline hemodynamic properties and the onset time to ischemic contracture***

Group (n = 6-8)	**LVDP (cmH_2O)**	**HR (bpm)**	**Flow rate (mL/min)**	**dP/dt_{max} (cmH_2O/sec)**	**dP/dt_{min} (cmH_2O/sec)**	**Time to contracture (min)**
FA	96.7 ± 9.6	306.6 ± 17.5	1.7 ± 0.3	3880 ± 208	-2744 ± 317	14.3 ± 1.9
UFCAPs	59.7 ± 15.4	270.3 ± 19.6	1.7 ± 0.5	2564 ± 825	-1452 ± 395	14.6 ± 1.8
UFCAPs + O_3	45.0 ± 9.3*	295.1 ± 18.5	2.0 ± 0.5	1975 ± 306*	-1323 ± 286*	15.1 ± 1.6
FCAPs	31.9 ± 6.7*	301.3 ± 27.6	5.7 ± 2.9	1397 ± 296*	-981 ± 259*	11.1 ± 0.5
FCAPs + O_3	88.0 ± 18.4	277.6 ± 15.7	1.7 ± 0.2	3034 ± 528	-2219 ± 394	12.9 ± 1.3
O_3	54.7 ± 12.6	246.3 ± 36.4	2.5 ± 0.6	2483 ± 480	-1520 ± 318	15.3 ± 1.6

Values are means ± SEM. Flow rate = coronary flow rate; dP/dt_{max} = maximum 1st derivative of the change in left ventricular pressure/time; dP/dt_{min} = minimum 1st derivative of the change in left ventricular pressure/time; time to contracture = onset time to ischemic contracture. *Significantly different from FA; $p < 0.05$; n = 5-8.

Table 4. ***Biochemical markers in the bronchoalveolar lavage and serum***

						Broncho alveolar lavage					**Blood**
O_3	**FCAPs + O_3**	**FCAPs**	**UFCAPs + O_3**	**UFCAPs**	**Air**	**Group**	**LDH (U/L)**	**MIA (µg/ml)**	**NAG (U/L)**	**Protein (µg/ml)**	**ACE (U/L)**	**CK (U/L)**	**CRP (µg/dl)**	**HBDH (U/L)**	**GTR (IU/ml)**	**LDH (U/L)**	**SOD (U/ml)**	**Protein (g/dl)**
53.9 ± 7.5	78.2 ± 313.7	58.5 ± 13.7	77.6 ± 20.3	50.7 ± 3.7	67.2 ± 12.4													
9.5 ± 0.4	10.2 ± 0.3	9.8 ± 0.5	10.6 ± 0.8	9.7 ± 0.2	10.3 ± 0.4													
5.0 ± 0.2	6.4 ± 0.3	6.0 ± 0.1	5.3 ± 0.5	6.7 ± 0.4	6.0 ± 0.2													
83.7 ± 6.3	127.4 ± 11.1	94.9 ± 14.3	120.2 ± 31.4	87.0 ± 11.8	131.0 ± 9.5													
142.4 ± 30.3	167.5 ± 22.2	191.3 ± 23.3	158.7 ± 25.3	250.3 ± 33.5	193.9 ± 26.4													
1.7E3 ± 334.0	1.9E3 ± 499.5	1.2E3 ± 124.2	1.0E3 ± 178.5	1.0E3 ± 32.9	2.3E3 ± 891.5													
71.9 ± 18.0	55.6 ± 9.6	86.9 ± 12.9	65.1 ± 6.6	73.2 ± 5.6	64.8 ± 10.0													
203.9 ± 36.4	170.1 ± 20.5	190.1 ± 32.0	239.5 ± 59.1	172.9 ± 6.5	216.3 ± 51.8													
0.016 ± 0.004*	0.019 ± 0.003	0.023 ± 0.005	0.015 ± 0.004*	0.022 ± 0.002	0.042 ± 0.014													
431.9 ± 86.1	382.4 ± 53.6	396.9 ± 71.5	441.8 ± 61.4	390.6 ± 30.2	551.4 ± 153.7													
2.6 ± 0.3	1.9 ± 0.2	1.8 ± 0.1	2.0 ± 0.2	1.6 ± 0.02	1.94 ± 0.2													
4.5 ± 0.3	4.3 ± 0.1	5.0 ± 0.2	4.7 ± 0.2	4.8 ± 0.2	4.4 ± 0.4													

Values are mean ± SEM. *p < 0.05; significantly different from FA.

LDH – Lactate dehydrogenase

MIA – Microalbumin

NAG - N-acetyl-b-d-glucosaminidase

ACE – angiotensin converting enzyme

CK – Creatine Kinase

CRP – C-reactive protein

HBDH - α-hydroxybutyrate dehydrogenase

GTR – glutathione-S-transferase

SOD – Superoxide dismutase

Table 5. ***Summary of effects***

Group	LVDP	dP/dt_{max}	dP/dt_{min}	LVDP recovery (post-ischemia)	Coronary flow rate (post-ischemia)	Heart rate	SDNN	RMSSD	LF/HF	QTc	NCPW
FA	--	--	--	--	--	--	--	--	--	--	--
UFCAPs	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
UFCAPs + O_3	→	→	→	NE	NE	NE	NE	NE	NE	←	←
FCAPs	→	→	→	NE	NE	NE	NE	NE	NE	NE	NE
FCAPs + O_3	NE	NE	NE	NE	NE	NE	→	NE	NE	NE	NE
O_3	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE

NE = No Effect

↓ = significant decrease

↑ = significant increase

NOTE: the above responses are compared to Air

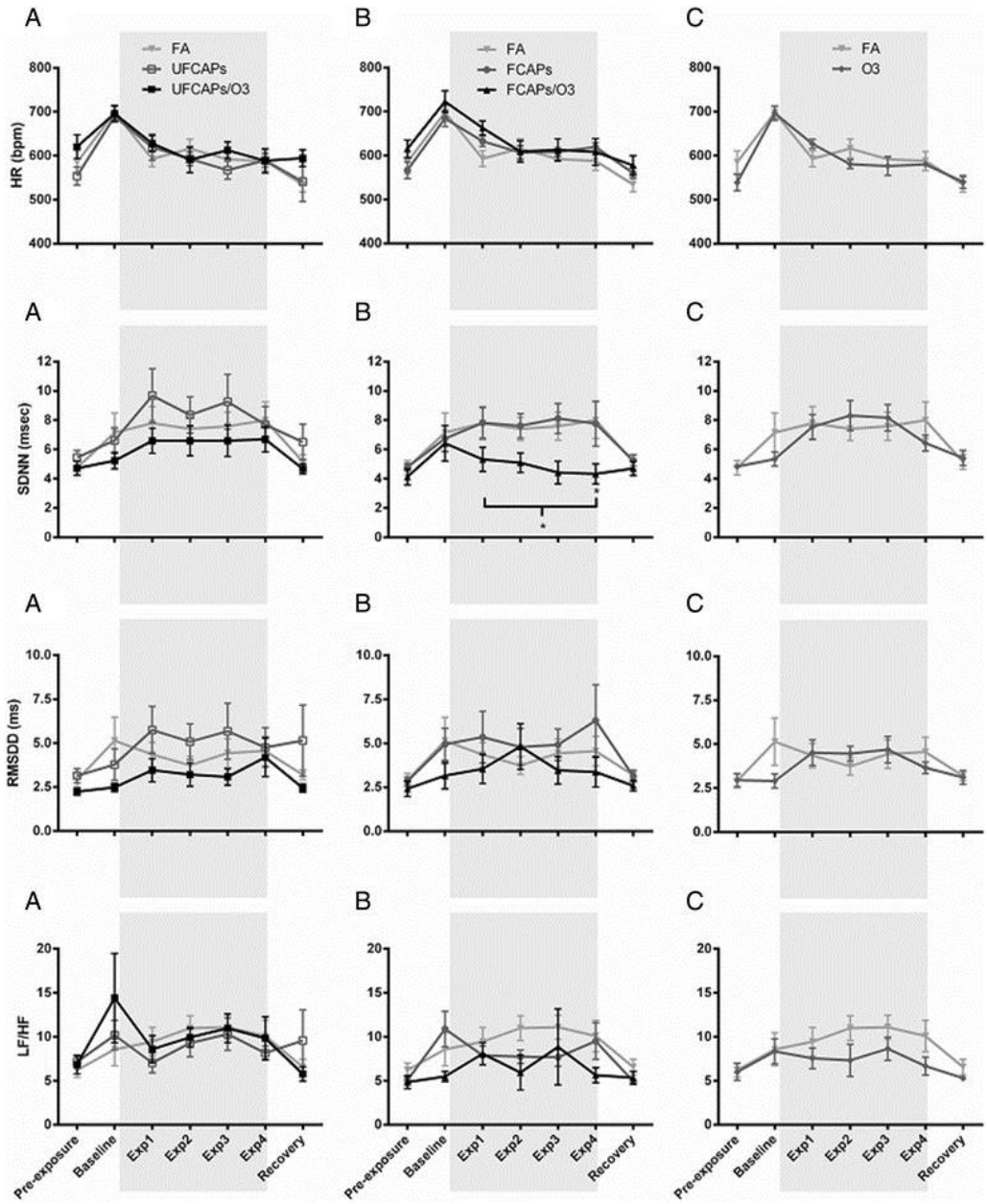


Figure 1. ***The effect of CAPs with and without O_3 on heart rate and heart rate variability.*** Animals were placed in the chambers and allowed to acclimate for 1 hr before exposure (Baseline), then exposed for 4 hrs (shaded). Compared to pre-exposure, all animals experienced an increase in HR during baseline, then a progressive decrease from baseline during exposure. UFCAPs alone (UF) or with ozone (UF/O_3) did not cause changes in HR or HRV at any time point (Column **A.**). Similarly, there was no effect of FCAPs alone (F) on any parameter; only exposure to FCAPs + O_3 (F/O_3) significantly decreased SDNN when compared to FA (Column **B.**). Exposure to ozone alone did not cause any significant effects (Column **C.**). Bracket indicates that each hour of the exposure period is significantly different. Values are mean ± SEM; *$p < 0.05$, significantly different from FA ($n = 6$).

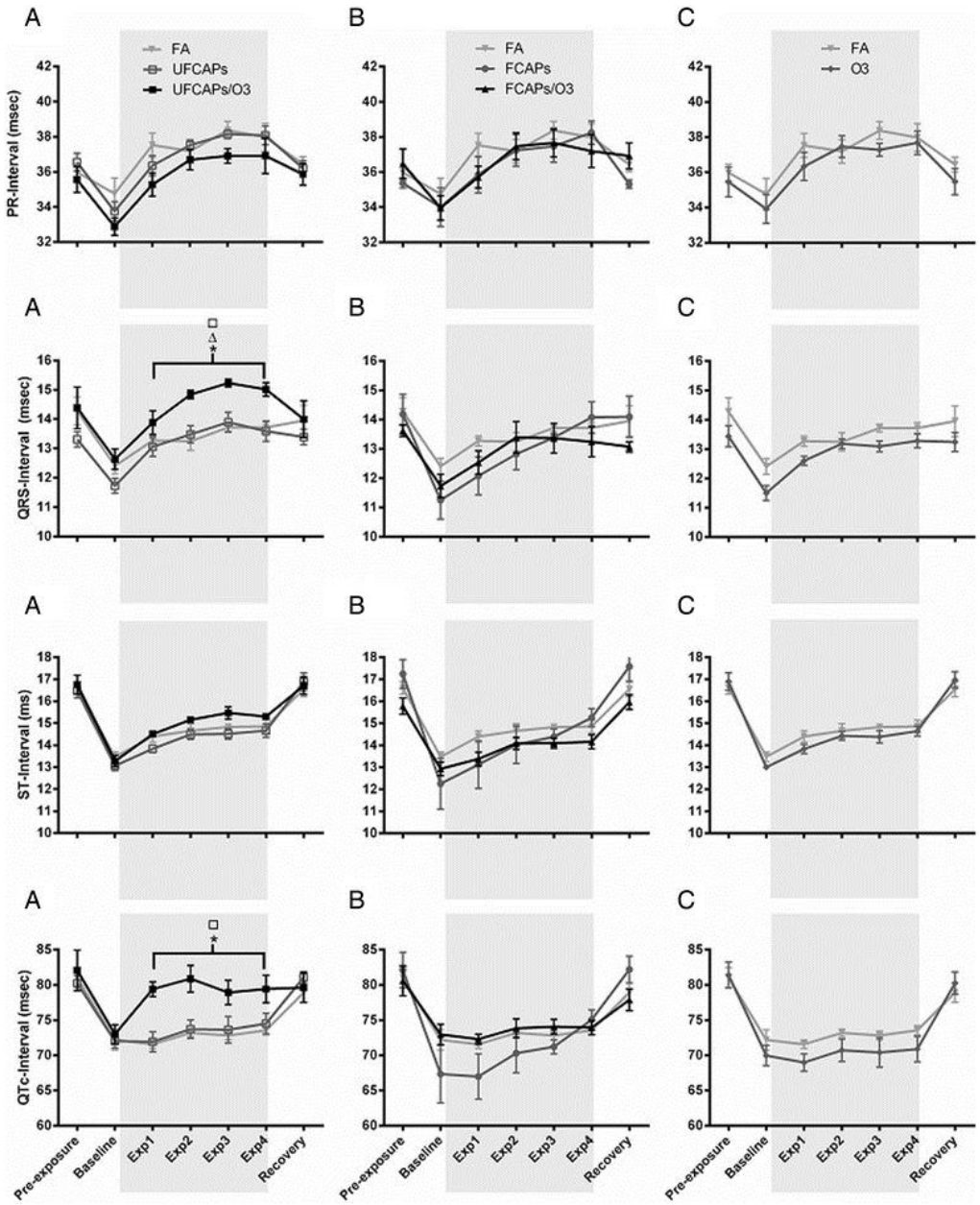


Figure 2. ***Electrocardiogram effects before, during and after exposure to CAPs alone or with O_3.*** Animals were placed in chambers acclimated for 1 hr before exposure (Baseline), then exposed for 4 hrs (shaded). All animals experienced a decrease in PR interval, QRS complex duration, ST interval and QTc from pre-exposure to baseline. Although both PR and ST intervals increased in all groups during exposure, there were no differences in either of these parameters among any of the groups. QRS also showed an increasing trend during exposure in all groups; however only mice exposed to UFCAPs + O_3 had a significant increase in QRS and QTc when compared to FA (Column **A.**). There were no significant effects of FCAPs, with or without O_3, or O_3 alone at any time points (Column **B.** and **C.**, respectively). Bracket indicates that each hour of the exposure period is significantly different. Values are mean ± SEM. $p < 0.05$; *significantly different from FA, Δ significantly different from UFCAPs alone, □ significantly different from O_3 alone (n = 6).

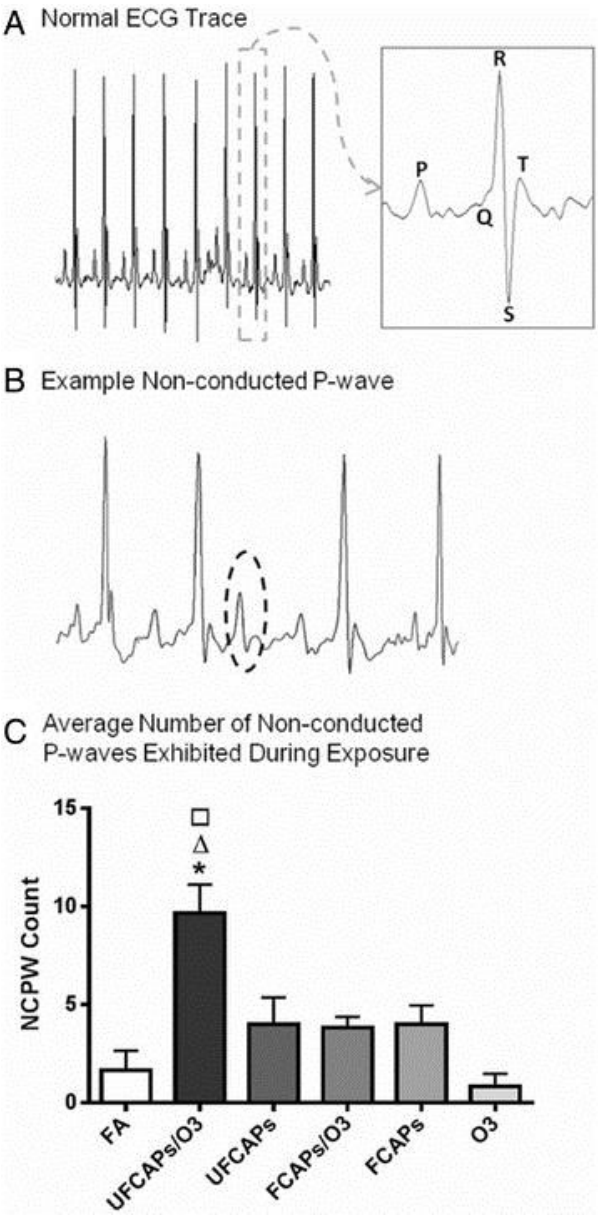


Figure 3. ***Typical mouse electrocardiogram and arrhythmia count during exposure.*** **A.** Typical mouse ECG during normal sinus rhythm and **B.** a non-conducted p-wave (NCPW) - represents a sudden loss of conduction from the atria to the ventricles. **C.** Non-conducted p-waves were significantly increased only in mice exposed to UFCAPs + O_3. Values are mean ± SEM. $p < 0.05$; *significantly different from FA, Δ significantly different from UFCAPs alone, □ significantly different from O_3 alone (n = 6).

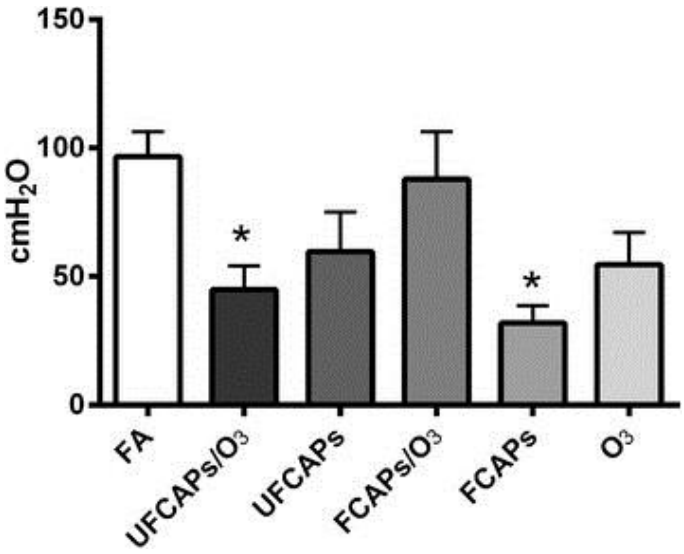


Figure 4. ***Effect of CAPs exposure on left ventricular developed pressure (LVDP).*** Exposure to FCAPs alone (F) significantly decreased LVDP at baseline (24 hrs after exposure - prior to ischemia) when compared to FA, however there was no effect with O_3 co-exposure. In contrast, UFCAPs alone had no effect on LVDP but with O_3 co-exposure caused a significant decrease when compared to FA. Values are means ± SEM (*n* = 5-8/group). *Significantly different from FA; $p < 0.05$.

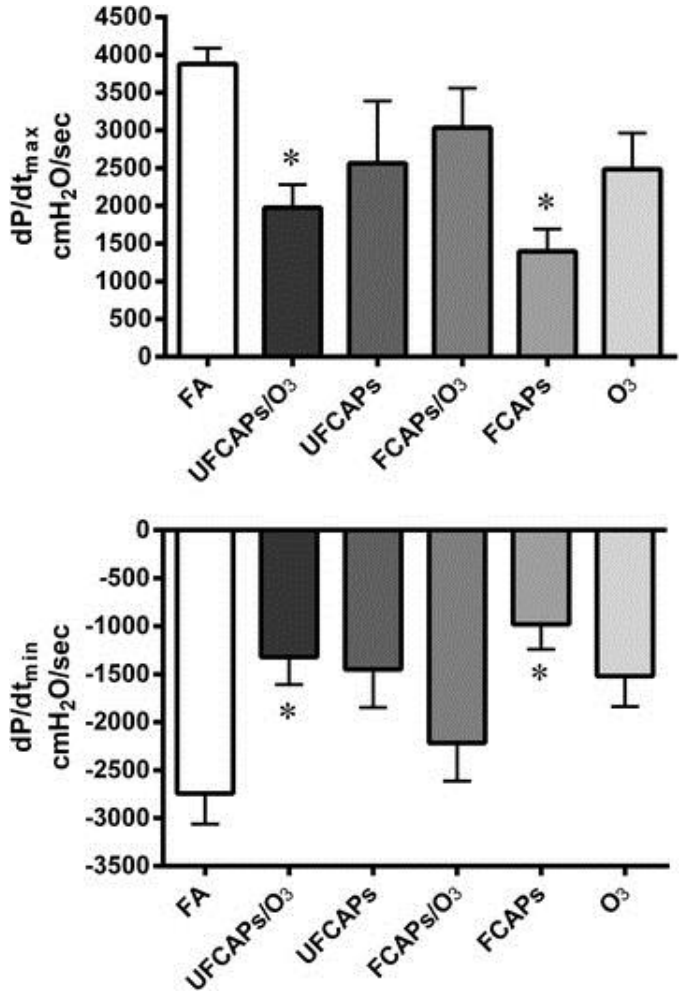


Figure 5. ***Effect of CAPs exposure with and without O_3 on rate of left ventricle contractility and relaxation*.** Assessment of contractility (dP/dt_{max} - upper panel) and lusitropy (dP/dt_{min} - lower panel) were carried out at baseline 24 hrs after exposure - prior to ischemia. Exposure to FCAPs alone significantly decreased dP/dt_{max} and dP/dt_{min} at baseline when compared to FA, however there was no effect with O_3 co-exposure (FCAPs/O_3). In contrast, UFCAPs alone had no effect; but, O_3 co-exposure with UFCAPs (UFCAPs/O_3) caused both dP/dt_{max} and dP/dt_{min} to significantly decrease when compared to FA. Values are means ± SEM (n = 5-8 in each group). *Significantly different from FA; $p < 0.05$.

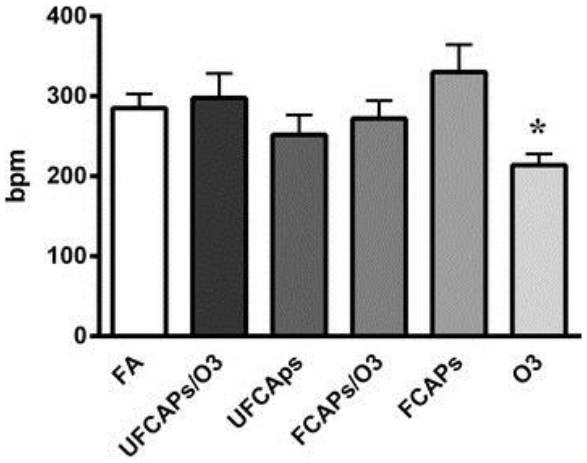


Figure 6. ***Heart rate twenty minutes after ischemia-reperfusion***. After 20mins of reperfusion, heart rate (HR) was significantly lower in animals exposed to O_3 when compared to FA. There were no other significant differences in post-ischemia HR between any groups or at any other time point. Values are means ± SEM (*n* = 5-8 in each group). *Significantly different from FA; $p < 0.05$.

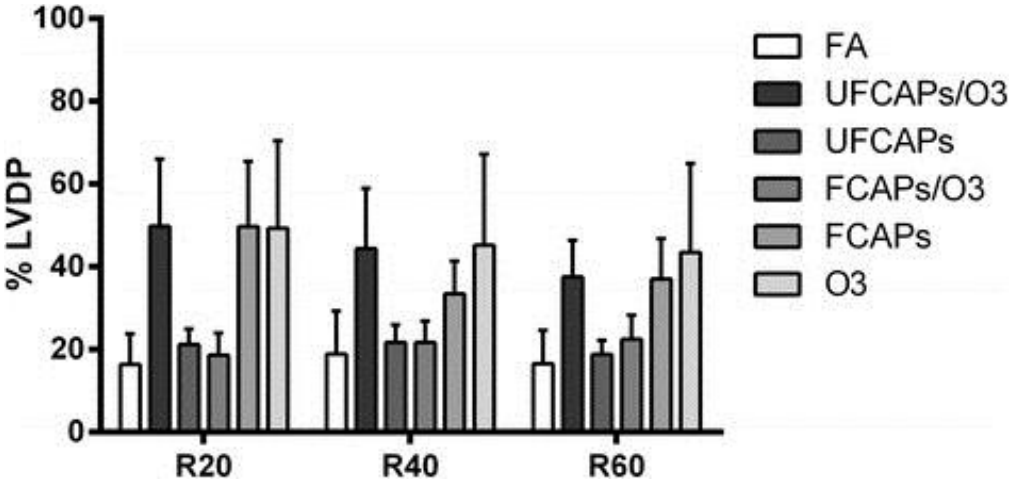


Figure 7. ***Post-ischemia recovery of LVDP.*** Following ischemia, all animals experienced a significant decrease in recovery LVDP (expressed as a percentage of pre-ischemia) when compared to pre-ischemia**.** There were no significant differences among any groups in the recovery LVDP at 20 (R20), 40 (R40) or 60 (R60) minutes post-ischemia; however there was a trend towards increased%LVDP in the FCAPs, O_3 and UFCAPs + O_3 groups at R20, R40 and R60 when compared to FA; consequently, these groups were the same ones demonstrating pre-ischemia changes. Values are means ± SEM (*n* = 5-8 in each group). *Significantly different from FA; $p < 0.05$.

CHAPTER III: TRPA1 MEDIATES CHANGES IN HEART RATE VARIABILITY AND CARDIAC MECHANICAL FUNCTION IN MICE EXPOSED TO ACROLEIN

Short-term exposure to ambient air pollution has been linked with adverse cardiovascular effects. While most studies from the last ten years have focused on particulate matter-induced responses, gaseous components of air pollution have also been shown to cause short-term cardiovascular effects. The mechanisms underlying such effects have still not been adequately described yet it is clear that irritant neural activation and downstream autonomic modulation are involved given the immediate nature of the response. Thus, this study examines the role of TRPA1, an irritant sensory receptor found in the airways, in the cardiac response of mice to acrolein and ozone. Conscious unrestrained wild-type C57BL/6 (WT) and TRPA1 knockout (KO) mice implanted with radiotelemeters were exposed once to 3 ppm acrolein, 0.3 ppm ozone, or filtered air. Heart rate (HR) and electrocardiogram (ECG) were recorded continuously before, during and after exposure. Analysis of ECG morphology, incidence of arrhythmia and heart rate variability (HRV) were performed. Cardiac mechanical function was assessed using a Langendorff perfusion preparation 24hrs post-exposure. Acrolein exposure increased HRV time and frequency domain parameters independent of HR, as well as incidence of arrhythmia. Acrolein also increased left ventricular developed pressure in WT mice at 24hrs post-exposure. Ozone did not cause any changes in cardiac function in WT mice. Neither gas caused any ECG effects nor was there any difference in HRV, arrhythmogenesis, or mechanical function in KO mice exposed to either acrolein or ozone. These data demonstrate that a single exposure to

acrolein causes cardiac dysfunction through TRPA1 activation and autonomic imbalance characterized by a shift towards parasympathetic modulation. Furthermore, it is clear from the lack of ozone effects that although gaseous irritants are capable of eliciting immediate cardiac changes, gas concentration and properties play an important role.

Introduction

Ambient air pollution has been linked to adverse cardiovascular effects by numerous epidemiological, human and animal studies. Although most of the research over the last decade have focused on particulate matter (PM), recent data suggest gaseous pollutants likely contribute significantly to acute decrements in cardiac function and result in increased overall risk for cardiovascular dysfunction (Campen, Robertson et al. 2014, Hazari, Griggs et al. 2014, Mills, Atkinson et al. 2015, Shah, Lee et al. 2015). In fact, the 2010 American Heart Association document on PM and cardiovascular disease clearly states that "Although $PM_{2.5}$ mass has rightfully attracted attention as a target for regulation and epidemiological study, more than 98% of the air pollutant mass in the mixture we breathe in urban settings is from gases or vapor-phase compounds…", not to mention there is still relatively little data describing the effects of gaseous pollutants on the cardiovascular system (Brook, Rajagopalan et al. 2010). Thus, more studies are needed to determine the effect of individual gases and their causative pathways, particularly given the mechanism underlying the response will undoubtedly differ for the gas alone and when combined with other pollutants such as PM.

In addition to "criteria" pollutant gases like ozone (O_3) for which the United States Environmental Protection Agency has set National Ambient Air Quality Standards

(NAAQS), non-NAAQS hazardous air pollutants (HAPs) such as acrolein are also frequently present in ambient air pollution, particularly in emissions from combustion processes including exhaust from automobiles, emissions from coal-fired power plants, and emissions from industrial sites (CDC ToxProfile). Acrolein is a gaseous irritant formed during the combustion of petrochemical fuels such as gasoline or diesel and it is considered to pose the greatest relative HAP hazard for non-cancer health effects (EPA 1996, Haussmann 2012). To that point, we previously showed that a single exposure to either O_3 or acrolein causes various cardiovascular effects in rodents; these range from electrocardiogram changes, autonomic imbalance, desensitization of baroreflex, to alterations in hypoxia responsiveness (Hazari, Haykal-Coates et al. 2009, Farraj, Hazari et al. 2012, Perez, Ledbetter et al. 2013, Hazari, Griggs et al. 2014). Moreover, many of these effects appear to be latent and are only evident if the body is challenged or encounters a subsequent trigger (Hazari, Callaway et al. 2012), which would suggest the underlying mechanisms involve the disruption of homeostatic controls which maintain equilibrium of vital bodily systems under changing conditions.

Numerous biological pathways have been described that link air pollution with cardiovascular dysfunction. These include systemic inflammation and oxidative stress, vasoconstriction, enhanced coagulation/thrombosis, autonomic effects due to sensory receptor activation, and the direct effects of translocated particles on the myocardium (Brook, Rajagopalan et al. 2010). Of these, airway sensory activation may be the most relevant when considering the short-term, reversible, and often latent effects of an acute ambient exposure. An examination of the role of airway sensory nerves in the body indicates that not only do they “sense” or become activated by specific gaseous pollutants but also cause internal

homeostatic changes that may predispose to respiratory and cardiovascular dysfunction (Lee and Pisarri 2001, Jordt and Ehrlich 2007).

Gaseous air pollutants such as ozone, acrolein, and other reactive aldehydes commonly found in combustion-derived exhaust, stimulate sensory nerves in the airways causing several well-described ventilatory and pulmonary effects (McDonnell, Horstman et al. 1983, Jordt 2011, Abbott-Banner, Poll et al. 2013, Büch, Schäfer et al. 2013). A subset of these responses is known to be mediated by airway sensors, particularly transient receptor potential cation channels A1 (TRPA1) and V1 (TRPV1), located on pulmonary C-fibers (Nassenstein, Kwong et al. 2008, Taylor-Clark, McAlexander et al. 2008). It has been suggested that these sensors may also mediate some cardiovascular responses as well (Hazari et al., 2011; Jones et al., 1995; L.-Y. Lee, 2010; Wang et al., 2000) and in fact, our previous studies have demonstrated that pharmacological inhibition of TRPA1 attenuates air pollution-induced cardiac arrhythmia produced by autonomic imbalance in rats (Hazari, Haykal-Coates et al. 2011).

Thus, in the present study we investigated the cardiac effects of exposure to acrolein or ozone inhalation with WT and TRPA1 KO mice. Acrolein was chosen because it is a common gaseous component of urban air pollution and a known TRPA1 activator (Bautista, Jordt et al. 2006). Only a single study has shown that ozone, albeit in solution and not inhaled, activates TRPA1 (Taylor-Clark and Undem 2010), yet its irritant properties suggest that such a finding is not unreasonable. Regardless, although both have been traditionally classified as respiratory irritants, data from the last five years have clearly shown that both gases not only also affect the cardiovascular system but other organ systems through

pathways which implicate a role for the intrinsic control mechanisms of the body (Bessac and Jordt 2010, DeJarnett, Conklin et al. 2014, Miller, Karoly et al. 2015). The concentration of acrolein used in this study was admittedly high to serve as a proof of concept but certainly there are extreme conditions (e.g. structural fires), occupational settings or cigarette smoke in which the levels of acrolein exceed those used here. Irrespective of this high concentration, the broader aim was to not only firmly establish a role for TRPA1 in air pollution-induced electrical and mechanical cardiovascular dysfunction but also clarify whether inhalation of acrolein and ozone produce cardiovascular effects through TRPA1.

Materials and Methods

Animals - Female C57BL/6 and TRPA1 -/- mice (22 ± 3.8 g) were used in this study (Jackson Laboratory - Bar Harbor, ME). Mice were initially housed five per cage and maintained on a 12-hr light/dark cycle at approximately 22°C and 50% relative humidity in an AAALAC–approved facility. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency and are in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The animals were treated humanely and with regard for alleviation of suffering. Background controls were used as appropriate.

Experimental Groups - Wild-type and TRPA1 knockout mice were randomly assigned to one of three exposure groups: (1) Acrolein (Acrl); (2) Ozone (O_3); or (3) filtered air (FA). There were 8-12 animals per group.

Surgical implantation of radiotelemeters - Animals were weighed and then anesthetized using inhaled isoflurane (Isothesia, Butler Animal Health Supply, Dublin OH). Anesthesia

was induced by spontaneous breathing of 2.5% isoflurane in pure oxygen at a flow rate of 1 L/min and then maintained by 1.5% isoflurane in pure oxygen at a flow rate of 0.5 L/min; all animals received the analgesic Buprenorphrine (0.03 mg/kg, i.p. manufacturer). Using aseptic technique, each animal was implanted subcutaneously with a radiotelemeter (ETA-F10, Data Sciences International, St Paul, MN); the transmitter was placed under the skin to the right of the midline on the dorsal side. The two electrode leads were then tunneled subcutaneously across the lateral dorsal sides; the distal portions were fixed in positions that approximated those of the lead II of a standard electrocardiogram (ECG). Body heat was maintained both during and immediately after the surgery. Animals were given food and water post-surgery and were housed individually. All animals were allowed 7-10 days to recover from the surgery and reestablish circadian rhythms.

Radiotelemetry data acquisition - Radiotelemetry methodology (Data Sciences International, Inc., St. Paul, MN) was used to track changes in cardiovascular function by monitoring heart rate (HR), and ECG waveforms immediately following telemeter implantation, through exposure until 24 hours post-exposure. This methodology provided continuous monitoring and collection of physiologic data from individual mice to a remote receiver. Sixty-second ECG segments were recorded every 5 minutes during the pre- and post-exposure periods. ECG was recorded continuously during exposure (baseline and hours 1-4); HR was automatically obtained from the waveforms (Dataquest ART Software, version 3.01, Data Sciences International, St. Paul, MN, USA).

Electrocardiogram (ECG) analysis - ECGAuto software (EMKA Technologies USA, Falls Church VA) was used to visualize individual ECG waveforms, analyze and quantify ECG segment durations and areas, as well as identify cardiac arrhythmias as previously described

(Hazari, Haykal-Coates et al. 2009). Briefly, using ECGAuto, P-wave, QRS complex, and T-wave were identified for individual ECG waveforms and compiled into a library. Analysis of all experimental ECG waveforms was then based on established libraries. The following parameters were determined for each ECG waveform: PR interval (P_{start}-R), QRS complex duration (Q_{start}-S), ST segment interval (S-T_{end}) and QT interval (Q_{start}-T_{end}). QT interval was corrected for HR using the correction formula for mice $QTc = QT/(RR/100)^{1/2}$ (Mitchell, Jeron et al. 1998). All ECG streams with less than 10 s of identifiable cardiac cycles were excluded from ECG parameter calculations. Figure 2 shows a typical ECG trace as well as examples of the types arrhythmia observed in this study.

HRV Analysis - Heart rate variability (HRV) was calculated as the mean of the differences between sequential RRs for the complete set of ECG waveforms using ECGAuto. For each 1-min stream of ECG waveforms, mean time between successive QRS complex peaks (RR interval), mean HR, and mean HRV-analysis–generated time-domain measures were acquired. The time-domain measures included standard deviation of the time between normal-to-normal beats (SDNN), and root mean squared of successive differences (RMSSD). SDNN represents overall HRV, while RMSSD represents parasympathetic influence over HRV. Analysis of HRV was also conducted in the frequency domain using a fast-Fourier transform. For frequency domain analysis, the signal was analyzed with a Hamming window for segment lengths of 512 samples with 50% overlapping. The spectral power obtained from this transformation represents the total harmonic variability for the frequency range being analyzed. In this study, the spectrum was divided into low-frequency (LF) and high-frequency (HF) regions with frequency bands assigned as 0.15-1.5 and 1.5-5 Hz respectively. LF is generally believed to represent a combination of sympathetic and parasympathetic

activity, while HF indicates cardiac parasympathetic (vagal) activity (Ori, Monir et al. 1992). All ECG streams with less than 1 min of identifiable RR intervals were excluded from HRV analysis. Additionally thorough visual inspection was conducted to identify and exclude arrhythmias and artifacts.

Exposure – The study protocol included two days of animal-to-chamber acclimatization prior to exposure. Three or four hour exposures to acrolein or ozone respectively (Exposure) started with 30 min of additional chamber acclimatization. All mice were moved back to their home-cages after the exposure (Post-exposure) and monitored for 24 hours (Post-exposure 24hr+) before necropsy.

Ozone exposure – Ozone (O_3) exposures took place in whole-body exposure chambers. O_3 was generated by passing extra dry oxygen past an arcing transformer in a model V5-0 ozone generator (Ozone Research & Equipment Corp., Phoenix, AZ). The chamber concentrations (0.3 ppm) were controlled by the computer program DASYLab (version 9.0; DasyTec USA, Amherst, NH), which controlled the opening and closing of a mass flow controller at each chamber. Ozone concentrations were monitored using continuous gas analyzers (model 49i, model 49, Thermo Fisher Scientific, Foxfire, MA), which fed a signal to a proportional, integral, derivative loop control, which then either opened or closed the mass flow controller to maintain the O_3 concentration in the chamber at the desired level. Filtered air control exposures were simultaneously conducted with all chamber conditions maintained and O_3 levels < 0.010 ppm

Acrolein exposure – Acrolein exposures took place in whole-body plethysmography chambers (Model PLY3213, Buxco Electronics, Inc., Wilmington, NC, USA. Acrolein gas was metered from a 1,000 ppm cylinder into a glass mixing chamber where the gas was

mixed and diluted with dry filtered air to achieve a final concentration of 3 ppm of acrolein with a total flow of 6 L/min. The actual chamber concentration was measured once per hour using an HP5890 gas chromatograph (GMI Inc., Ramsey, MN, USA) equipped with manual injection, a flame ionization detector and a DB-VRX capillary column.

Cardiac Perfusion - The procedure for cardiac perfusion has been previously described (Tong et al. 2010). Briefly, 24 hours after exposure, mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.). Heparin (100 units) was injected intravenously before removal of heart. The hearts were rapidly removed and placed in ice-cold Krebs-Henseleit buffer, after which the aortas were cannulated. Retrograde perfusion via the aorta was performed under constant pressure (100 cmH_2O) above the heart. The non-recirculating perfusate was a Krebs-Henseleit buffer containing (in mmol/L) 120 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.75 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose. The buffer was aerated with 95% O_2—5% CO_2 and maintained at pH 7.4 and a temperature of 37°C.

For assessment of contractile function, a latex balloon on the tip of a polyethylene catheter was inserted through the left atrium into the left ventricle. The catheter was connected to a pressure transducer (Argon Medical Devices, Athens, TX) at the same height as the heart. The pressure of the left ventricular balloon was inflated to 0-5 cmH_2O. A PowerLab system was used to collect and process the heart rate, left ventricular developed pressure (LVDP), and contractility (dP/dt) data (AD Instruments, Milford, MA). All hearts were perfused for 25 min; we then initiated 20 min of global no-flow ischemia by stopping the flow of oxygenated perfusion buffer, followed by 1 hr of reperfusion. Onset of ischemic contracture was measured as the time from the start of ischemia until initial contracture (at least 5 cmH_2O increase in left ventricular pressure). Recovery of LVDP, expressed as a

percentage of the initial pre-ischemic LVDP, was measured at 20, 40 and 60 min of reperfusion after 20 min of ischemia.

Statistics - All data are expressed as means ± SEM. Statistical analyses of the data were performed with GraphPad Prism 5 (GraphPad software, San Diego CA). A minimum power of 0.8 was used to determine sample size. For HR, ECG intervals and HRV, two-way analysis of variance (ANOVA) for repeated-measures and Bonferroni *post hoc* tests were used to determine statistical differences. A one-way ANOVA was used to analyze arrhythmia counts. For Langendorff cardiac perfusion data, comparisons between groups were performed by one-way ANOVA followed by Bonferroni *post hoc* test for multiple comparisons. Comparisons were made across all groups taking into account the multiple endpoints, exposure groups and time points as well as any interactions.

Results

Heart Rate – Table 1 lists the average heart rates before, during and after exposure. The Pre-exposure period represents a 4 hr time-matched period one day before exposure. The Post-exposure period represents the 4 hrs directly following exposure, and the 24 hr Post-exposure represents a 4 hr time-matched period one day after exposure. All animals experienced an increase in HR when initially placed in the exposure chamber and all progressively decreased over the course of the exposure. There were no significant differences in HR within the strains among any of the exposure groups during any time period, however the WT animals demonstrated a more robust increase in HR in every exposure group compared with the KO animals. Exposure to ozone did produce a decrease

in HR in the KO animals compared with the pre-exposure period and compared with ozone-exposed WT animals.

Electrocardiogram (ECG) - Table 2 shows the ECG parameters before, during and after exposure. There were no significant differences in ECG interval durations between animals exposed to acrolein or ozone compared with filtered air during any time period. When compared to the pre-exposure period, WT animals had no significant changes in ECG interval durations during the exposure period. In contrast, KO mice demonstrated slight increases in QRS and QTc interval lengths during acrolein, ozone, and filtered air exposure indicating that these were likely a physiological response of the KOs to placement in the exposure chamber rather than a response to the pollutant.

Heart Rate Variability (HRV) - Exposure to acrolein caused significant increases in all measures of HRV in WT animals, including the time domain measurements SDNN and RMSSD (Figure 1A and B), as well as the high and low frequency domain measurements (Figure 1C and D) when compared with filtered air and KO mice. There was no effect of acrolein on KO mice nor was there any effect of ozone exposure on either WT or KO mice.

Cardiac arrhythmia - Typical ECG and cardiac arrhythmia observed in mice exposed to acrolein and ozone are shown in Figure 2. There was a significant increase in the number of arrhythmia during the 3 hour exposure period to acrolein (27.8 ± 10.8) when compared with filtered air (2.2 ± 0.9) (Figure 3). No other significant differences in arrhythmias were observed between any exposure groups at any time period.

Cardiac mechanical effects - Post-exposure (pre-ischemic) and post-ischemic left ventricular pressure measurements are summarized in Table 3. Exposure to acrolein significantly increased LVDP 24 hrs post-exposure (129.9 ± 10.3 cm H_20) compared with filtered air-exposed controls (86.4 ± 7.8 cm H_20) (Figure 4) There were no other significant differences in cardiac mechanical function between any other groups at any other time points.

Discussion

The results of this study demonstrate that TRPA1 channels found on airway sensory nerves, which can be described as sensors for a broad array of environmental irritants, play a role in the cardiovascular response of mice to acrolein. The data presented here also suggests that a single exposure to air pollution causes not only cardiac electrical disturbances, which are commonly observed in humans (Peters, Liu et al. 2000), but acute, nearly imperceptible changes in cardiac mechanical function as well; these findings corroborate not only our previous work (Kurhanewicz, McIntosh-Kastrinsky et al. 2014) but the work of others (Tong, Cheng et al. 2010). More importantly, the current study confirms that gaseous air pollutants have effects beyond the respiratory system affecting not only cardiac function but also the homeostatic control of the cardiovascular system as indicated by changes in HRV. In order to elucidate how gaseous air pollutants may cause this cardiac dysfunction, we investigated the role of TRPA1 by using high concentrations of acrolein as a putative positive control and lower concentrations of ozone as a more relevant and real-world exposure. Acrolein produced abnormal modulation of autonomic balance and an increased incidence of arrhythmia through TRPA1, which also mediated an increase in LVDP. In contrast, exposure to ozone did not appear to cause any significant effects despite the fact that mild

cardiovascular effects of ozone at similar levels have been documented by our lab in the past and by others (Wu, Kuo et al. 2010, Farraj, Hazari et al. 2012, Barath, Langrish et al. 2013, Wang, Jiang et al. 2013, Kurhanewicz, McIntosh-Kastrinsky et al. 2014, Farraj, Walsh et al. 2015).

Sensory receptors in the respiratory system, from the nose down to the lower lungs, respond to gaseous irritants and initiate visceral (*i.e*. autonomic) reflexive changes which impact the function of the cardiovascular system. While it is unlikely that inhalation of these gases affects the heart or vasculature by causing direct toxicity or damage, there is certainly enough data that suggests signals initiated in the airways can lead to altered cardiovascular regulation, compensatory deficits, and increased sensitivity to subsequent exposures (Widdicombe and Lee 2001). Thus, these irritant gases may not be overtly deleterious but rather may increase the risk of an adverse response if another stressor is encountered and the body is unable to maintain homeostasis. We previously showed that acrolein exposure increases risk by desensitizing the baroreflex (Hazari, Griggs et al. 2014), which maintains blood pressure, and therefore systemic perfusion, by altering heart rate. As such, the baroreflex, as an internal sensor of blood pressure, operates and exerts its effects through the sympathetic and parasympathetic branches of the autonomic nervous system. Therefore any exposure-related changes in autonomic balance, which can be measured through HRV, would result in altered, and possibly impaired, regulation of the cardiovascular system. Although yet unsubstantiated, TRPA1 activation by acrolein may represent the initiating event for this baroreflex desensitization given it leads to autonomic imbalance as we have shown here.

While the results from this study do not provide direct evidence of serious cardiac morbidity or premature mortality as a result of acute exposure to airway irritants, they may reflect a transient instability that can worsen if exposure continues over a longer period. Moreover, such dysregulation in individuals with pre-existing regulatory issues or disease resulting in poor compensatory capacity could be more seriously impacted. Performing studies in at-risk humans is problematic, so as a result these fundamental relationships in autonomic cardiac dysfunction may only be dissected in rodent models. As data examining autonomic function in rodents in the past had to be interpreted cautiously due to the use of anesthesia, the current results were produced from conscious unrestrained mice that could be monitored continuously during exposure, thus improving discernibility of toxicological effects relevant to human scenarios. Still, HRV data in rodents is not easily interpretable given that several dynamic factors contribute to it (Rowan, Campen et al. 2007).

Exposure to acrolein produced significant increases in SDNN, RMSSD, LF power and HF power in WT mice; this response was not present in acrolein-exposed TRPA1 KO animals. While decreased HRV is commonly accepted as an indicator of heightened cardiovascular risk in humans (Gold, Litonjua et al. 2000), increased HRV has also been linked to adverse cardiovascular outcomes (Stein, Domitrovich et al. 2005). Indeed, other reports note increased HRV as an indicator of heightened risk. For example, increased RMSSD has been shown to be associated with elevated risk of air pollution-induced arrhythmia (Davoodi, Sharif et al. 2010) and increases in HRV have been demonstrated preceding post-operative atrial fibrillation (Amar, Zhang et al. 2003). Additionally, increases in vagal tone have been associated with adverse cardiovascular events in type II diabetics (Eguchi, Schwartz et al. 2010), and linked with increased mortality in heart failure patients

and the elderly (de Bruyne, Kors et al. 1999, Stein, Domitrovich et al. 2005). With regard to the animal models, we have consistently shown that exposure to different types of air pollutants such as residual oil fly ash (Farraj, Hazari et al. 2011, Carll, Haykal-Coates et al. 2015), diesel exhaust (Hazari, Haykal-Coates et al. 2011, Carll, Lust et al. 2013), ozone (Farraj, Hazari et al. 2012) and acrolein (Hazari, Griggs et al. 2014) all lead to an increase in HRV in rodent models. This is not entirely surprising given increased HRV, and in particular RMSSD and HF, indicate parasympathetic modulation which is a well-characterized reflex response to airway sensory activation (Lee and Pisarri 2001). Thus, it is clear the relationship between HRV and cardiac dysfunction cannot be overly simplified to say increased HRV is good whereas decreased HRV is bad. Instead, it is the imbalance of this homeostatic mechanism, regardless of direction of change, which likely produces the increase in risk. In fact, the presence of simultaneous electrical disturbances in the ECG may further indicate the underlying shift from normal function.

In our studies, increased HRV, and therefore parasympathetic modulation, was observed with an increased incidence of sinus node dysfunction and dysrhythmia (Figure 2) during acrolein exposure in normal animals. Increased vagal tone can cause such changes in the P-wave to P-wave interval independent of normal breathing. This condition is known as non-respiratory sinus arrhythmia and it differs from respiratory sinus arrhythmia (RSA) in that it is not associated with respiration but rather occurs when the outflow of parasympathetic tone predominates over sympathetic tone (Deboor, Pelter et al. 2005, McMullen, Whitehouse et al. 2012) and unlike RSA is grossly irregular. Furthermore, non-respiratory sinus arrhythmia or dysrhythmia when observed with sinus node dysfunction can indicate sick sinus syndrome (Keller and Lemberg 2006), which is a group of abnormal heart

rhythms that appear not only as a blocked sinus signal but also as random intervals of bradycardia and tachycardia. Although these parameters fit what we observed with acrolein, it still remains to be determined whether it represents a toxicological effect that leads to adverse systemic outcomes; yet it would not be unreasonable to suspect that such a change might predispose an individual to subsequent triggered responses. Increases in HRV associated with increased randomness of the heart rate, that is, a high degree of non-respiratory sinus arrhythmia have been found to be strongly associated with risk of mortality (Stein 2004, McMullen, Whitehouse et al. 2012).

Interpreting the frequency domain of the HRV data is a bit more challenging. The cardiac cycle and R-R interval variability are affected by multiple control mechanisms including autonomic modulation at the SA node, the dynamic regulation of the vasculature, as well as endocrine/paracrine, endothelial and mechanical factors. Additionally, complex control mechanisms including baroreflex and respiratory sinus arrhythmia can also drive changes in these parameters. Numerous studies have shown that the HF power component of HRV is strongly associated with cardiovagal activity (Chess, Tam et al. 1975, Piccirillo, Ogawa et al. 2009, Billman 2013, Heathers 2014). In contrast, there is a growing body of evidence directly countering the claim that LF power is proportional to cardiac sympathetic nerve activity (Goldstein, Bentho et al. 2011, Billman 2013, Reyes del Paso, Langewitz et al. 2013). In fact, a number of studies suggest that LF power may better reflect other mechanisms exerting regulatory control over the cardiac cycle such as baroreflex activity in response to vasomotor tone (Moak, Goldstein et al. 2007, Goldstein, Bentho et al. 2011, Rahman, Pechnik et al. 2011). Investigators have also used LF/HF, which was popularized in the 1980's, as a measure of sympatho-vagal balance (Pagani, Lombardi et al. 1984), however

more recent analysis of this metric has cast doubt on its interpretation (Billman 2013, Reyes del Paso, Langewitz et al. 2013, Heathers 2014). Although the two branches of the autonomic nervous system may act reciprocally, they can also be co-activated or completely uncoupled and function independently of one-another (Amar, Zhang et al. 2003, Reyes del Paso, Langewitz et al. 2013). Thus, the HF and LF power measurements may provide information relating to different physiological control mechanisms. Namely that HF power may be more strongly related to vagal influence while LF may provide information about a mix of sympathetic and other factors such as blood pressure control mechanisms including modulation of vasomotor tone, but with parasympathetic factors accounting for the largest portion of the variability in this frequency range (Billman 2013, Reyes del Paso, Langewitz et al. 2013).

Notably, in this study exposure to ozone produced no discernable adverse cardiac effects in either WT or KO mice. While these results corroborate both our previous findings and those of others (Wu, Kuo et al. 2010, Farraj, Hazari et al. 2012, Barath, Langrish et al. 2013, Wang, Jiang et al. 2013, Farraj, Walsh et al. 2015) they also highlight the fact that the cardiovascular response to low level or near-ambient ozone is quite variable and requires further investigation. We assumed a role for TRPA1 given the findings of Taylor-Clark and Undem (Taylor-Clark and Undem 2006)2006) who showed activation of TRPA1 on airway C-fibers by high concentrations of ozone in an ex-vivo system. Therefore, the lack of discernable effects in this study may be due to the lower concentrations of ozone applied in our study as well as differences intrinsic to the in-vivo system which would certainly have changed the exposure profile (i.e. presence of epithelium, lining fluid, etc). To that point, the activity level of the subjects also contributes to the dose of ozone received as exercise has

been shown to increase the dose of ozone delivered to the lungs (Hatch, Slade et al. 1994). In this study, where the mice were primarily at rest, the effective dose of ozone delivered to the lower airways would be relatively low. Additionally, phenotypic differences between the subtypes of C-fibers being targeted by acrolein and ozone may also account for some of the differences observed in our responses. Acrolein is known to primarily impact the upper airways and not penetrate much past the larynx while ozone can reach deep into the lung. As such, C-fibers demonstrate phenotypic differences in their sensitivities and responses to chemical and mechanical stimuli based on location (Lee and Pisarri 2001) (Coleridge and Coleridge 1984). Hence, differences in activation of C-fiber subtypes may account for the disparate responses observed.

Our study demonstrates that autonomic nervous system function can be modulated by TRPA1, and appears to also play a role in the acrolein-induced cardiac arrhythmia and mechanical changes. Stimulation of the nasal or laryngeal mucosa with an irritant such as acrolein has been shown to cause a slowing of the HR in combination with an irregular rhythm (Kratschmer 2001), however there are very little data demonstrating a link between airway irritation and cardiac dysrhythmia. TRPA1 activation increases the release of glutamate, a centrally-acting neurotransmitter that facilitates transmission in the nucleus tractus solitarius (Sun, Bang et al. 2009). Although yet unproven, it is conceivable that this modulation of central neurotransmission can result in autonomic modulation and altered cardiovascular function. Similar mechanisms have already been demonstrated in models of cigarette smoke irritation (Mutoh, Joad et al. 2000) as well as others (Paton and Nolan 2000, Simms, Paton et al. 2006), while TRPA1's role in modulating other "autonomic" activity (e.g. breathing) in the brainstem is also known (Tani, Yazawa et al. 2015). TRPA1 also

appears to play a role in the increase in LVDP 24hrs after exposure. We have also shown myocardial dysynchrony and increased left ventricular stroke volume in C57BL/6 mice 24hrs after acrolein. Similarly, Tong et al. (Tong, Cheng et al. 2010) showed a trend toward increasing LVDP after particulate matter exposure. It is possible that the increase in LVDP observed here is a compensatory response to increased stroke volume to maintain cardiac output

Conclusion

We showed here that a single acute exposure to acrolein disrupts normal autonomic balance and produces arrhythmia in healthy, young mice. Disruption of autonomic homeostasis can increase the risk of subsequent adverse cardiovascular events as was seen here with the increased incidence of arrhythmia. Acute exposure to airway irritants may not cause overt functional effects, but rather may produce latent or subclinical effects that are only manifested when the host is challenged by a subsequent stressor or disease state. However, the likelihood of this occurring clearly depends on not only the level (i.e. concentration, duration, etc) of exposure, but the characteristics of the pollutant as well. The underlying health of the host must also be considered because the greatest risk may be in subjects with eroded or impaired compensatory capacity as is seen with chronic respiratory and cardiovascular disease. Despite a somewhat better understanding of this mechanism, all of these factors must be accounted for if a proper assessment of risk is to be made.

Tables and Figures

Table 1. The Effect of Acrolein or Ozone Exposure on Heart Rate

WT	**Filtered Air**	**Acrolein**	**Ozone**
Pre-exposure	575.9 ± 19.5	607.3 ± 34.4	563.1 ± 11.3
Exposure	617.1 ± 9.9	638.3 ± 6.6	626.6 ± 10.5
Post-exposure	546.5 ± 13.2	573.5 ± 7.1	548 ± 9.8
24hr Post-exposure	525.9 ± 14.2	552.4 ± 14.7	540.3 ± 8.8
KO			
Pre-exposure	570.9 ± 19.3	575 ± 16.2	573.3 ± 9.6
Exposure	587.9 ± 14.3	600.8 ± 12.6	562.2 ± 20.7 †‡
Post-exposure	584.8 ± 15.8	607.5 ± 9.5	594.8 ± 13.4
24hr Post-exposure	552.5 ± 17	573.2 ± 15.4	571.5 ± 9.4

Values are mean (beats/min) ± SEM. *$p < 0.05$; significantly different from FA. †$p < 0.05$; significantly different from Pre-exposure period. ‡$p < 0.05$; significantly different from WT strain. (n=8-12)

Table 2. The Effect of Acrolein or Ozone Exposure on Electrocardiogram Parameters

PR (msec)

WT	**Filtered Air**	**Acrolein**	**Ozone**
Pre-exposure	35.16 ± 0.59	32.65 ± 0.51	34.14 ± 0.74
Exposure	37.02 ± 0.4	34.33 ± 0.73	35.26 ± 0.58
Post-exposure	35.34 ± 0.54	32.82 ± 0.52	34.25 ± 0.59
KO			
Pre-exposure	30.34 ± 0.65	29.35 ± 0.35	30.17 ± 0.69 ‡

Exposure	30.95 ± 0.77	31.36 ± 0.75 ‡	31.97 ± 0.75 ‡
Post-exposure	32.33 ± 0.98	31.84 ± 0.75	32.75 ± 0.74

QRS (msec)

WT	**Filtered Air**	**Acrolein**	**Ozone**
Pre-exposure	13.41 ± 0.52	11.9 ± 0.08	12.45 ± 0.3
Exposure	12.4 ± 0.35	11.14 ± 0.28	12.23 ± 0.18
Post-exposure	13.28 ± 0.48	11.74 ± 0.02	12.34 ± 0.28
KO			
Pre-exposure	11.96 ± 0.34	12.43 ± 0.31	11.66 ± 0.25
Exposure	13.64 ± 0.56 †	14.21 ± 0.58 †‡	13.45 ± 0.5 †
Post-exposure	11.78 ± 0.35	12.39 ± 0.33	11.71 ± 0.36

QTc (msec)

WT	**Filtered Air**	**Acrolein**	**Ozone**
Pre-exposure	74.58 ± 3.07	65.94 ± 2.08	71.58 ± 2.69
Exposure	67.98 ± 2.14	68.1 ± 1.73	69.39 ± 1.34
Post-exposure	73.93 ± 3.93	63.36 ± 2.15	69.95 ± 2.95
KO			
Pre-exposure	72.38 ± 1.18	71.81 ± 1.34	72.58 ± 3.11
Exposure	82.91 ± 3.39 †‡	84.74 ± 4.07 †‡	81.93 ± 4.02 †‡
Post-exposure	67.68 ± 1.76	70.18 ± 1.21	67.29 ± 2.38

Values are mean ± SEM. *$p < 0.05$; significantly different from FA. †$p < 0.05$; significantly different from Pre-exposure period. ‡$p < 0.05$; significantly different from WT strain. (n=8-12)

Table 3. Cardiac Mechanical Effects of Exposure to Acrolein or Ozone

Strain/Exposure	WT/FA	WT/Acrl	WT/O_3	KO/FA	KO/Acrl	KO/O_3
LVDP Baseline (cm H_20)	86.39 ± 7.86	**129.9 ± 10.33 ***	74.22 ± 9.60	101.9 ± 8.72	83.62 ± 7.32	80.85 ± 16.96
dP/dT(+) (cm H_20/sec)	3527 ± 243	4501 ± 415	3209 ± 379	5297 ± 721	3980 ± 500	3830 ± 601
dP/dT(-) (cm H_20/sec)	-2794 ± 296	-3411 ± 363	-2363 ± 351	-3267 ± 268	-2416 ±209	-3560 ± 1214
LVDP Post-ischemia Recovery (% $LVDP_{baseline}$)						
20 min	32.06 ± 9.3	34.14 ± 5.6	50.52 ± 12.7	14.4 ± 3.8	35.08 ± 13.3	30.37 ± 6.9
40 min	38.07 ± 10.7	37.25 ± 6.3	51.88 ± 15.4	23.39 ± 7.1	58.63 ± 34.9	41.94 ± 12.0
60 min	44.00 ± 13.7	39.92 ± 9.6	50.55 ± 15.6	24.38 ± 8.4	34.74 ± 12.8	49.01 ± 17.7

dP/dt_{max} = maximum 1st derivative of the change in left ventricular pressure/time; dP/dt_{min} = minimum 1st derivative of the change in left ventricular pressure/time. Values are mean ± SEM. *$p < 0.05$; significantly different from FA (n=8-12)

Table 4. Summary of Exposure Effects

Exposure Group	HR	SDNN	RMSSD	LF	HF	PR	QRS	QTc	Arrhythmia	LVDP baseline	LVDP Post-ischemia recovery	dP/dT (+)	dP/dT (-)
Filtered Air	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
Ozone	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
Acrolein	NE	↑	↑	↑	↑	NE	NE	NE	↑	↑	NE	NE	NE

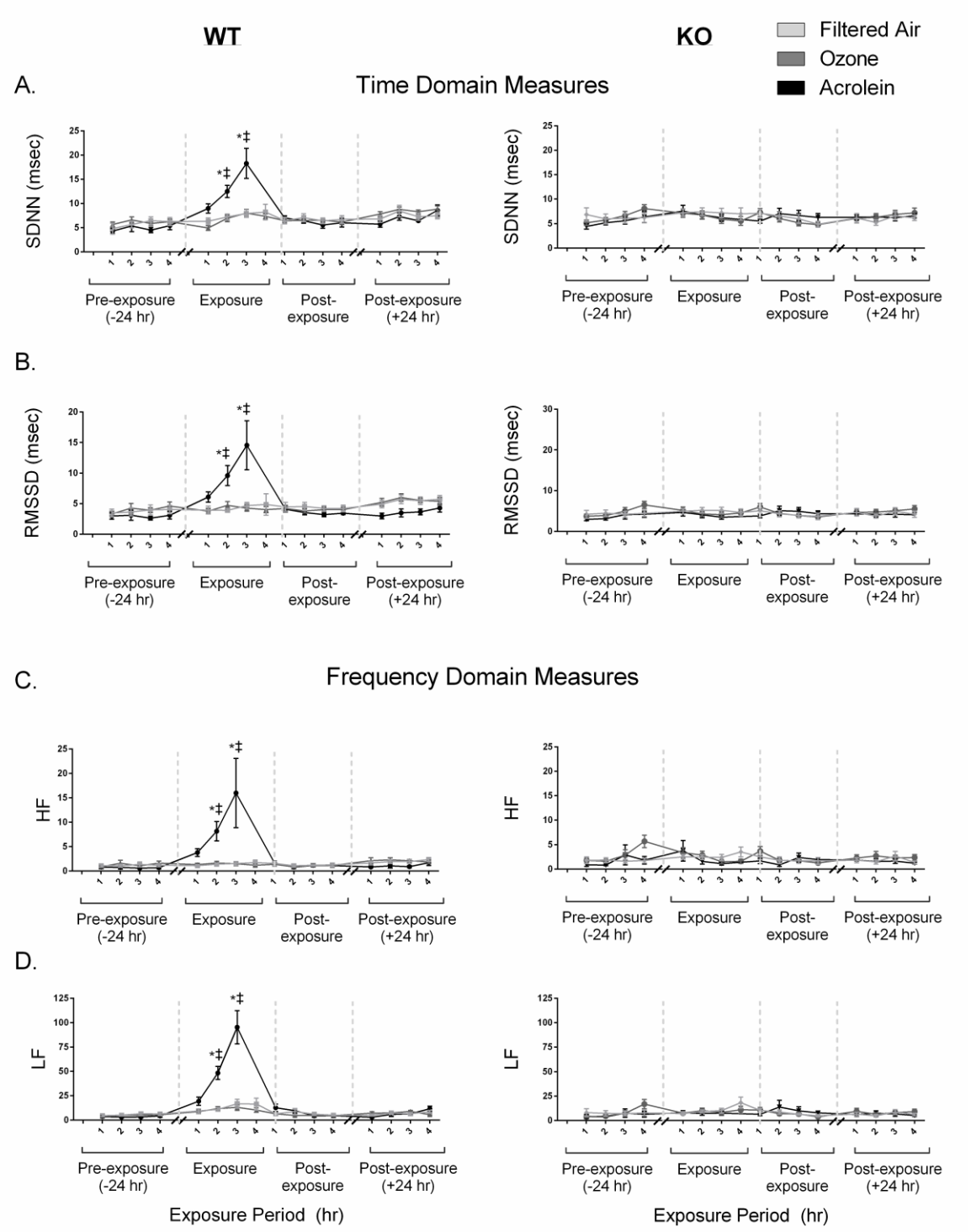


Figure 1. *Exposure to acrolein but not ozone increases all measures of HRV*. WT mice experienced an increase in SDNN, RMSSD, LF/HF, LF and HF during exposure to acrolein. These increases in HRV were not observed in TRPA1 KO animals. There was no effect of ozone exposure on either the WT or KO animals. Values are mean ± SEM. *$p < 0.05$; significantly different from FA. ‡$p < 0.05$; significantly different from KO strain. (n=8-12)

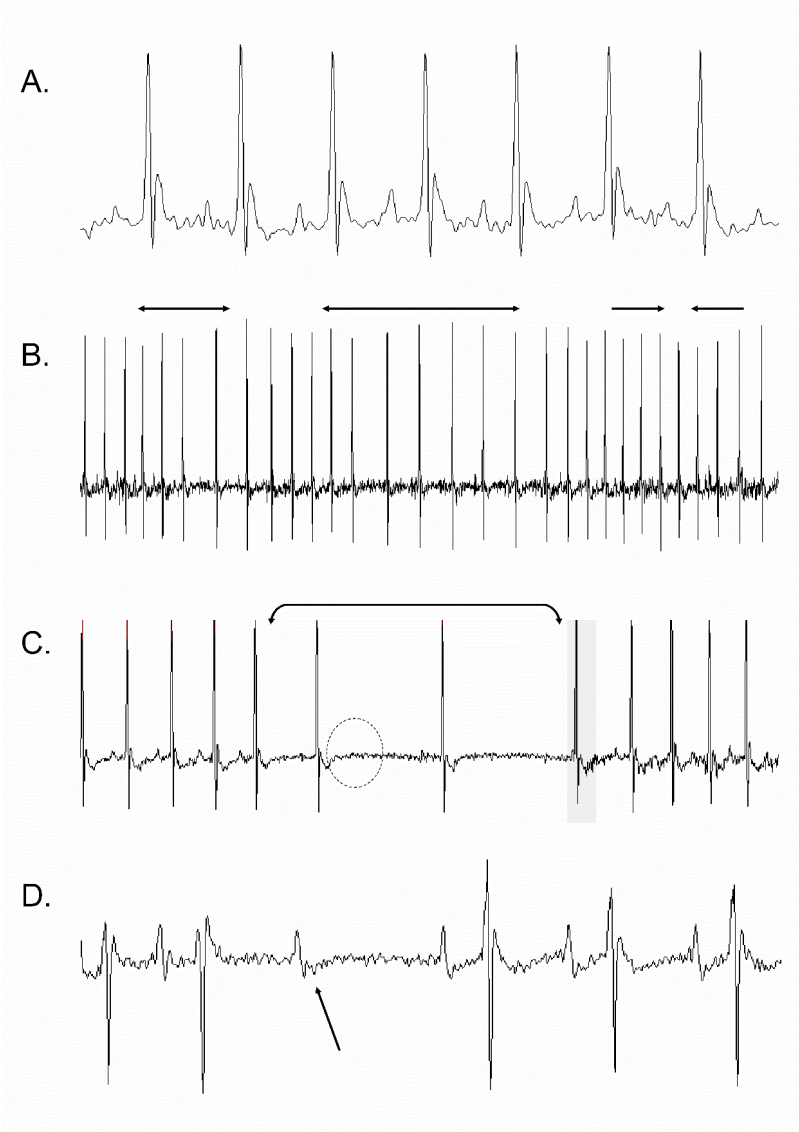


Figure 2. *Typical Electrocardiogram and Cardiac Arrhythmia in Mice Exposed to Acrolein and Ozone.* **A.** Typical mouse ECG during normal sinus rhythm. **B.** Dysrhythmia characterized by brief periods of bradycardia dispersed between periods of normal heart rate. **C.** Sino-atrial node dysfunction characterized by elongation of the R-R interval and loss of discernable p-waves. **D.** Non-conducted p-waves, which represent a loss of conduction from the atria to the ventricles. All of these arrhythmia are typical of vagal dominance.

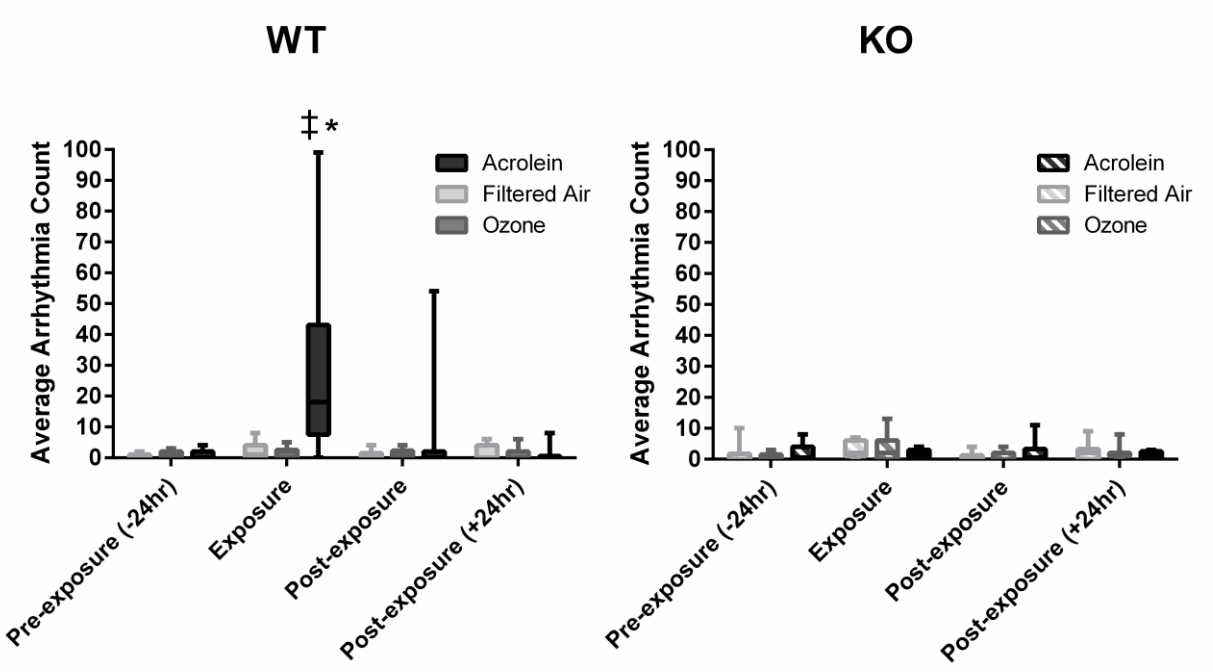


Figure 3. *Exposure to acrolein increases cardiac arrhythmias in wildtype mice*. WT mice experienced an increase in arrhythmia during exposure to acrolein. Values are total number of arrhythmia occurances during the pre-exposure, exposure and post-exposure periods. There were no significant differences in arrhythmia counts between any of the groups during any other period. *$p < 0.0001$; significantly different from FA. ‡$p < 0.0001$; significantly different from KO strain (n=8-12)

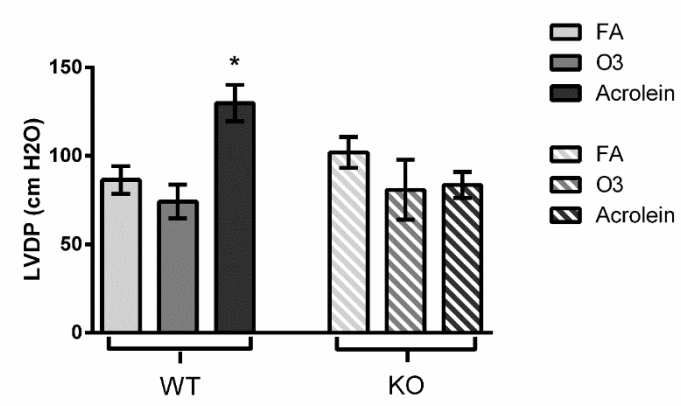


Figure 4. *Exposure to acrolein increases left ventricular developed pressure 24hrs post-exposure.* WT animals exposed to acrolein experienced an increase in LVDP 24hrs post-exposure when compared to FA controls. Exposure to ozone did not produce any changes in LVDP. *$p < 0.05$; significantly different from FA (n=8-12)

CHAPTER IV: EXPOSURE TO ACROLEIN PRODUCES CARDIAC EFFECTS MEDIATED BY PARASYMPATHETIC DOMINANCE BUT ALSO SYMPATHETIC MODULATION IN MICE

Numerous studies show that short-term air pollution exposure modulates heart rate variability (HRV). HRV is a marker of autonomic influence on the heart and represents homeostatic control mechanisms which dynamically regulate cardiovascular function. Our previous studies show that a single exposure to air pollution increases HRV and produces arrhythmia in wild-type mice in a manner implicating autonomic imbalance. In addition, these changes appeared to be mediated through the airway irritant sensor TRPA1. Thus, the goal of this study was to characterize the autonomic changes due to exposure as reflected by HRV in both wild-type and knockout mice. We hypothesized that pharmacological inhibition of the parasympathetic and sympathetic effects on the heart would block the cardiac response to air pollution in wild-type mice, and that TRPA1 knockout mice would demonstrate neither modulated HRV nor cardiovascular dysfunction. Conscious, unrestrained C57BL/6 and TRPA1 knockout mice were exposed to 3 ppm acrolein for 3 hours. Immediately prior to exposure separate cohorts were treated with either atropine (2 mg/kg) or atenolol (2 mg/kg) to block the parasympathetic or sympathetic influence on the heart, respectively, hexamethonium (80 mg/kg) to block both, or saline. Electrocardiogram (ECG) and HR were recorded continuously before, during and after exposure. Prior to exposure treatment with atenolol decreased HR but had no effect on any measure of HRV. Treatment with atropine

increased HR and decreased SDNN. Treatment with hexamethonium decreased both HR and SDNN. Exposure to acrolein produced significant increases in SDNN, RMSSD and LF measures of HRV, as well as an increase in arrhythmia in WT mice. TRPA1 KO mice did not demonstrate any increases in HRV with exposure to acrolein, however there was a minor increase in arrhythmia. Treatment with atenolol reduced this response while treatment with atropine enhanced it. Treatment with hexamethonium blunted the acrolein-induced increases in HRV. Treatment with all three pharmacological agents blocked the acrolein-induced increase in arrhythmia. A generalized stress response in early exposure and pharmacological wash-out near mid-exposure compounded the complexity of this analysis. However it is clear from these data that acute exposures to air pollution, which may not cause observable symptoms, can disrupt normal autonomic balance in healthy young animals and that while these changes are characterized by parasympathetic dominance, sympathetic modulation also appears to play a role

Introduction

Autonomic reflex arcs activated by sensory receptors in the airways are believed to mediate some of the acute cardiovascular effects of air pollution. In relation to this, there is evidence to suggest that 1) nociceptive nerve fibers in the airways are activated by irritant gaseous components of ambient air pollution (Bessac & Jordt 2010), 2) activation of these fibers produces afferent signaling to the brainstem and activity in specific areas known to play a role in the regulation of cardiovascular and ventilatory function (Mutoh, Bonham, et al. 2000; Bonham et al. 2006), and 3) modulation of efferent autonomic activity in the brainstem occurs as a result of neuropeptide release and can persist for some time after the

initial stimulation (i.e. plasticity)(Carr & Undem 2003; Bonham et al. 2001). In fact, we previously demonstrated that the airway irritant receptor transient receptor potential A1 channel (TRPA1) mediates cardiac arrhythmia through autonomic modulation one day after diesel exhaust exposure (Hazari, Callaway, Winsett, Lamb, Haykal-coates, et al. 2012). Similarly, altered heart rate variability (HRV) and cardiac function during acrolein exposure was abolished using TRPA1 knockout mice further confirming the link between sensory activation and altered autonomic modulation (Chapter II).

Numerous biological pathways have been described that link air pollution with cardiac dysfunction, including systemic inflammation and oxidative stress, vasoconstriction, enhanced coagulation/thrombosis, autonomic effects due to sensory receptor activation, and the direct effects of translocated particles on the myocardium (Simkhovich et al. 2008; Lee & Widdicombe 2001); of these, airway sensory activation resulting in changes in autonomic nervous system function may be the most relevant when considering the short-term, reversible, and often latent effects of an acute exposure. In fact, epidemiological studies have repeatedly shown that exposure to air pollution causes changes in HRV (Gold et al. 2000; Pope et al. 1999), which is an indicator of autonomic influence on the heart. Yet, data characterizing HRV changes during exposure are limited, particularly focused examinations of the relative influence of the parasympathetic and sympathetic branches which dynamically control cardiac function on a beat-by-beat basis. The results of this study will address some of those questions and fill the data gaps related to HRV responses during exposure, particularly as they relate to TRPA1 and irritant-induced activation in the airways.

HRV is often used to address the influence of the autonomic nervous system on the heart. It is an indirect measure which represents input from homeostatic control mechanisms that regulate, among other things, cardiovascular and respiratory function (Anon 1996). As such, the cardiac cycle and R-R interval variability are continuously adjusted by multiple controls including the modulation of autonomic outflow at the sinoatrial (SA) node, the dynamic regulation of the vasculature, as well as endocrine/paracrine, endothelial and mechanical factors. Additionally, dynamic mechanisms like baroreflex, which monitors blood pressure changes, chemoreflex, which measures oxygen and carbon dioxide in the body, and respiratory sinus arrhythmia, which originates from the cyclical changes in thoracic pressure during breathing, can also drive changes in HRV. Despite this complexity, several measures of HRV have been shown to correlate with modulation of one or both arms of the autonomic nervous system and thus indicate changes in the neural inputs to the heart

Thus, the current study focuses on the role of the parasympathetic and sympathetic branches of the autonomic nervous system in HRV, which is still not completely characterized in mice. In particular, it is not always clear whether a given response is related to the normal dynamic tendency of HRV or if it is actually an exposure-induced effect. In addition, the fluctuation of autonomic influences on the heart during an air pollution exposure have not been delineated. This is particularly important given certain changes in HRV have been strongly associated with increased cardiovascular risk. Whether this relates to an increased level of sympathetic influence due to the environmental stressor or vagal (i.e. parasympathetic) dominance because of irritant activation in the airways remains to be determined. Hence, in this study, pharmacological blockade was used to determine the relative contribution of the parasympathetic and sympathetic branches to HRV changes

observed during acrolein exposure. We hypothesized that pharmacological blockade of parasympathetic neurotransmission would block the HRV effects of acrolein in mice. Ventilatory function was also measured during exposure to get a general idea of the acrolein effects on breathing and whether they may affect HRV.

Materials and Methods

Animals - Female C57BL/6 (21 ± 1.1 g) and TRPA1 -/- mice (27 ± 3.8 g) between 15 and 30 weeks old were used in this study (Jackson Laboratory - Bar Harbor, ME). Mice were initially housed five per cage and maintained on a 12-hr light/dark cycle at approximately 22°C and 50% relative humidity in an AAALAC–approved facility. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency and are in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The animals were treated humanely and with regard for alleviation of suffering. Background controls were used as appropriate.

Experimental Groups and Pharmacological Agents - Wild-type and TRPA1 knockout mice were randomly assigned to one of four pharmacological treatment groups: (1) Saline vehicle (0.9% sodium chloride), (2) Atropine (2 mg/kg), (3) Atenolol (2 mg/kg), or (3) Hexamethonium chloride (80 mg/kg). All drugs were purchased from Sigma-Aldrich, St. Louis MO. There were 6 animals per group. Animals were pre-treated a total of 3 times prior to exposure (days -14, -10, -7) to assure they were responding predictably and reproducibly to pharmacological agents, then given a week in order to avoid possible complications due to

habituation to the drug and for any residual effects of the drugs to wear off. On day 0 animals were treated immediately prior to filtered air exposure, and on day 1 animals were treated immediately prior to acrolein exposure.

Surgical implantation of radiotelemeters - Animals were weighed and then anesthetized using inhaled isoflurane (Isothesia, Butler Animal Health Supply, Dublin OH). Anesthesia was induced by spontaneous breathing of 2.5% isoflurane in pure oxygen at a flow rate of 1 L/min and then maintained by 1.5% isoflurane in pure oxygen at a flow rate of 0.5 L/min; all animals received the analgesic Buprenorphrine (0.03 mg/kg, i.p. manufacturer). Using aseptic technique, each animal was implanted subcutaneously with a radiotelemeter (ETA-F10, Data Sciences International, St Paul, MN); the transmitter was placed under the skin to the right of the midline on the dorsal side. The two electrode leads were then tunneled subcutaneously across the lateral dorsal sides; the distal portions were fixed in positions that approximated those of the lead II of a standard electrocardiogram (ECG). Body heat was maintained both during and immediately after the surgery. Animals were given food and water post-surgery and were housed individually. All animals were allowed two weeks to recover from the surgery and reestablish circadian rhythms.

Radiotelemetry data acquisition - Radiotelemetry methodology (Data Sciences International, Inc., St. Paul, MN) was used to track changes in cardiovascular function by monitoring heart rate (HR), and ECG waveforms immediately following telemeter implantation, through exposure until 24 hours post-exposure. This methodology provided continuous monitoring and collection of physiologic data from individual mice to a remote receiver. Sixty-second ECG segments were recorded every 5 minutes during the pre- and

post-exposure periods. ECG was recorded continuously during exposure (baseline and hours 1-4); HR was automatically obtained from the waveforms (Dataquest ART Software, version 3.01, Data Sciences International, St. Paul, MN, USA).

HRV Analysis - Heart rate variability (HRV) was calculated as the mean of the differences between sequential RRs for the complete set of ECG waveforms using ECGAuto. For each 1-min stream of ECG waveforms, mean time between successive QRS complex peaks (RR interval), mean HR, and mean HRV-analysis–generated time-domain measures were acquired. The time-domain measures included standard deviation of the time between normal-to-normal beats (SDNN), and root mean squared of successive differences (RMSSD). Analysis of HRV was also conducted in the frequency domain using a fast-Fourier transform. For frequency domain analysis, the signal was analyzed with a Hamming window for segment lengths of 512 samples with 50% overlapping. The spectral power obtained from this transformation represents the total harmonic variability for the frequency range being analyzed. In this study, the spectrum was divided into low-frequency (LF) and high-frequency (HF) regions with frequency bands assigned as 0.15-1.5 and 1.5-5 Hz respectively. All ECG streams with less than 1 min of identifiable RR intervals were excluded from HRV analysis. Additionally thorough visual inspection was conducted to identify and exclude arrhythmias and artifacts.

Exposure – The study protocol included two days of animal-to-chamber acclimatization prior to exposure. 3 hour exposures to both filtered air and acrolein started with 30 min of additional chamber acclimatization. Mice served as their own FA control: They were exposed first to filtered air, then exposed to acrolein 24 hours later. Mice were moved back

to their home-cages and monitored immediately following both exposures. Necropsies were performed 24 hours after acrolein exposure.

Acrolein exposure – Acrolein exposures took place in whole-body plethysmography chambers (Model PLY3213, Buxco Electronics, Inc., Wilmington, NC, USA. Acrolein gas was metered from a 1,000 ppm cylinder into a glass mixing chamber where the gas was mixed and diluted with dry filtered air to achieve a final concentration of 3 ppm of acrolein with a total flow of 6 L/min. The actual chamber concentration was measured once per hour using an HP5890 gas chromatograph (GMI Inc., Ramsey, MN, USA) equipped with manual injection, a flame ionization detector and a DB-VRX capillary column.

Statistics - All data are expressed as means ± SEM. Statistical analyses of the data were performed with GraphPad Prism 6 (GraphPad software, San Diego CA). A minimum power of 0.8 was used to determine sample size. For HR, ECG intervals and HRV, two-way analysis of variance (ANOVA) for repeated-measures and Bonferroni *post hoc* tests were used to determine statistical differences. A one-way ANOVA was used to analyze arrhythmia counts. Comparisons were made across all groups taking into account the multiple endpoints, exposure groups and time points as well as any interactions.

Statistical Power - We had a sample size of 6, which allows for sufficient power in the statistical analysis of various biological/molecular (lung lavage indicators of inflammation and toxicity, serum and plasma indicators of coagulation, inflammation, and toxicity, heart and lung tissue indicators of inflammation and toxicity, and heart and lung pathology) and physiological (electrocardiogram, blood pressure, heart rate, ventilation) endpoints.

Results

Cardiac Responses

Characterization of HR and HRV in TRPA1 KO vs WT mice - Control WT and TRPA1 KO mice were injected with saline and exposed to filtered air. HR and HRV were measured during pre-exposure, exposure, and post-exposure time periods (Figure 1.). WT mice demonstrated an elevated HR at the beginning of exposure which decreases steadily before returning to baseline levels around mid-exposure. TRPA1 KO mice also had increased HR during the first fifteen minutes of exposure, however KO mice demonstrated a more rapid return of HR to baseline, producing a statistically significant decrease in HR compared with WT mice during early exposure. Additionally TRPA1 KO mice demonstrated increases in SDNN and RMSSD concurrent with their decrease in HR. There were no other differences between the strains for any other measure of HRV or occurrence of arrhythmia

Effect of pharmacological treatment on HR and HRV in WT and TRPA1 KO mice - Treatment with atenolol decreased HR but had no effect on any measure of HRV in WT or TRPA1 KO animals. Treatment with atropine increased HR in both WT and TRPA1 KO mice, however this only reached statistical significance in KO mice. Atropine also significantly decreased SDNN in both WT and TRPA1 KO mice. Treatment with hexamethonium slightly decreased HR and SDNN in WT mice but the effect was considerably more profound and reached statistical significance only in the TRPA1 KO mice (Figure 2). There was no other pharmacological effects on any measures of HR or HRV in either strain (Figure 2. B)

Effect of acrolein exposure on HR, HRV and arrhythmia in WT and TRPA1 KO mice - Exposure to acrolein produced significant increases in SDNN, RMSSD and LF measures of HRV (Figure 3A), as well as an increase in arrhythmia (6.33 ± 2.56) compared with filtered air controls (1.33 ± 0.98) and pre-exposure arrhythmia counts (0 ± 0) in WT mice (Figure 3B). TRPA1 KO mice did not demonstrate any increases in HRV with exposure to acrolein, however there was an increase in arrhythmia (3.66 ± 3.47) compared with pre-exposure counts (0 ± 0). This increase was not significant compared with the TRPA1 KO filtered air control group and it was driven by increased arrhythmia from a single animal in the acrolein-exposed TRPA1 KO group.

Combined effect of pharmacological treatment and acrolein exposure on HR, HRV and arrhythmia in WT and TRPA1 KO mice - Treatment with atenolol blocked acrolein-induced increases in SDNN and RMSSD but did not affect the acrolein-induced increase in LF (Figure 4A and B). Treatment with atropine enhanced acrolein-induced increases in HR, SDNN, RMSSD and LF, and increased HF as well (Figure 4A and C). Acrolein exposure enhanced the decrease in HR observed with hexamethonium treatment alone during early exposure. Treatment with hexamethonium also blunted the acrolein-induced increases in SDNN and RMSSD but did not affect the acrolein-induced increase in LF. Lastly, hexamethonium treatment increased HF during the final half hour of exposure (Figure 4A and D). Treatment with all three pharmacological agents blocked the acrolein-induced increase in arrhythmia (Figure 5).

Ventilatory Responses

Characterization of ventilatory timing in TRPA1 KO vs WT mice - Control WT and TRPA1 KO mice were injected with saline and exposed to filtered air. Ventilatory parameters were measured during exposure (Figure 6). Compared with WT mice TRPA1 KO mice demonstrated increased inspiratory and expiratory time periods, as well as decreased breathing frequency. TRPA1 KO mice also demonstrated increased enhanced pause values throughout the exposure but only reaching statistical significance during the final hour.

Effect of acrolein exposure on ventilation in WT and TRPA1 KO mice - Exposure to acrolein produced changes in measures of ventilatory timing (Figure 7A) as well as qualitative differences in the shape and amplitude of the ventilatory flow trace (Figure 7B) in WT mice. TRPA1 KO mice did not demonstrate any changes in ventilatory timing with exposure to acrolein with the exception of an increase in enhanced pause during the final 15 minutes of exposure (Figure 7A and C).

Effect of pharmacological treatment on ventilation in WT and TRPA1 KO mice - WT and TRPA1 KO mice did not produce significant responses to atenolol or atropine treatment alone. Hexamethonium treatment however produced minor changes in ventilatory timing in KO mice; namely an early decrease in inspiratory and expiratory time as well as a decrease in breathing frequency and minute volume.

Combined effect of pharmacological treatment and acrolein exposure on ventilation in WT and TRPA1 KO mice - In WT mice treatment with atenolol blunted the acrolein-

induced changes in ventilatory timing, including blocking the increase in expiratory time and decrease in breathing frequency. Atenolol treatment with acrolein also attenuated the acrolein-induced increase in peak expiratory flow and reduced relaxation time (Figure 8A and B). Treatment with atropine also blunted the acrolein-induced changes in ventilatory timing including blocking the decrease in breathing frequency, as well as attenuating the acrolein-induced increases in expiratory time and tidal volume. Atropine treatment also decreased relaxation time during early exposure (Figure 8A and C). Hexamethonium treatment produced a biphasic response where inspiration time, expiration time and relaxation time were only increased during early exposure, while peak expiratory flow, tidal volume and enhanced pause were reduced at the earliest time points but quickly rose within the first hour and remained elevated. Hexamethonium treatment also reduced breathing frequency during early exposure and elevated it during late exposure (Figure 8A and D). TRPA1 animals exposed to acrolein demonstrated an increase in enhanced pause only during the final fifteen minutes of exposure; this was blocked by treatment with atropine (Table#).

Biochemical markers and inflammatory cells in BAL - Biochemical analysis of BAL fluid showed that TRPA1 KO mice had lower SOD_{total} as well as MnSOD compared with WT mice. Treatment with atropine and atenolol blocked this effect. There were no other significant differences between any other groups.

Discussion

Building on our previous findings, this study demonstrates that TRPA1 mediates acrolein-induced cardiovascular changes through mechanisms involving both the parasympathetic and sympathetic branches of the autonomic nervous system. In addition,

TRPA1 also plays a role in acute breathing changes indicative of airway irritation in these healthy mice. Although, one of the proposed pathways for the cardiovascular effects of air pollution includes the activation of irritant reflex arcs and modulation of autonomic function there has been very little data characterizing this phenomenon to date. Part of the challenge of clarifying these mechanisms has been due to the complexity of autonomic cardiovascular control from a systemic perspective; namely, the fact that it is governed by several regulatory processes whose interplay can vary on a beat-to-beat basis. In particular, the body is continuously monitored by internal sensory apparatuses (i.e. baro- and chemosensory) that send information to the parasympathetic and sympathetic branches which in turn alter the activity of the heart and vasculature. The regular beating of the heart is also modulated in this manner by external "sensors" which respond to environmental stimuli by activating cardiopulmonary reflex arcs upon exposure and reseting autonomic function and control of the heart for a certain amount of time thereafter (Perez et al. 2015). Although these data demonstrate how autonomic nervous system function is modulated by exposure to inhaled irritants to produce reflexive changes in not only ventilatory function, but cardiovascular function as well, they certainly do not address all the possible mechanisms underlying these effects.

We previously demonstrated that TRPA1 mediates increased HRV and incidence of arrhythmia during exposure to acrolein (Chapter II). These results were reproduced in the current study in which we observed increased SDNN, RMSSD and LF measures of HRV, as well as an increase in brady-arrhythmias with exposure to acrolein which were not apparent in the TRPA1 KO animals. Overall, these data suggested that the response to acrolein, which is a ubiquitous air pollutant and activator of TRPA1, was characterized solely by

parasympathetic dominance. However cardiac and ventilatory responses during exposure to acrolein following pharmacological blockade of one or both branches of the autonomic nervous system demonstrated a role for both sympathetic and parasympathetic modulation. Atenolol, which blocks β1-adrenergic receptors and ostensibly sympathetic neurotransmission at the level of the heart, significantly reduced both the time-domain HRV response to acrolein exposure, whereas atropine, which prevents parasympathetic neurotransmission by blocking muscarinic receptors on the heart, enhanced all of the acrolein-induced HRV responses and in particular increased HF which was not evident in animals without pharmacological blockade. Thus, even though an increase in HRV, specifically RMSSD and HF, generally reflects parasympathetic modulation, sympathetic effects also appear to play a role.

As such, the response of un-exposed mice to the pharmacological drugs might help to clarify what is happening during exposure. Blockade of parasympathetic, sympathetic or both confirmed two things in general: (1) baseline heart rate in mice is greatly influenced by the sympathetic branch, hence the significant reduction in heart rate with atenolol and similar trend with hexamethonium, and minimal effect with atropine, and (2) although short-term HRV is under the control of both branches it is mostly regulated by parasympathetic modulation (Gehrmann et al. 2000; Pham et al. 2009); this could be seen by the atropine-induced decrease in SDNN and similar trend in RMSSD and LF, and lack of effect of atenolol on any HRV parameter. These characteristics need to be carefully considered when assessing the HRV effects of acrolein exposure, particularly when combined with pharmacological blockade.

The entire 3-hour exposure to acrolein appears to produce a bi-phasic response characterized by early sympathetic modulation followed by enhanced vagal modulation at later time-points. The fact that we observed an early elevated heart rate in air-exposed mice as well as in those exposed to acrolein suggests that a generalized stress response, one potentially due to handling, injection and placement in the exposure chamber, may have contributed to the sympathetic modulation early during the exposure. This appears to be unresolvable, even with acclimatization (Andreev-Andrievskiy et al. 2014). However, midway through the exposure, acrolein appears to prevent the decline in heart rate, albeit without further increase, when compared to air, an effect which coincides with a significant increase in SDNN, RMSSD and LF. Thus, this time point likely represents the period of acrolein effects without the generalized stress. Furthermore, it is apparent from our pharmacological treatment approach (i.e. immediately prior to acrolein exposure) that most of the physiological effects of the drugs occurred within the first half of the exposure period and so what was observed in the second half of the exposure was a rebound response to the dissipation of the drug effect. This physiological "compensation" when the drug washes out has been reported by others (Fuder & Muscholl 1995; Farah et al. 2006) and in our case likely contributes to blocking the HRV effects of acrolein.

It is notable that during the first half of the exposure, atenolol blocked the generalized stress-induced increase in HR and likely prevented any blood pressure increases as well. On the other hand, atenolol does not block the stress-induced increased sympathetic nerve activity given it blocks neurotransmission at the level of the heart. Thus, increased sympathetic nerve activity would continue to inhibit acetylcholine release from parasympathetic nerves keeping SDNN and RMSSD low. Once again, this has been observed

with other models of stress in the same strain of mice (Andreev-Andrievskiy et al. 2014; Fuder & Muscholl 1995; Farah et al. 2006). As far as the second half of the exposure is concerned, atenolol's ability to block the acrolein-induced SDNN/RMSSD increase may be due to the recovery of sympathetic influence on the heart as the drug washes out which ostensibly balanced out the increased parasympathetic shift which occurred due to irritant inhalation (Farraj et al. 2015; Carll et al. 2015; Hazari et al. 2014). To some degree this is likely related to baroreflex on autonomic balance, since the atenolol-induced effects in the first half of exposure likely result in reflex sympathetic activation later (Just et al. 2000).

The aforementioned conclusion is also supported by the fact that cholinergic blockade with atropine resulted in a further increase in heart rate and decrease in SDNN and RMSSD in the first half of acrolein exposure. These results are in accordance with the concept that the cardiac stress response in mice is due to activation of sympathetic and withdrawal of parasympathetic activity. This also reiterates the point that HRV in mice is dominated by parasympathetic tone especially when considered with atenolol's lack of SDNN and RMSSD effects. Subsequently, as atropine washed out in the second half of the exposure, recovery of parasympathetic influence further increased SDNN, RMSSD and even the frequency domain measures. In fact, the increase in LF during the second half of exposure, which was not blocked by any treatment, may in part represent the respective recovery of sympathetic and parasympathetic influence with atenolol and atropine treatment. This is a reasonable assertion given LF indicates not only sympathetic and parasympathetic modulation but baroreflex input as well (Reyes del Paso et al. 2013).

Hexamethonium blocks both braches of the autonomic nervous system and this appears to reduce the acrolein-induced HRV response overall, however there is still some effect of exposure confirming that parasympathetic activity dominates mouse HRV and indicating homeostatic control mechanisms which function outside of direct autonomic modulation of heart rate but still contribute to HRV measures (Sayers 1973; Parati et al. 1995). Additional control mechanisms include baroreflex but also respiratory sinus arrhythmia (i.e. breathing patterns), which can also drive changes in HRV (Sayers 1973; Reyes del Paso et al. 2013). In fact, early work on upper airway irritants such as acrolein suggested a biphasic response to irritants such as acrolein whereby there is early sympathetic modulation followed by a parasympathetic dominance (Alarie 1973). It is this later vagal dominance phase that is being suppressed by atenolol and enhanced by atropine as detailed above.

Exposure to acrolein produced an increase in arrhythmia in WT animals, however the total number of arrhythmia identified was very low with a group average 6.3 ± 2.5 occurances over the entire 3 hour exposure period. It is notable that 4 of the 6 animals in the WT group were responders, however mild, while apparent increases in arrhythmia occurring in the KO animals were driven by a single animal. This result did reach statistical significance, and may represent a slight arrhythmia response to acrolein exposure, nevertheless the physiological significance of this finding is questionable.

In an effort to characterize the effect of acrolein on breathing patterns, ventilatory parameters were also measured during exposure using whole-body plethysmography. Exposure to acrolein produced striking changes in the quality and rhythm of ventilation in

WT mice but not TRPA1 mice. The primary changes observed in the ventilatory parameters such as decrease in respiratory rate, increased expiratory pause and peak expiratory time, increased tidal volume, and increased enhanced pause or ventilatory timing are all hallmarks of an upper airway irritant response which is mediated by stimulation of the trigeminal nerve (Alarie 1973; Morris et al. 2003; Vijayaraghavan et al. 1993). The decrease in respiratory rate and elongated expiratory phase specifically are linked with the phenomena of ventilatory "braking" whereby reflexive glottal closure and inhibition of the phrenic nerve impair expiratory flow at the onset of expiration" (Alarie, 1973; Vijayaraghavan et al., 1993). The results presented here corroborate the findings of others (Bessac & Jordt 2008) that TRPA1 mediates these responses. Interestingly, pharmacological blockade with atenolol and atropine generally only blocked the ventilatory changes related to frequency and tidal volume whereas enhanced pause remained elevated. On the other hand, hexamethonium worsened the acrolein-induced changes in frequency and tidal volume. Although the role of the autonomic branches in these ventilatory effects is unclear, it appears the activity of one or the other is necessary to maintain normal breathing. Yet, any effects observed due to the pharmacological drugs may also be secondary to the cardiovascular changes. Further work connecting irritant ventilatory responses to cardiovascular changes is needed in order for us to understand this better.

At baseline WT and TRPA1 KO mice demonstrate differences in cardiac and ventilatory function. Control saline-injected mice exposed to filtered air produce a sympathetically mediated stress response to being handled and injected, one indicator of this was an increase in heart rate in both strains of mouse at the earliest exposure time points. TRPA1 KO mice HR's returned to baseline much more quickly than WT mice. This fast

decrease in HR occurred simultaneously with increases in HRV indicating enhanced cardiac parasympathetic modulation (Stein et al. 1994). TRPA1 KO mice also responded more robustly to pharmacological agents blocking cardiac parasympathetic neurotransmission than WT mice. Lastly, control TRPA1 KO mice demonstrated decreased inspiratory and expiratory times as well as decreased breathing frequency during exposure compared with WT mice. Overall the TRAP1 KO mice demonstrated a calmer phenotype than the WT mice characterized by a quicker return to baseline HR after stress and slower breathing, both of which are likely mediated through a baseline elevation in parasympathetic modulation. Considering the baseline differences in HRV between the WT and TRPA1 KO mice, direct comparisons across strains may not be valuable. Inter strain comparisons of exposure and pharmacological effect however should be valid. In addition, there are a number of other elements in this study which contribute to the difficulty/complexity in analyzing the results. Chief of which is that the temporal natures of the stress response, the exposure response and the pharmacodynamics of the agents used to block cardiac autonomic neurotransmission were not fully considered, or understood, until after the completion of this study. In retrospect it may have been beneficial to allow the animals to acclimate in the chamber, begin exposure and then infuse the pharmacological agents (rather than rely on a single injection immediately prior to exposure). In this way the stress response, exposure response and pharmacological interventions would have been parsed in to distinct stages rather than overlaid on top of each other.

Taken together these data indicate TRPA1 as a mediator of the cardiac response to airway irritants via autonomic modulation. This response is likely centrally-driven rather than being mediated through local release of neuropeptides. The cardiac effects we observed

were driven by changes in both branches of the autonomic nervous system, with a stress response producing sympathetic modulation and airway irritation producing parasympathetic modulation. With prolonged exposure there is the potential for compensatory effects as one branch is activated, dominates, and then turns off, the other branch may rebound.

These data demonstrate that exposure to air pollution can disrupt normal autonomic balance in healthy young animals, producing marked changes in both cardiac and ventilatory function. Both the sympathetic and parasympathetic branches of the autonomic nervous system appear to play a role in the physiological responses observed, likely in a biphasic manner characterized by early sympathetic activation followed by a prolonged period of vagal dominance. This study highlights the inextricable link between the cardiac and pulmonary systems, and begins to describe how a complex interplay between sympathetic and parasympathetic arms of the autonomic nervous system may modulate their function during exposure to air pollution. The increase in risk inherent to autonomic imbalance is not obvious. Rather these changes represent a latent risk; a loss of compensatory capacity in the animal, which may predispose them to adverse cardiovascular events only when challenged. This type of risk is particularly pronounced in susceptible sub-populations; those with already impaired compensatory capacity such as people with underlying cardiac or respiratory disease.

Tables and Figures

Table 1. *Summary of acrolein exposure and pharmacological treatment effects on measures of HRV and occurrence of arrhythmia in WT and TRPA1 KO mice*

WT: Treatment	Heart Rate	SDNN	RMSSD	LF	HF	Arrhythmia
Saline + Acrolein	NE	↑	↑	↑	NE	↑
Atenolol + Acrolein	NE	NE	NE	↑	NE	NE
Atropine + Acrolein	NE	↑↑	↑↑	↑↑	↑	NE
Hexamethonium + Acrolein	↓↑	↑	↑	↑	↑	NE

WT: Treatment	Heart Rate	SDNN	RMSSD	LF	HF	Arrhythmia
Saline + Acrolein	NE	NE	NE	NE	NE	↑
Atenolol + Acrolein	NE	NE	NE	NE	NE	NE
Atropine + Acrolein	NE	NE	NE	NE	NE	NE
Hexamethonium + Acrolein	NE	NE	NE	NE	NE	NE

Compared with filtered air-exposed controls

Table 2. *Summary of acrolein exposure and pharmacological treatment on ventilatory parameters in WT and TRPA1 KO mice.*

WT: Treatment	Ti	Te	PIF	PEF	TV	RT	MV	Freq	PenH
Saline + Acrolein	NE	↑	NE	↑	↑	NE	NE	↓	↑
Atenolol + Acrolein	NE	NE	NE	↑	NE	↓	NE	NE	↑
Atropine + Acrolein	NE	↑	NE	↑	↑	↓	NE	NE	↑
Hexamethonium + Acrolein	↑ early	↑ early	↑	↑	↑↑	↑ early	↑	↓↑	↑

KO: Treatment	Ti	Te	PIF	PEF	TV	RT	MV	Freq	PenH
Saline + Acrolein	NE	NE	NE	NE	NE	NE	NE	NE	↑ at 180min
Atenolol + Acrolein	NE	NE	NE	NE	NE	NE	NE	NE	↑ at 180min
Atropine + Acrolein	NE	NE	NE	NE	NE	NE	NE	NE	NE
Hexamethonium + Acrolein	NE	NE	NE	NE	NE	NE	NE	NE	↑ at 180min

Compared with filtered air-exposed controls

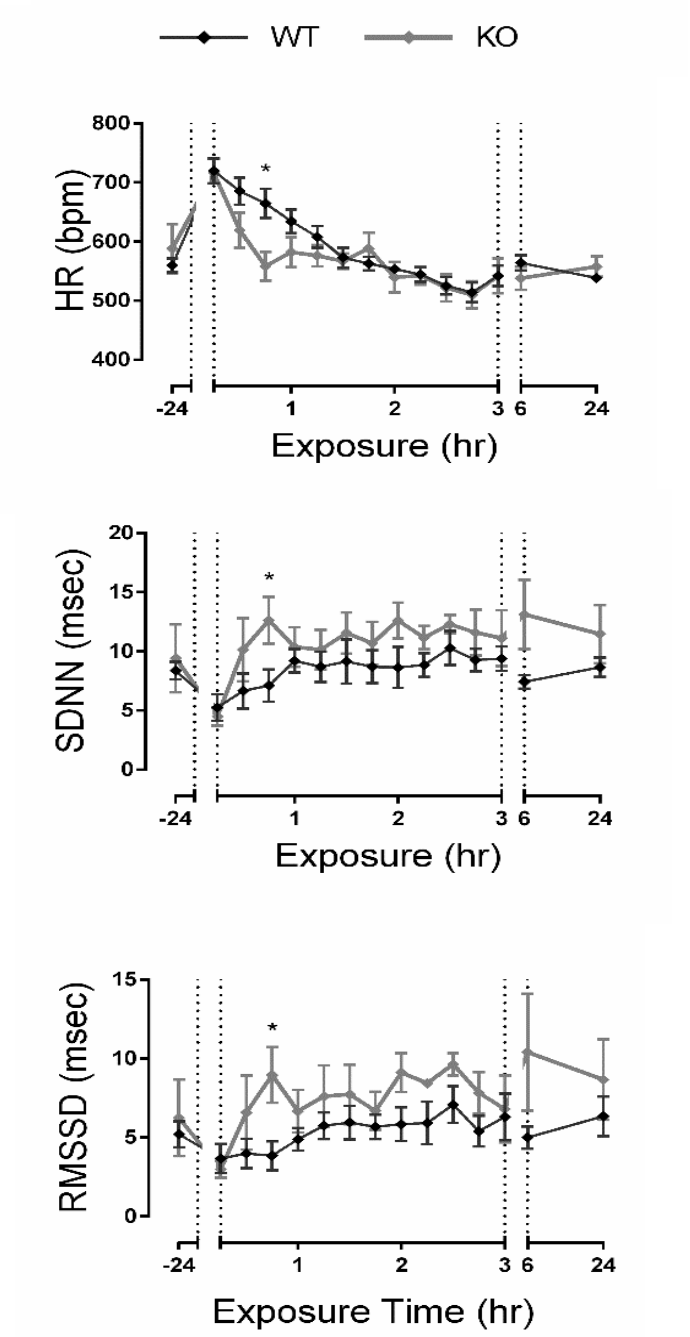
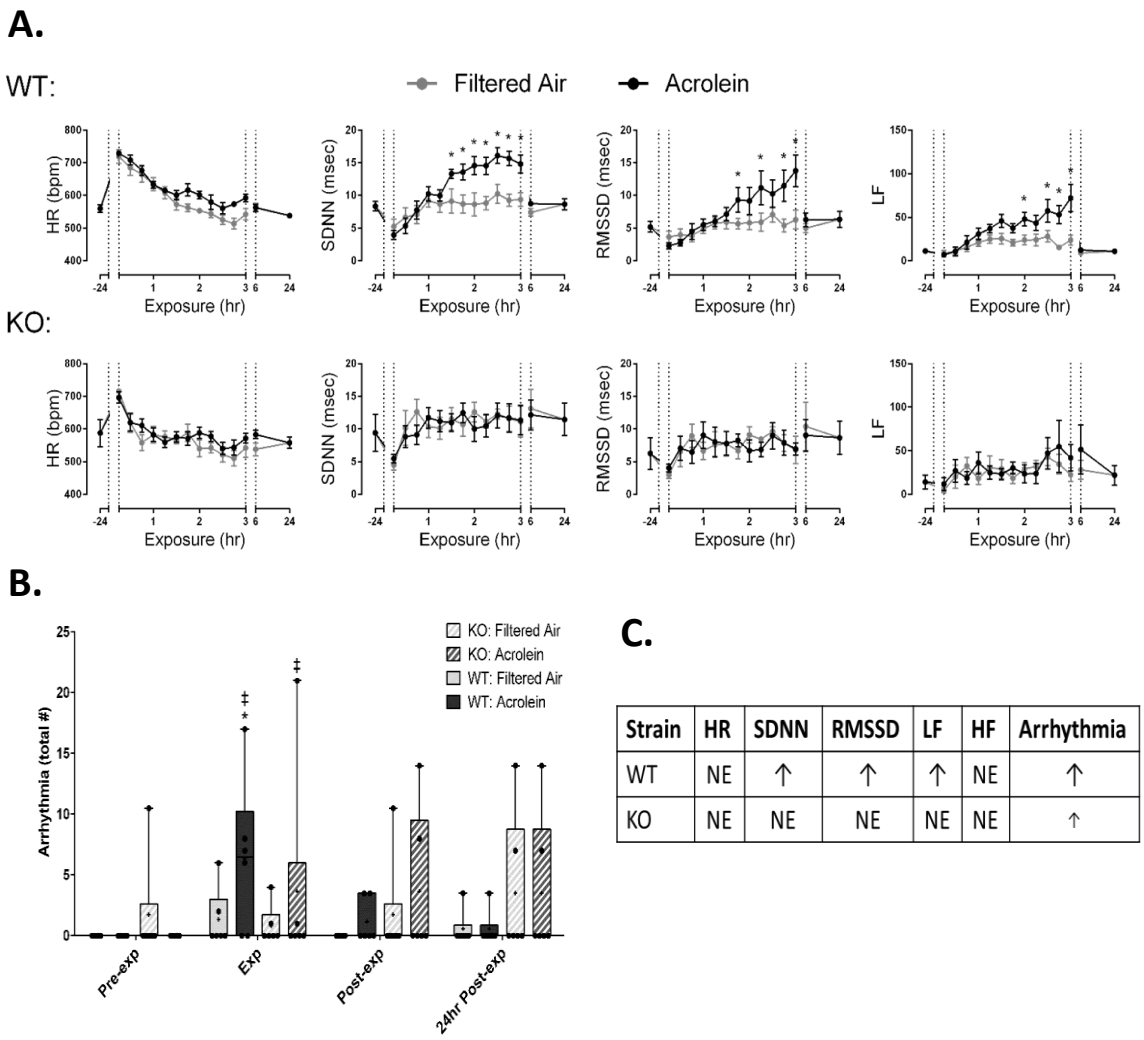


Figure 1.*TRPA1 KO mice demonstrate altered cardiac autonomic modulation compared with WT mice.* HR, SDNN and RMSSD measurements during pre-exposure period (-24hr), exposure (1-3hr), immediately post-exposure (6hr), and one day post-exposure (24hr). Data are from saline-injected, filtered air-exposed control animas, both WT and TRPA1 KO. Data points are mean ± SEM.*$p \leq 0.05$; significantly different from WT mice (n=6)

Figure 2. *WT and TRPA1 KO mice respond similarly and predictably to pharmacological agents affecting autonomic nervous system function.* A. HR and SDNN measurements during pre-exposure period (-24hr), exposure (1-3hr), immediately post-exposure (6hr), and one day post-exposure (24hr). Data are from saline or drug-injected, filtered air-exposed animas; both WT and TRPA1 KO. Data points are mean ± SEM. †$p \leq 0.05$; significantly different from saline-injected controls (n=6). B. Summary of pharmacological treatment effects on measures of HRV in WT and TRPA1 KO mice compared with saline-injected controls.



Strain	HR	SDNN	RMSSD	LF	HF	Arrhythmia
WT	NE	↑	↑	↑	NE	↑
KO	NE	NE	NE	NE	NE	↑

Figure 3. *TRPA1 mediates acrolein-induced increases in HRV and arrhythmia*. HR and HRV measurements during pre-exposure period (-24hr), exposure (1-3hr), immediately post-exposure (6hr), and one day post-exposure (24hr). Saline-injected, filtered air or acrolein-exposed animas; both WT and TRPA1 KO. Data points are mean ± SEM. B. Total arrhythmia counts during a time-matched pre-exposure period (Pre-exp), exposure (Exp), a post-exposure period immediately following exposure (Post-exp), and a time-matched post-exposure period one day later (24hr Post-exp). All exposure periods were 3 hours in duration. Individual data points per group are plotted and "+" indicates group mean. C. Summary of acrolein exposure effects on measures of HRV and arrhythmia in WT and TRPA1 KO mice compared with filtered air-exposed controls. *$p \leq 0.05$; significantly different from filtered air-exposed controls (n=6). ‡$p \leq 0.05$; significantly different from Pre-exp

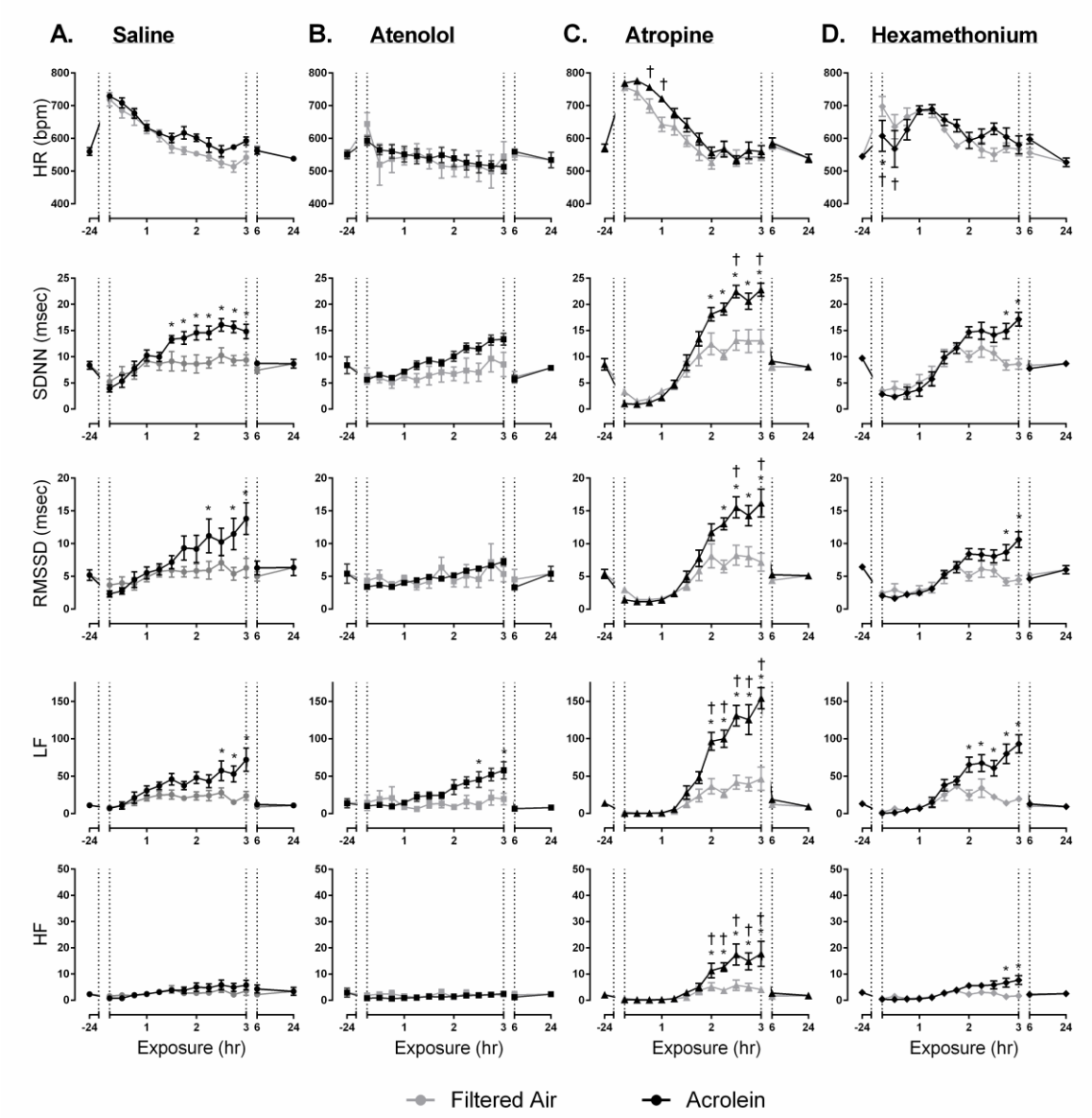


Figure 4. *Pharmacological treatments affecting cardiac autonomic modulation alter the HR and HRV response to acrolein exposure.* HR and HRV measurements in WT animals exposed to filtered air or acrolein and treated with saline or one of three pharmacological agents affecting cardiac autonomic function. Atenolol blocks sympathetic neurotransmission in the heart. Atropine blocks parasympathetic neurotransmission in the heart. Hexamethonium blocks both sympathetic and parasympathetic peripheral neurotransmission. Exposure periods are defined as follows: pre-exposure period (-24hr), exposure (1-3hr), immediately post-exposure (6hr), and one day post-exposure (24hr). Data points are mean ± SEM. *$p \leq 0.05$; significantly different from filtered air-exposed controls (n=6). †$p \leq 0.05$; significantly different from saline-treated controls.

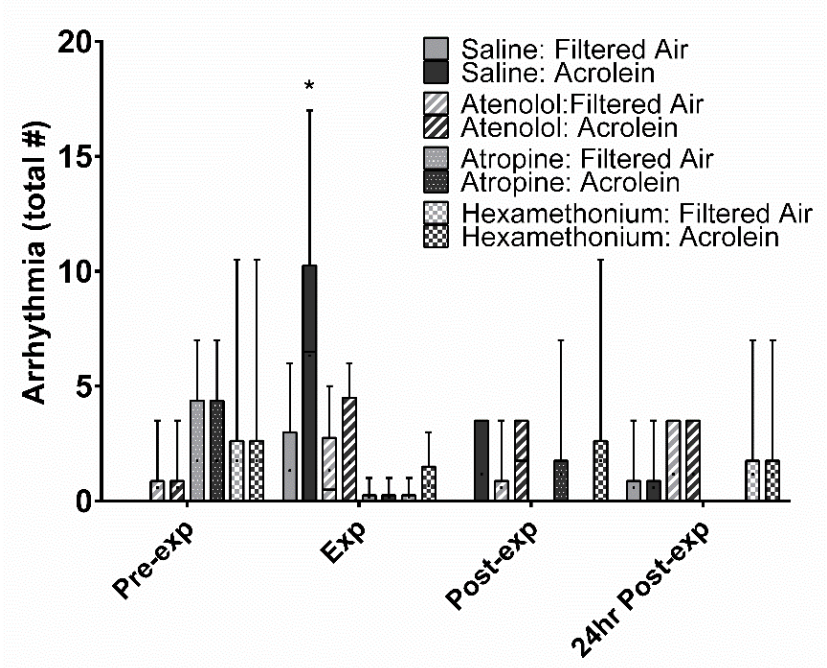


Figure 5. *Pharmacological treatments affecting cardiac autonomic modulation prevent acrolein-induced increases in arrhythmia in WT mice.* Total arrhythmia counts during a time-matched pre-exposure period (Pre-exp), exposure (Exp), a post-exposure period immediately following exposure (Post-exp), and a time-matched post-exposure period one day later (24hr Post-exp). All exposure periods were 3 hours in duration. "+" indicates group mean. *p ≤ 0.05; significantly different from filtered air-exposed controls (n=6).

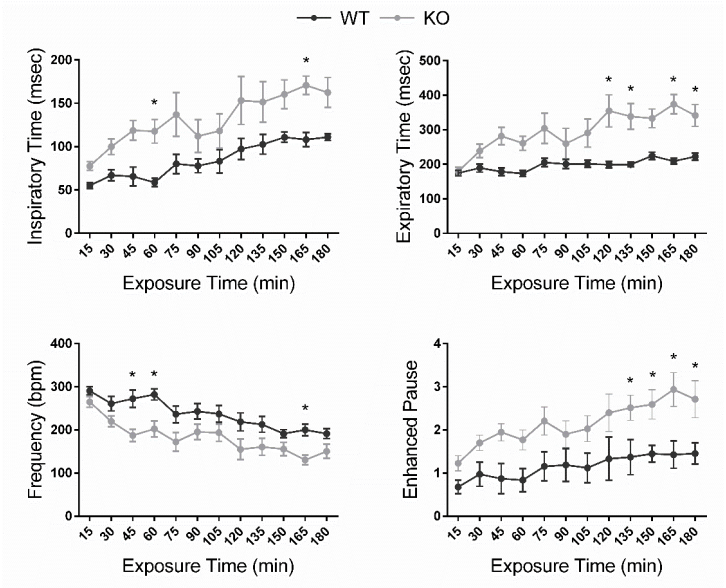
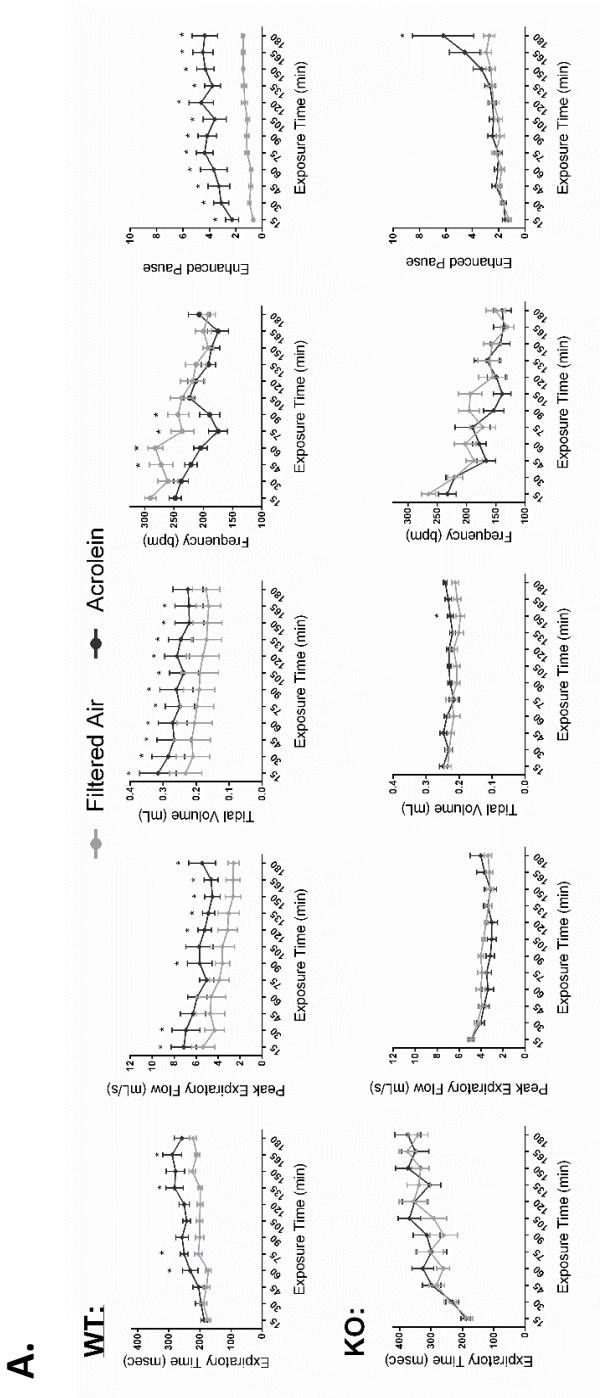
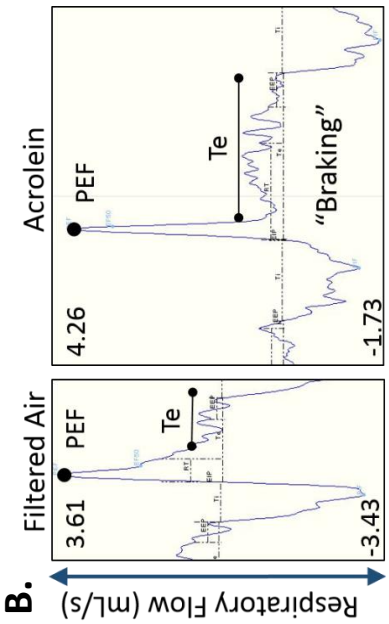


Figure 6. *TRPA1 KO mice demonstrate altered ventilatory timing compared with WT mice.* Ti, Te, breathing frequency and enhanced pause during exposure period. Data are from saline-injected, filtered air-exposed control animas, both WT and TRPA1 KO. Data points are mean ± SEM.*p ≤ 0.05; significantly different from WT mice (n=6)





C.

Strain	Ti	Te	PIF	PEF	TV	RT	MV	Freq	PenH
WT	NE	↑	NE	↑	↑	NE	NE	↓	↑
KO	NE	NE	NE	NE	NE	NE	NE	NE	↑ At 180 min

Figure 7. *TRPA1 mediates acrolein-induced changes in ventilatory timing*. Data are from saline-injected, filtered air or acrolein-exposed animas; both WT and TRPA1 KO. Data points are mean ± SEM. B. Representative traces from saline-injected control animals exposed to either filtered air or acrolein demonstrating ventilatory "braking" in response to acrolein exposure. C. Summary of ventilatory response to acrolein exposure in WT and TRPA1 KO mice compared with filtered air-exposed controls. *$p \leq 0.05$; significantly different from filtered air-exposed controls (n=6).

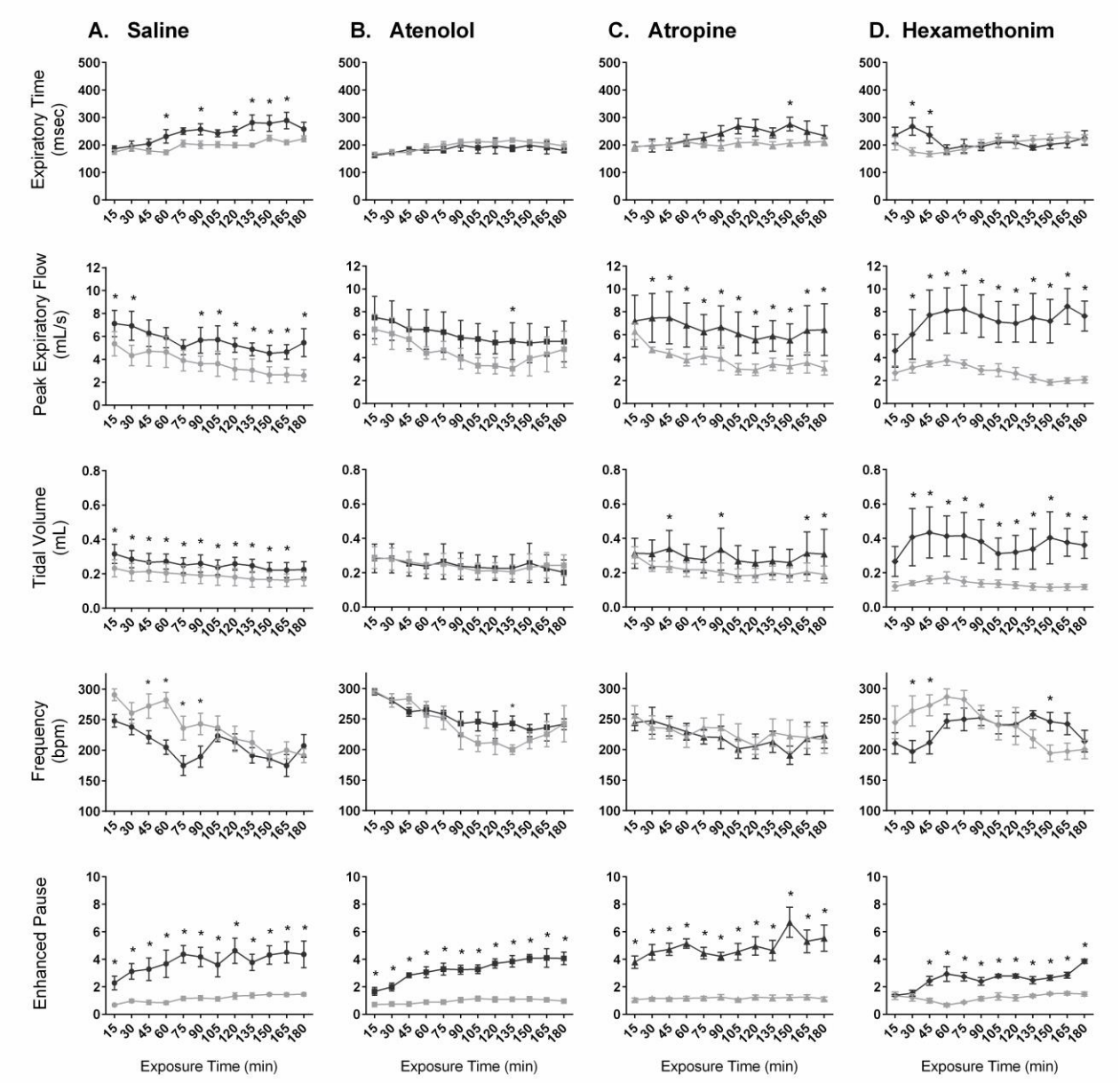


Figure 8. *Pharmacological treatments affecting cardiac autonomic modulation alter the ventilatory response to acrolein exposure.* Ventilatory parameters during exposure. WT animals exposed to filtered air or acrolein and treated with saline or one of three agents affecting cardiac autonomic function. Atenolol blocks sympathetic neurotransmission in the heart. Atropine blocks parasympathetic neurotransmission in the heart. Hexamethonium blocks both. Data points are mean ± SEM. *$p \leq 0.05$; significantly different from filtered air-exposed controls (n=6). †$p \leq 0.05$; significantly different from saline-treated control

CHAPTER V: CONCLUSIONS AND IMPLICATIONS

Early air pollution regulations were focused on reducing emissions and ambient air pollution levels in order to decrease the risk of large-scale public health emergencies due to sudden extreme events such as the ones in London and Donora, PA in the mid-20th century. Over the past 50 years ambient air pollution levels have been significantly reduced. However 60 million people in the U.S. still lives in areas that meet or exceed at least one NAAQS (U.S. EPA n.d.). Additionally, the public health impact of ambient air pollution still remains significant, producing an estimated 200,000 additional deaths each year in the US (Fann et al. 2013). There is also a growing rate of chronic diseases, especially cardiovascular disease, in the United States. In fact, a report from the American Heart Association estimates that by 2030 ~40% of the US population will have some form of cardiovascular disease (Heidenreich et al. 2011). Thus a large segment of the population has a heightened risk of adverse health effects due to environmental exposure and should be treated as a susceptible sub-group. Future regulatory decisions need to consider these susceptible populations. Research presented in this dissertation, exploring the health effects of low level or acute exposures, exposures to pollutant mixtures, and elucidation of the specific mechanisms underlying the increased risk for adverse cardiovascular events in both normal and susceptible populations will contribute greatly to these regulatory decisions and aid in the identification of air pollution levels which produce no observable adverse effect

Susceptibility is defined as "the state of being likely or liable to be influenced or harmed by a particular thing."(Oxford Online 2010). In the case of air pollution-induced adverse cardiovascular events, susceptibility usually refers to the presence of existing disease or other conditions which predispose a person to deleterious effect and allows for the development of decrements in cardiovascular function over time. Other factors known to contribute to air pollution-induced cardiovascular susceptibility include genetic polymorphisms (Yang et al. 2009; Kleeberger 2003) and life stage (Mead 2011). Socioeconomic status, which encompasses a variety of additional modifiable factors including nutritional status and body mass index, social stressors, and access to healthcare, also contributes to the overall health state of the individual, which could in turn modify their response to air pollution (Clougherty & Kubzansky 2009; Schwartz et al. 2011). Thus, susceptibility to air pollution-induced cardiotoxicity in terms of pre-existing conditions describes an organism already existing in a state of homeostatic imbalance, where further challenge by air pollution may then shift the organism past the bounds of their compensatory capacity and produce cardiovascular dysfunction. The key challenge exists in measuring these conditions such that an accurate picture of the existing risk can be determined.

Methodologies employed in the detection of early physiological and biochemical changes have rapidly advanced in the past few decades alone. As such we are now able to detect changes in physiological and biochemical function which in fact precede the development of symptoms related to injury or disease (Bates 2005). We have progressed well beyond mortality as a primary indicator of the effects of air pollution on a population, and now have the tools to effectively identify the level, if any, at which air pollution is no longer producing any adverse effects. In the least, we have become keenly aware of the fact

that not all the toxicological effects of air pollution are overt or clinical but rather more than likely occur latently in most people. Understanding the mechanisms underlying air-pollution induced cardiovascular toxicity allows for the potential development of interventions, but more importantly contributes to the identification of sensitive sup-populations who are more susceptible to air-pollution induced injury due genetic background, existing injury, disease state, or the influence of other environmental factors such as diet or general stress level.

The research described here was conducted to investigate previously undescribed toxicological mechanisms underlying adverse cardiovascular events associated with acute air pollution exposure. As such, the basis for this work was predicated on a wealth of epidemiological studies showing that exposure to air pollution causes increased cardiovascular morbidity and mortality. In particular, large-scale studies have shown that air pollution causes changes in heart rate variability, which although derived from heart rate, signifies subtle shifts in the internal autonomic controls of the body and a potential increase in cardiovascular risk. These autonomic controls are regulated by an intricate network of neural sensors, which monitor not only the normal physiological function of the body (e.g. blood pressure) but also external exposure to potentially harmful environmental substances. Yet the effects that result from changes to these components are not necessarily overt and often lack a simultaneous relevant clinical symptom that points to the health threat. Thus, the underlying assumption is a change in heart rate variability reflects a state of homeostatic imbalance, where further stress on the body may shift the body past the bounds of its compensatory capacity and produce cardiovascular dysfunction. There are numerous clinical and experimental data that demonstrate autonomic imbalance plays a role in the adverse cardiovascular effects of acute air pollution exposure (Davoodi et al. 2010; Link & Dockery

2010; Hazari et al. 2011). However, to date, there are very few studies that have specifically characterized the neural link between the targeting of the respiratory system by air pollution and altered autonomic control of the cardiovascular system. Consequently, several studies have shown that the initiating event for a certain class of inhaled irritants, several of which are found in complex air pollution mixtures, is the activation of TRPA1 and subsequent signaling through the central nervous system.

This dissertation explores the hypothesis that TRPA1 mediates air pollution induced autonomic imbalance and cardiac dysfunction (Figure 1.2). To this end, cardiac responses to air pollution exposure, both electrical and mechanical, were characterized in the wild-type mouse. Then TRPA1 knockout mice were incorporated into subsequent studies to determine 1) what role if any TRPA1 plays in the mediation of air pollution induced cardiac dysfunction 2) whether autonomic changes are associated with adverse cardiac effects, and 3) how exactly autonomic nervous system function is modulated by exposure to inhaled irritants to produce cardiac effects. These data describe mechanisms underlying cardiovascular toxicity associated with acute exposure to air pollution, and aid in the identification of potentially sensitive subpopulations and periods of heightened risk. Taken together these results contribute to the assessment of risk to public health due to air pollution exposure, not only among sensitive groups but among the seemingly healthy as well.

Principal Conclusions

Exposure to particulate and gaseous air pollution produces electrocardiogram and mechanical responses in the mouse heart - Exposure to common constituents of air

pollution produce decrements in cardiac electrical and mechanical function in the mouse heart similar to those observed in humans including arrhythmia (Peters et al. 2000). Although PM has long been the focus of most epidemiological and experimental air pollution research and has been associated with myocardial infarction, stroke, arrhythmia, and exacerbation of heart failure (Brook et al. 2010), air pollution is a complex mixture with volatile gaseous components as well. Furthermore, the majority of ambient air pollution by mass is in the gaseous or vapor phase (Brook et al. 2010). Ozone, which along with PM is another ubiquitous pollutant, has been associated with decreased HR, alteration of cardiac repolarization and increased inflammation (Farraj et al. 2012; McIntosh-Kastrinsky et al.; Balmes et al. 2011). Therefore research needs to examine the combined effects of PM and ozone, particularly to clarify where the interactions are synergistic, antagonistic or additive. We found disparate effects between size-fractionated PM with fine CAPs altering autonomic balance and ultrafine CAPs increasing the incidence of cardiac arrhythmia and producing decrements in left ventricular mechanical function. These effects were only apparent in ozone co-exposed animals, suggesting that ozone interactions contribute to the cardiovascular response to PM, however the results suggest that it is not generalizable to the particle size modes. Previous work examining PM and ozone co-exposure primarily focused on pulmonary inflammation endpoints (Bouthillier et al. 1998; Farraj et al. 2010; Vincent et al. 1997; Kumarathasan et al. 2015) and did not examine cardiovascular function. Thus, this study fills an existing gap in the research describing how ozone may contribute to or otherwise modify the cardiovascular response to PM inhalation. O3 may have enhanced the response to PM via direct interaction with airway tissues producing damage and oxidative stress, additionally ozone may have interacted with PM constituents forming potent reactive

species and indirectly contributed to oxidative stress and enhancing the PM effect. Furthermore, the size fraction of PM is a major determinant in the location of airway deposition (i.e. pulmonary vs extra-thoracic), and sub-types of airway sensory receptors impacted, all of which may contribute to the disparate responses observed between particulate modes. Finally, the fact that these latent effects were observed in the absence of overt toxicity at very low ambient concentrations demonstrates the subtle homeostatic changes that can occur as a result of air pollution exposure.

TRPA1 mediates changes in heart rate variability and arrhythmia following exposure to acrolein - TRPA1 has been previously described as an airway irritant sensor which is known to mediate some reflexive respiratory responses to air pollution exposure, particularly certain highly-reactive noxious compounds (Bessac & Jordt 2008). It has also been suggested that TRPA1 may act as a direct target of many constituents of air pollution including components of wood smoke (Shapiro et al. 2013) and diesel exhaust (Deering-Rice et al. 2011), heavy metals including zinc, cadmium and copper (Hu et al. 2009; Gu & Lin 2010), as well as gaseous air pollutants ozone (Taylor-Clark & Undem 2010), and acrolein (Andrè et al. 2008; Hinman et al. 2006). The majority of this research has been done *in-vitro* or utilized isolated organ perfusion systems. While these techniques provide a great deal of insight regarding channel activation and elucidation of the neural pathways potentially involved in the adverse cardiopulmonary response, physiological endpoints in the whole organism can only be inferred. The adverse effects ascribed to air pollution exposure result from the interplay of responses across multiple organ systems, with deficits in one organ system affecting the others. Thus, it is necessary that whole animals were used in these studies. I measured

physiological endpoints, relevant to the development of serious cardiovascular events, such as arrhythmia, along with more subtle alterations in autonomic and mechanical function. Our previous studies in rats demonstrated that pharmacological inhibition of TRPA1 attenuated air pollution-induced cardiac arrhythmia (Hazari et al. 2011). This study, using both C57BL/6 wild-type and TRPA1 knockout mice demonstrated that acrolein produced autonomic imbalance and an increased incidence of arrhythmia through TRPA1, which also mediated an increase in LVDP. In contrast, exposure to ozone did not appear to cause any significant effects despite the fact that mild cardiovascular effects of ozone at similar levels have been documented by our lab in the past and by others (Wu et al. 2010; Farraj et al. 2012; Barath et al. 2013; Wang et al. 2013; Kurhanewicz et al. 2014; Farraj et al. 2015). This result highlights the fact that the cardiovascular response to near-ambient ozone exposure *in-vivo* is variable and warrants further study. With this work we have demonstrated that autonomic nervous system function can be modulated by airway TRPA1 activity, and appears to also play a role in acrolein-induced cardiac arrhythmia and mechanical changes. A likely mechanism linking airway irritation and cardiac dysrhythmia is the activation of C-fibers which triggers the release of centrally-acting neurotransmitters including glutamate and substance P in the NTS. This process is known to modify autonomic regulation of cardiac and respiratory function(Mutoh, Bonham, et al. 2000; Bonham et al. 2001). Similar mechanisms have already been demonstrated in models of cigarette smoke irritation (Mutoh, Joad, et al. 2000) as well as others (Paton & Nolan 2000; Simms et al. 2006), while TRPA1's role in modulating other "autonomic" activity (e.g. breathing) in the brainstem is also known (Tani et al. 2015). This is the first study however

to directly demonstrate a role for airway TRPA1 in mediating autonomic imbalance and changes in cardiac function due to air pollution.

Exposure to acrolein produces changes in heart rate variability and increased incidence of arrhythmia indicative of increased vagal modulation, however sympathetic modulation also plays a role. - In our studies, increased HRV, and therefore parasympathetic modulation, was observed with an increased incidence of sinus node dysfunction and bradyarrhythmia during acrolein exposure in normal animals. Unlike respiratory sinus arrhythmia, which occurs regularly/periodically according to cyclic ventilatory patterns, these acrolein-induced arrhythmias occurred randomly albeit with increasing frequency through the course of exposure. Increased vagal tone is known to produce changes in the P-wave to P-wave interval in the electrocardiogram independent of normal breathing. This condition is known as non-respiratory sinus arrhythmia and it differs from respiratory sinus arrhythmia (RSA) in that it is not associated with respiration but rather occurs when the outflow of parasympathetic tone predominates over sympathetic tone (Deboor et al. 2005; McMullen et al. 2012). In humans, intermittent periods of bradycardia, and bradyarrhythmia indicating sinus node dysfunction along with parasympathetic dominance are all features included in a group of abnormal heart rhythms termed sick sinus syndrome (Keller & Lemberg 2006). It is interesting that acrolein exposure produces comparable effects in the mouse. While this does not indicate the development of sick sinus syndrome in acrolein exposed individuals per se, it does highlight the underlying feature of autonomic imbalance and strengthen the case for plausibility of this mechanism in humans as well as mice. Alterations from baseline HRV, both increases and decreases, have been implicated in the development of adverse

cardiovascular events indicating that any change in autonomic function which shifts the animal out of homeostatic balance represents an increase in risk due to impaired compensatory capacity to respond to subsequent challenges or stressors. These effects would be all the more apparent in populations with existing impairments in compensatory capacity (e.g. susceptible individuals such as those with underlying cardiovascular disease)

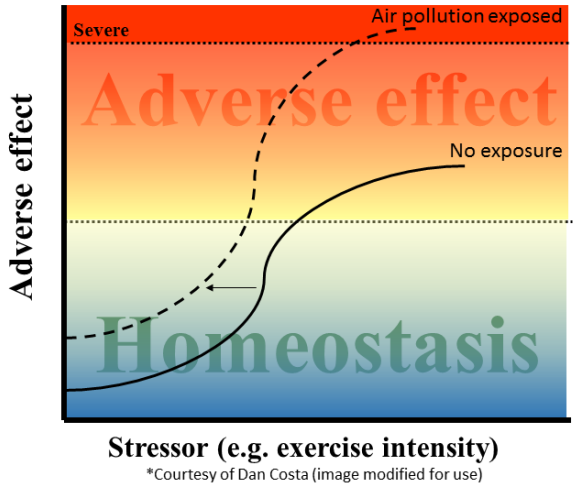
On the whole these results suggest that the autonomic response to acrolein is characterized solely by parasympathetic dominance. However cardiac responses during exposure to acrolein following pharmacological blockade of one or both branches of the autonomic nervous system demonstrated a role for both sympathetic and parasympathetic modulation. Sympathetic neurotransmission blockade by atenolol reduced the HRV response to acrolein, while parasympathetic neurotransmission blockade by atropine enhanced HRV response to acrolein overall. There is limited existing research examining exactly how functional changes in autonomic nervous system function results in specific changes in measures of HRV, especially with regard to air pollution exposure done using whole animals. As such, this research adds a wealth of new data that better characterizes autonomic imbalance as a risk factor for cardiac dysfunction in air pollution exposures (Figure 1).

In general, when addressing the cardiovascular effects of air pollution, further exploration is needed addressing factors contributing to HRV such as breathing patterns, stimuli-driven changes in blood pressure, and the effects of modifying factors like as nutritional status. Additionally, research focusing on common components of air pollution and their individual contributions to the types of autonomic and cardiovascular responses described herein would be valuable for informing the public about specific risks inherent to specific varieties of air pollution (e.g. high PM days vs high oxidant days)

Summary and Broad Perspectives on Findings

This research helps to improve our current understanding of the effects of air pollution on cardiovascular health. All of the studies described herein used healthy, young to middle aged mice, with no known co-morbidities. That adverse cardiovascular responses, however subtle, could be elicited in these cohorts following a single acute exposure to common air pollutants is not trivial as it implies that while epidemiological studies have demonstrated populations with underlying cardiovascular and pulmonary diseases represent the most sensitive group, nobody is completely protected from potentially adverse homeostatic shifts toward increased risk for adverse cardiovascular events following exposure to air pollution (Figure 1), particularly when the components include airway irritants like acrolein.

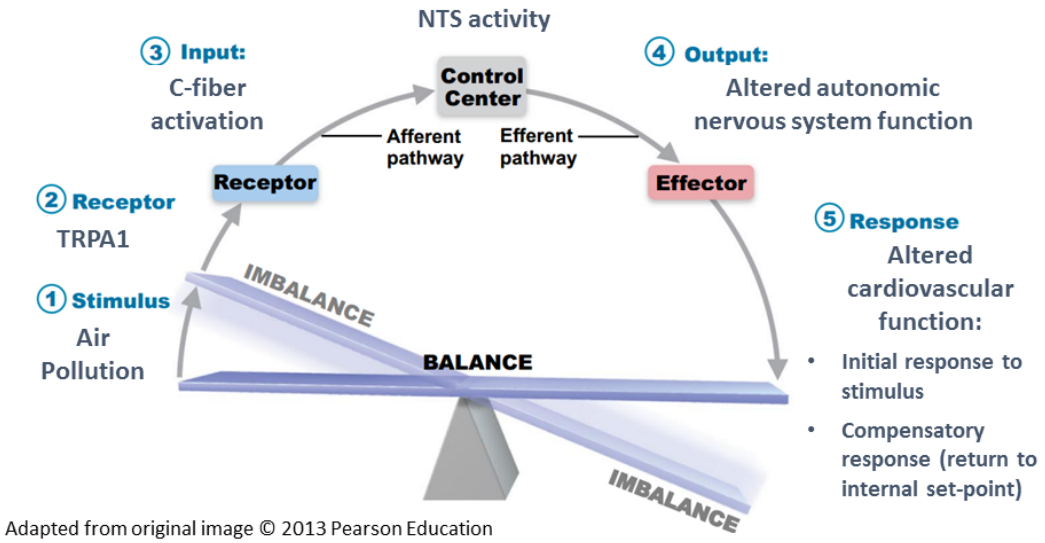
Figure 1. Autonomic Imbalance Shifts the Individual Toward an Increased Risk for Adverse Cardiac Events



This project contributes to the scientific literature addressing the mechanisms underlying the association between exposure to air pollution and cardiovascular dysfunction and confirms the utility of animal models in extrapolating human responses. As such, we

demonstrate that mice produce similar decrements in cardiac electrical and mechanical function as humans following exposure to air pollution and that reflexive cardiovascular responses to airway irritants in particular are mediated by TRPA1, a mechanism which is likely similar in humans. In addition, while the cardiovascular response to airway irritants appears to be characterized primarily by enhanced vagal modulation, sympathetic nervous system activity also plays a role (Figure 2). These novel findings support and extend important information regarding mechanisms by which airway irritant exposure results in adverse health effects and provides a tangible piece of evidence that can be used for applied purposes like risk assessment and potentially the setting of standards. With heart disease as the number one cause of death worldwide, understanding these contributing factors paves the way for the development of interventions, or may help shape future regulatory measures, which could produce drastic public health benefits.

Figure 2. Cardiac Dysfunction Following a Single Acute Exposure to Air Pollution is the Result of Altered Autonomic Nervous System Function, which is Mediated by TRPA1



REFERENCES

Adamson, I.Y., Prieditis, H. & Vincent, R., 1999. Pulmonary toxicity of an atmospheric particulate sample is due to the soluble fraction. *Toxicology and applied pharmacology*, 157(1), pp.43–50.

Adán, V. & Crown, L.A., 2003. Diagnosis and treatment of sick sinus syndrome. *American family physician*, 67(8), pp.1725–32.

Agostini, E. et al., 1957. Functional and histological studies of the vagus nerve and its branches to the heart, lungs and abdominal viscera in the cat. *The Journal of physiology*, 135(1), pp.182–205.

Alarie, Y., 1973. Sensory irritation by airborne chemicals. *CRC critical reviews in toxicology*, 2(3), pp.299–363.

Allen, R.W. et al., 2011. An air filter intervention study of endothelial function among healthy adults in a woodsmoke-impacted community. *American journal of respiratory and critical care medicine*, 183(9), pp.1222–30.

Alonso, A. et al., 2014. Association of sick sinus syndrome with incident cardiovascular disease and mortality: the Atherosclerosis Risk in Communities study and Cardiovascular Health Study. *PloS one*, 9(10), p.e109662.

Amar, D. et al., 2003. Competing autonomic mechanisms precede the onset of postoperative atrial fibrillation. *Journal of the American College of Cardiology*, 42(7), pp.1262–8.

Amatullah, H. et al., 2012. Comparative cardiopulmonary effects of size-fractionated airborne particulate matter. *Inhalation toxicology*, 24(3), pp.161–71.

Andrè, E. et al., 2008. Cigarette smoke-induced neurogenic inflammation is mediated by alpha,beta-unsaturated aldehydes and the TRPA1 receptor in rodents. *The Journal of clinical investigation*, 118(7), pp.2574–2582.

Andreev-Andrievskiy, A.A. et al., 2014. Stress-associated cardiovascular reaction masks heart rate dependence on physical load in mice. *Physiology & behavior*, 132, pp.1–9.

Anjilvel, S. & Asgharian, B., 1995. A multiple-path model of particle deposition in the rat lung. *Fundamental and applied toxicology : official journal of the Society of Toxicology*, 28(1), pp.41–50.

Anon, 1996. Heart rate variability. Standards of measurement, physiological interpretation, and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. *European heart journal*, 17(3), pp.354–81.

Anon, National Ambient Air Quality Standards (NAAQS) | Air and Radiation | US EPA.

Appel, M.L. et al., 1989. Beat to beat variability in cardiovascular variables: noise or music? *Journal of the American College of Cardiology*, 14(5), pp.1139–48.

Arjomandi, M. et al., 2015. Exposure to medium and high ambient levels of ozone causes adverse systemic inflammatory and cardiac autonomic effects. *American journal of physiology. Heart and circulatory physiology*, 308(12), pp.H1499–509.

Aubdool, A.A. et al., 2014. TRPA1 is essential for the vascular response to environmental cold exposure. *Nature Communications*, 5, p.5732.

Bagate, K. et al., 2006. Ambient particulate matter affects cardiac recovery in a Langendorff ischemia model. *Inhalation toxicology*, 18(9), pp.633–43.

Baja, E.S. et al., 2010. Traffic-related air pollution and QT interval: modification by diabetes, obesity, and oxidative stress gene polymorphisms in the normative aging study. *Environmental health perspectives*, 118(6), pp.840–6.

Balmes, J. et al., 2011. *Effects of Ozone Exposure on Cardiovascular Responses in Healthy and Susceptible Humans*, San Francisco, CA.

Barath, S. et al., 2013. Short-term exposure to ozone does not impair vascular function or affect heart rate variability in healthy young men. *Toxicological sciences : an official journal of the Society of Toxicology*, 135(2), pp.292–9.

Bates, D. V, 2005. A perspective on air pollution. *The Journal of allergy and clinical immunology*, 115(2), pp.432–4.

Baumert, M. et al., 2006. Changes in heart rate variability of athletes during a training camp. *Biomedizinische Technik. Biomedical engineering*, 51(4), pp.201–4.

Bautista, D.M. et al., 2006. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell*, 124(6), pp.1269–1282.

Beauchamp, R.O. et al., 1985. A critical review of the literature on acrolein toxicity. *Critical reviews in toxicology*, 14(4), pp.309–80.

Bein, K. & Leikauf, G.D., 2011. Acrolein - a pulmonary hazard. *Molecular nutrition & food research*, 55(9), pp.1342–60.

Bell, M.L. & Davis, D.L., 2001. Reassessment of the lethal London fog of 1952: novel indicators of acute and chronic consequences of acute exposure to air pollution. *Environmental health perspectives*, 109 Suppl , pp.389–94.

Ben-Jebria, A. et al., 1994. Human bronchial smooth muscle responsiveness after in vitro exposure to acrolein. *American journal of respiratory and critical care medicine*, 149(2 Pt 1), pp.382–6.

Bessac, B.F. et al., 2008. TRPA1 is a major oxidant sensor in murine airway sensory

neurons. *The Journal of clinical investigation*, 118(5), pp.1899–910.

Bessac, B.F. & Jordt, S.-E., 2008. Breathtaking TRP channels: TRPA1 and TRPV1 in airway chemosensation and reflex control. *Physiology (Bethesda, Md.)*, 23, pp.360–370.

Bessac, B.F. & Jordt, S.-E., 2010. Sensory detection and responses to toxic gases: mechanisms, health effects, and countermeasures. *Proceedings of the American Thoracic Society*, 7(4), pp.269–277.

Billman, G.E., 2013. The LF/HF ratio does not accurately measure cardiac sympatho-vagal balance. *Frontiers in physiology*, 4, p.26.

Bodkin, J. V et al., 2014. Investigating the potential role of TRPA1 in locomotion and cardiovascular control during hypertension. *Pharmacology research & perspectives*, 2(4), p.e00052.

Bodkin, J. V & Brain, S.D., 2011. Transient receptor potential ankyrin 1: emerging pharmacology and indications for cardiovascular biology. *Acta physiologica (Oxford, England)*, 203(1), pp.87–98.

Bonham, A.C. et al., 2001. Lung C-fiber CNS reflex: role in the respiratory consequences of extended environmental tobacco smoke exposure in young guinea pigs. *Environmental health perspectives*, 109 Suppl , pp.573–578.

Bonham, A.C. et al., 2006. Plasticity in the nucleus tractus solitarius and its influence on lung and airway reflexes. *Journal of applied physiology (Bethesda, Md. : 1985)*, 101(1), pp.322–7.

Bortkiewicz, A. et al., 2014. Cardiovascular changes in workers exposed to fine particulate dust. *International journal of occupational medicine and environmental health*, 27(1), pp.78–92.

Bouthillier, L. et al., 1998. Acute effects of inhaled urban particles and ozone: lung morphology, macrophage activity, and plasma endothelin-1. *The American journal of pathology*, 153(6), pp.1873–84.

Boyd, J.T., 1960. Climate, air pollution, and mortality. *British journal of preventive & social medicine*, 14, pp.123–35.

Brederson, J.-D., Kym, P.R. & Szallasi, A., 2013. Targeting TRP channels for pain relief. *European journal of pharmacology*, 716(1-3), pp.61–76.

Brignole, M., 2002. Sick sinus syndrome. *Clinics in geriatric medicine*, 18(2), pp.211–27.

Brook, R.D. et al., 2004. Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation*, 109(21), pp.2655–2671.

Brook, R.D., 2008. Cardiovascular effects of air pollution. *Clinical science (London, England : 1979)*, 115(6), pp.175–87.

Brook, R.D. et al., 2002. Inhalation of fine particulate air pollution and ozone causes acute arterial vasoconstriction in healthy adults. *Circulation*, 105(13), pp.1534–6.

Brook, R.D. et al., 2009. Insights into the mechanisms and mediators of the effects of air pollution exposure on blood pressure and vascular function in healthy humans. *Hypertension*, 54(3), pp.659–67.

Brook, R.D. et al., 2010. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation*, 121(21), pp.2331–2378.

Caiazzo, F. et al., 2013. Air pollution and early deaths in the United States. Part I: Quantifying the impact of major sectors in 2005. *Atmospheric Environment*, 79, pp.198–208.

Calderón-Garcidueñas, L. et al., 2007. Pediatric respiratory and systemic effects of chronic air pollution exposure: nose, lung, heart, and brain pathology. *Toxicologic pathology*, 35(1), pp.154–62.

Campen, M.J. et al., 2006. Fresh gasoline emissions, not paved road dust, alter cardiac repolarization in ApoE-/- mice. *Cardiovascular toxicology*, 6(3-4), pp.199–210.

Canning, B.J. & Chou, Y.-L., 2009. Cough sensors. I. Physiological and pharmacological properties of the afferent nerves regulating cough. *Handbook of experimental pharmacology*, (187), pp.23–47.

Carll, A.P. et al., 2015. Cardiomyopathy confers susceptibility to particulate matter-induced oxidative stress, vagal dominance, arrhythmia and pulmonary inflammation in heart failure-prone rats. *Inhalation toxicology*, 27(2), pp.100–12.

Carll, A.P. et al., 2013. Diesel exhaust inhalation increases cardiac output, bradyarrhythmias, and parasympathetic tone in aged heart failure-prone rats. *Toxicological sciences : an official journal of the Society of Toxicology*, 131(2), pp.583–95.

Carll, A.P. et al., 2010. Particulate matter inhalation exacerbates cardiopulmonary injury in a rat model of isoproterenol-induced cardiomyopathy. *Inhalation toxicology*, 22(5), pp.355–68.

Carr, M.J. & Undem, B.J., 2003. Bronchopulmonary afferent nerves. *Respirology (Carlton, Vic.)*, 8(3), pp.291–301.

Cassee, F.R. et al., 2002. Particle size-dependent total mass deposition in lungs determines inhalation toxicity of cadmium chloride aerosols in rats. Application of a multiple path dosimetry model. *Archives of toxicology*, 76(5-6), pp.277–86.

Caterina, M.J., 2007. Chemical biology: Sticky spices. *Nature*, 445(7127), pp.491–492.

Caterina, M.J. et al., 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*, 389(6653), pp.816–24.

CDC, 2016. FASTSTATS - Heart Disease. Available at: http://www.cdc.gov/nchs/fastats/heart.htm [Accessed February 3, 2014].

CDC-ASTDR, 2007. *Toxicological Profile for Acrolein*,

Chen, J. et al., 2011. In Vitro and in Vivo Assays for the Discovery of Analgesic Drugs Targeting TRP Channels - PubMed - NCBI. In M. Zhu, ed. *TRP Channels*. Boca Raton (FL): CRC Press/Taylor & Francis.

Chen, L.C. & Hwang, J.-S., 2005. Effects of subchronic exposures to concentrated ambient particles (CAPs) in mice. IV. Characterization of acute and chronic effects of ambient air fine particulate matter exposures on heart-rate variability. *Inhalation toxicology*, 17(4-5), pp.209–16.

Chess, G.F., Tam, R.M. & Calaresu, F.R., 1975. Influence of cardiac neural inputs on rhythmic variations of heart period in the cat. *The American journal of physiology*, 228(3), pp.775–80.

Chuang, K.-J. et al., 2007. Associations between particulate sulfate and organic carbon exposures and heart rate variability in patients with or at risk for cardiovascular diseases. *Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine*, 49(6), pp.610–7.

Clougherty, J.E. & Kubzansky, L.D., 2009. A framework for examining social stress and susceptibility to air pollution in respiratory health. *Environmental health perspectives*, 117(9), pp.1351–8.

Colais, P. et al., 2012. Particulate air pollution and hospital admissions for cardiac diseases in potentially sensitive subgroups. *Epidemiology (Cambridge, Mass.)*, 23(3), pp.473–81.

Coleridge, H.M., Coleridge, J.C. & Roberts, A.M., 1983. Rapid shallow breathing evoked by selective stimulation of airway C fibres in dogs. *The Journal of physiology*, 340, pp.415–33.

Coleridge, J.C. & Coleridge, H.M., 1984. Afferent vagal C fibre innervation of the lungs and airways and its functional significance. *Reviews of physiology, biochemistry and pharmacology*, 99, pp.1–110.

Costa, D.L. et al., 2006. Comparative pulmonary toxicological assessment of oil combustion particles following inhalation or instillation exposure. *Toxicological sciences : an official journal of the Society of Toxicology*, 91(1), pp.237–46.

Cozzi, E. et al., 2006. Ultrafine particulate matter exposure augments ischemia-reperfusion

injury in mice. *American journal of physiology. Heart and circulatory physiology*, 291(2), pp.H894–903.

Davoodi, G. et al., 2010. Comparison of heart rate variability and cardiac arrhythmias in polluted and clean air episodes in healthy individuals. *Environmental health and preventive medicine*, 15(4), pp.217–21.

Deboor, S.S., Pelter, M.M. & Adams, M.G., 2005. Nonrespiratory sinus arrhythmia. *American journal of critical care : an official publication, American Association of Critical-Care Nurses*, 14(2), pp.161–2.

Deering-Rice, C.E. et al., 2011. Electrophilic components of diesel exhaust particles (DEP) activate transient receptor potential ankyrin-1 (TRPA1): a probable mechanism of acute pulmonary toxicity for DEP. *Chemical research in toxicology*, 24(6), pp.950–9.

DeJarnett, N. et al., 2014. Acrolein exposure is associated with increased cardiovascular disease risk. *Journal of the American Heart Association*, 3(4).

Devlin, R.B. et al., 2003. Elderly humans exposed to concentrated air pollution particles have decreased heart rate variability. *The European respiratory journal. Supplement*, 40, p.76s–80s.

Dishman, R.K. et al., 2000. Heart rate variability, trait anxiety, and perceived stress among physically fit men and women. *International journal of psychophysiology : official journal of the International Organization of Psychophysiology*, 37(2), pp.121–33.

Dockery, D.W. et al., 1993. An association between air pollution and mortality in six U.S. cities. *The New England journal of medicine*, 329(24), pp.1753–9.

Domej, W., Oettl, K. & Renner, W., 2014. Oxidative stress and free radicals in COPD--implications and relevance for treatment. *International journal of chronic obstructive pulmonary disease*, 9, pp.1207–24.

Earley, S., 2012. TRPA1 channels in the vasculature. *British journal of pharmacology*, 167(1), pp.13–22.

Eguchi, K. et al., 2010. Increased heart rate variability during sleep is a predictor for future cardiovascular events in patients with type 2 diabetes. *Hypertension research : official journal of the Japanese Society of Hypertension*, 33(7), pp.737–42.

Eid, S.R. & Cortright, D.N., 2009. Transient receptor potential channels on sensory nerves. *Handbook of experimental pharmacology*, (194), pp.261–81.

Elder, A.C. et al., 2000. Pulmonary inflammatory response to inhaled ultrafine particles is modified by age, ozone exposure, and bacterial toxin. *Inhalation toxicology*, 12 Suppl 4, pp.227–46.

Esterbauer, H., Schaur, R.J. & Zollner, H., 1991. Chemistry and biochemistry of 4-

hydroxynonenal, malonaldehyde and related aldehydes. *Free radical biology & medicine*, 11(1), pp.81–128.

Fakhri, A.A. et al., 2009. Autonomic effects of controlled fine particulate exposure in young healthy adults: effect modification by ozone. *Environmental health perspectives*, 117(8), pp.1287–1292.

Fann, N., Fulcher, C.M. & Baker, K., 2013. The recent and future health burden of air pollution apportioned across U.S. sectors. *Environmental science & technology*, 47(8), pp.3580–9.

Farah, V.M.A., Joaquim, L.F. & Morris, M., 2006. Stress cardiovascular/autonomic interactions in mice. *Physiology & behavior*, 89(4), pp.569–75.

Farraj, A.K. et al., 2015. Cardiac effects of seasonal ambient particulate matter and ozone co-exposure in rats. *Particle and fibre toxicology*, 12, p.12.

Farraj, A.K. et al., 2010. Increased lung resistance after diesel particulate and ozone co-exposure not associated with enhanced lung inflammation in allergic mice. *Inhalation toxicology*, 22(1), pp.33–41.

Farraj, A.K. et al., 2009. Increased non-conducted P-wave arrhythmias after a single oil fly ash inhalation exposure in hypertensive rats. *Environmental health perspectives*, 117(5), pp.709–15.

Farraj, A.K. et al., 2012. Overt and latent cardiac effects of ozone inhalation in rats: evidence for autonomic modulation and increased myocardial vulnerability. *Environmental health perspectives*, 120(3), pp.348–54.

Farraj, A.K., Hazari, M.S., Haykal-Coates, N., et al., 2011. ST depression, arrhythmia, vagal dominance, and reduced cardiac micro-RNA in particulate-exposed rats. *American journal of respiratory cell and molecular biology*, 44(2), pp.185–96.

Farraj, A.K., Hazari, M.S. & Cascio, W.E., 2011. The utility of the small rodent electrocardiogram in toxicology. *Toxicological sciences : an official journal of the Society of Toxicology*, 121(1), pp.11–30.

Fischer, M.J.M. et al., 2014. Direct evidence for functional TRPV1/TRPA1 heteromers. *Pflügers Archiv : European journal of physiology*, 466(12), pp.2229–41.

Franchini, M. & Mannucci, P.M., 2007. Short-term effects of air pollution on cardiovascular diseases: outcomes and mechanisms. *Journal of thrombosis and haemostasis : JTH*, 5(11), pp.2169–74.

Fuder, H. & Muscholl, E., 1995. Heteroreceptor-mediated modulation of noradrenaline and acetylcholine release from peripheral nerves. *Reviews of physiology, biochemistry and pharmacology*, 126, pp.265–412.

Furuyama, A. et al., 2009. Extrapulmonary translocation of intratracheally instilled fine and ultrafine particles via direct and alveolar macrophage-associated routes. *Archives of toxicology*, 83(5), pp.429–37.

Gang, Y. & Malik, M., 2003. Heart rate variability analysis in general medicine. *Indian Pacing and Electrophysiology Journal*, 3(1), pp.34–40.

Gehrmann, J. et al., 2000. Phenotypic screening for heart rate variability in the mouse. *American journal of physiology. Heart and circulatory physiology*, 279(2), pp.H733–40.

Ghilarducci, D.P. & Tjeerdema, R.S., 1995. Fate and effects of acrolein. *Reviews of environmental contamination and toxicology*, 144, pp.95–146.

Gill, E.A. et al., Air pollution and cardiovascular disease in the Multi-Ethnic Study of Atherosclerosis. *Progress in cardiovascular diseases*, 53(5), pp.353–60.

Gladuli, A. et al., 2011. Poincaré plots and tachograms reveal beat patterning in sick sinus syndrome with supraventricular tachycardia and varying AV nodal block. *Journal of veterinary cardiology : the official journal of the European Society of Veterinary Cardiology*, 13(1), pp.63–70.

Godleski, J.J. et al., 2000. Mechanisms of morbidity and mortality from exposure to ambient air particles. *Research report (Health Effects Institute)*, (91), pp.5–88; discussion 89–103.

Gold, D.R. et al., 2000. Ambient pollution and heart rate variability. *Circulation*, 101(11), pp.1267–73.

Goldberg, M.S. et al., 2001. Identification of persons with cardiorespiratory conditions who are at risk of dying from the acute effects of ambient air particles. *Environmental health perspectives*, 109 Suppl , pp.487–94.

Goldstein, D.S. et al., 2011. Low-frequency power of heart rate variability is not a measure of cardiac sympathetic tone but may be a measure of modulation of cardiac autonomic outflows by baroreflexes. *Experimental physiology*, 96(12), pp.1255–61.

Gong, H. et al., 2004. Altered heart-rate variability in asthmatic and healthy volunteers exposed to concentrated ambient coarse particles. *Inhalation toxicology*, 16(6-7), pp.335–43.

Graepel, R. et al., 2011. 4-oxo-2-nonenal (4-ONE): evidence of transient receptor potential ankyrin 1-dependent and -independent nociceptive and vasoactive responses in vivo. *The Journal of pharmacology and experimental therapeutics*, 337(1), pp.117–24.

Grunig, G. et al., 2014. Perspective: ambient air pollution: inflammatory response and effects on the lung's vasculature. *Pulmonary circulation*, 4(1), pp.25–35.

Gu, Q. & Lin, R.-L., 2010. Heavy metals zinc, cadmium, and copper stimulate pulmonary

sensory neurons via direct activation of TRPA1. *Journal of applied physiology (Bethesda, Md. : 1985)*, 108(4), pp.891–7.

Guimaraes, M. & Jordt, S., 2007. TRPA1 : A Sensory Channel of Many Talents - PubMed - NCBI. In W. Liedtke & S. Heller, eds. *Frontiers in Neuroscience.* Boca Raton (FL): Taylor & Francis Group, LLC.

Gurgueira, S.A. et al., 2002. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environmental health perspectives*, 110(8), pp.749–55.

Hales, C.A. et al., 1989. Bronchial artery ligation modifies pulmonary edema after exposure to smoke with acrolein. *Journal of applied physiology (Bethesda, Md. : 1985)*, 67(3), pp.1001–6.

Hazari, M.S. et al., 2014. A single exposure to acrolein desensitizes baroreflex responsiveness and increases cardiac arrhythmias in normotensive and hypertensive rats. *Cardiovascular toxicology*, 14(1), pp.52–63.

Hazari, M.S., Callaway, J., Winsett, D.W., Lamb, C., Haykal-Coates, N., et al., 2012. Dobutamine "stress" test and latent cardiac susceptibility to inhaled diesel exhaust in normal and hypertensive rats. *Environmental health perspectives*, 120(8), pp.1088–93.

Hazari, M.S. et al., 2011. TRPA1 and sympathetic activation contribute to increased risk of triggered cardiac arrhythmias in hypertensive rats exposed to diesel exhaust. *Environmental health perspectives*, 119(7), pp.951–7.

Heathers, J.A.J., 2014. Everything Hertz: methodological issues in short-term frequency-domain HRV. *Frontiers in physiology*, 5, p.177.

Heidenreich, P.A. et al., 2011. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation*, 123(8), pp.933–44.

Hillebrand, S. et al., 2013. Heart rate variability and first cardiovascular event in populations without known cardiovascular disease: meta-analysis and dose-response meta-regression. *Europace : European pacing, arrhythmias, and cardiac electrophysiology : journal of the working groups on cardiac pacing, arrhythmias, and cardiac cellular electrophysiology of the European Society of Cardiology*, 15(5), pp.742–9.

Hinman, A. et al., 2006. TRP channel activation by reversible covalent modification. *Proceedings of the National Academy of Sciences of the United States of America*, 103(51), pp.19564–8.

Hooper, J.S. et al., 2015. Characterization of cardiovascular reflexes evoked by airway stimulation with allylisothiocyanate, capsaicin and ATP in Sprague Dawley rats. *Journal of applied physiology (Bethesda, Md. : 1985)*, p.jap.00944.2015.

Hu, H. et al., 2009. Zinc activates damage-sensing TRPA1 ion channels. *Nature chemical biology*, 5(3), pp.183–90.

Hwang, H. et al., 2008. Direct and acute cardiotoxic effects of ultrafine air pollutants in spontaneously hypertensive rats and Wistar--Kyoto rats. *Journal of cardiovascular pharmacology and therapeutics*, 13(3), pp.189–98.

Ismahil, M.A. et al., 2011. Chronic oral exposure to the aldehyde pollutant acrolein induces dilated cardiomyopathy. *American journal of physiology. Heart and circulatory physiology*, 301(5), pp.H2050–60.

Jaquemar, D., Schenker, T. & Trueb, B., 1999. An ankyrin-like protein with transmembrane domains is specifically lost after oncogenic transformation of human fibroblasts. *The Journal of biological chemistry*, 274(11), pp.7325–33.

Johnson, R.L., 2004. Relative effects of air pollution on lungs and heart. *Circulation*, 109(1), pp.5–7.

Jones, J.F., Wang, Y. & Jordan, D., 1995. Heart rate responses to selective stimulation of cardiac vagal C fibres in anaesthetized cats, rats and rabbits. *The Journal of physiology*, 489 (Pt 1, pp.203–14.

Just, A., Faulhaber, J. & Ehmke, H., 2000. Autonomic cardiovascular control in conscious mice. *American journal of physiology. Regulatory, integrative and comparative physiology*, 279(6), pp.R2214–21.

Kachel, D.L. & Martin, W.J., 1994. Cyclophosphamide-induced lung toxicity: mechanism of endothelial cell injury. *The Journal of pharmacology and experimental therapeutics*, 268(1), pp.42–6.

Kehrer, J.P. & Biswal, S.S., 2000. The molecular effects of acrolein. *Toxicological sciences : an official journal of the Society of Toxicology*, 57(1), pp.6–15.

Keller, K.B. & Lemberg, L., 2006. The Sick Sinus Syndrome. *Am. J. Crit. Care.*, 15(2), pp.226–229.

Kleeberger, S.R., 2003. Genetic aspects of susceptibility to air pollution. *The European respiratory journal. Supplement*, 40, p.52s–56s.

Kodavanti, U.P., 2015. Air pollution and insulin resistance: do all roads lead to Rome? *Diabetes*, 64(3), pp.712–4.

Kodavanti, U.P. et al., 2011. Vascular and cardiac impairments in rats inhaling ozone and diesel exhaust particles. *Environmental health perspectives*, 119(3), pp.312–8.

Kollarik, M., Ru, F. & Brozmanova, M., 2010. Vagal afferent nerves with the properties of nociceptors. *Autonomic neuroscience : basic & clinical*, 153(1-2), pp.12–20.

Kop, W.J. et al., 2001. Changes in heart rate and heart rate variability before ambulatory ischemic events(1). *Journal of the American College of Cardiology*, 38(3), pp.742–9.

Kozumbo, W.J. & Agarwal, S., 1990. Induction of DNA damage in cultured human lung cells by tobacco smoke arylamines exposed to ambient levels of ozone. *American journal of respiratory cell and molecular biology*, 3(6), pp.611–8.

Kubin, L. et al., 2006. Central pathways of pulmonary and lower airway vagal afferents. *Journal of applied physiology (Bethesda, Md. : 1985)*, 101(2), pp.618–27.

Kumarathasan, P. et al., 2015. Nitrative stress, oxidative stress and plasma endothelin levels after inhalation of particulate matter and ozone. *Particle and fibre toxicology*, 12, p.28.

Kurhanewicz, N. et al., 2014. Ozone co-exposure modifies cardiac responses to fine and ultrafine ambient particulate matter in mice: concordance of electrocardiogram and mechanical responses. *Particle and fibre toxicology*, 11(1), p.54.

Kutzman, R.S. et al., 1985. Changes in rat lung structure and composition as a result of subchronic exposure to acrolein. *Toxicology*, 34(2), pp.139–51.

Kwan, K.Y. et al., 2006. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron*, 50(2), pp.277–89.

Lawrence, A.J. & Jarrott, B., 1996. Neurochemical modulation of cardiovascular control in the nucleus tractus solitarius. *Progress in Neurobiology*, 48(1), pp.21–53.

Lee, B.P., Morton, R.F. & Lee, L.Y., 1992. Acute effects of acrolein on breathing: role of vagal bronchopulmonary afferents. *Journal of applied physiology (Bethesda, Md. : 1985)*, 72(3), pp.1050–6.

Lee, L.-Y., 2010. TRPA1 ion channels: a gateway to airway irritation and reflex responses induced by inhaled oxidants. *The Journal of physiology*, 588(Pt 5), pp.747–8.

Lee, L.Y. & Pisarri, T.E., 2001. Afferent properties and reflex functions of bronchopulmonary C-fibers. *Respiration physiology*, 125(1-2), pp.47–65.

Lee, L.Y. & Widdicombe, J.G., 2001. Modulation of airway sensitivity to inhaled irritants: role of inflammatory mediators. *Environmental health perspectives*, 109 Suppl , pp.585–589.

Leikauf, G.D. et al., 1989. Bronchial responsiveness and inflammation in guinea pigs exposed to acrolein. *Journal of applied physiology (Bethesda, Md. : 1985)*, 66(1), pp.171–8.

Link, M.S. & Dockery, D.W., 2010. Air pollution and the triggering of cardiac arrhythmias. *Current opinion in cardiology*, 25(1), pp.16–22.

Lipsitz, L.A. & Goldberger, A.L., 1992. Loss of "complexity" and aging. Potential

applications of fractals and chaos theory to senescence. *JAMA*, 267(13), pp.1806–9.

Liu, J. et al., 2014. Heart rate and heart rate variability assessment identifies individual differences in fear response magnitudes to earthquake, free fall, and air puff in mice. *PloS one*, 9(3), p.e93270.

Logan, W.P., 1956. Mortality from fog in London, January, 1956. *British medical journal*, 1(4969), pp.722–5.

Macpherson, L.J. et al., 2007. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature*, 445(7127), pp.541–5.

Madden, M.C. et al., 1993. Chemical nature and immunotoxicological properties of arachidonic acid degradation products formed by exposure to ozone. *Environmental health perspectives*, 101(2), pp.154–64.

Madden, M.C. et al., 2000. Effect of ozone on diesel exhaust particle toxicity in rat lung. *Toxicology and applied pharmacology*, 168(2), pp.140–8.

McIntosh-Kastrinsky, R. et al., 2013. Photochemically altered air pollution mixtures and contractile parameters in isolated murine hearts before and after ischemia. *Environmental health perspectives*, 121(11-12), pp.1344–8.

McMullen, M.K. et al., 2012. Respiratory and non-respiratory sinus arrhythmia: implications for heart rate variability. *Journal of clinical monitoring and computing*, 26(1), pp.21–8.

Mead, M.N., 2011. Who's at risk? Gauging susceptibility to air pollutants. *Environmental health perspectives*, 119(4), p.A176.

Méndez, L.B., Gookin, G. & Phalen, R.F., 2010. Inhaled aerosol particle dosimetry in mice: a review. *Inhalation toxicology*, 22(12), pp.1032–7.

Milan, D.J. & MacRae, C.A., 2005. Animal models for arrhythmias. *Cardiovascular research*, 67(3), pp.426–37.

Mills, N.L. et al., 2009. Adverse cardiovascular effects of air pollution. *Nature clinical practice. Cardiovascular medicine*, 6(1), pp.36–44.

Mills, N.L. et al., 2011. Diesel exhaust inhalation does not affect heart rhythm or heart rate variability. *Heart (British Cardiac Society)*, 97(7), pp.544–50.

Mills, N.L. et al., 2007. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. *The New England journal of medicine*, 357(11), pp.1075–82.

Mitchell, G.F., Jeron, A. & Koren, G., 1998. Measurement of heart rate and Q-T interval in the conscious mouse. *The American journal of physiology*, 274(3 Pt 2), pp.H747–51.

Moak, J.P. et al., 2007. Supine low-frequency power of heart rate variability reflects baroreflex function, not cardiac sympathetic innervation. *Heart rhythm : the official journal of the Heart Rhythm Society*, 4(12), pp.1523–1529.

Morilak, D.A., Morris, M. & Chalmers, J., 1988. Release of substance P in the nucleus tractus solitarius measured by in vivo microdialysis: response to stimulation of the aortic depressor nerves in rabbit. *Neuroscience letters*, 94(1-2), pp.131–7.

Morris, J.B. et al., 2003. Immediate sensory nerve-mediated respiratory responses to irritants in healthy and allergic airway-diseased mice. *Journal of applied physiology (Bethesda, Md. : 1985)*, 94(4), pp.1563–71.

Mutoh, T., Bonham, A.C. & Joad, J.P., 2000. Substance P in the nucleus of the solitary tract augments bronchopulmonary C fiber reflex output. *American journal of physiology. Regulatory, integrative and comparative physiology*, 279(4), pp.R1215–23.

Mutoh, T., Joad, J.P. & Bonham, A.C., 2000. Chronic passive cigarette smoke exposure augments bronchopulmonary C-fibre inputs to nucleus tractus solitarii neurones and reflex output in young guinea-pigs. *The Journal of physiology*, 523 Pt 1, pp.223–233.

Nadziejko, C. et al., 2002. Immediate effects of particulate air pollutants on heart rate and respiratory rate in hypertensive rats. *Cardiovascular toxicology*, 2(4), pp.245–52.

Nemmar, A. et al., 2002. Passage of inhaled particles into the blood circulation in humans. *Circulation*, 105(4), pp.411–4.

Nemmar, A. et al., 2004. Possible mechanisms of the cardiovascular effects of inhaled particles: systemic translocation and prothrombotic effects. *Toxicology letters*, 149(1-3), pp.243–53.

O'Toole, T.E. et al., 2009. Acrolein activates matrix metalloproteinases by increasing reactive oxygen species in macrophages. *Toxicology and applied pharmacology*, 236(2), pp.194–201.

Oxford English Dictionary Online A. Stevenson, J. Elliott, & R. Jones, eds. *Oxford English Dictionary*, 2010(07/07), p.http://dictionary.oed.com/.

Osier, M. & Oberdörster, G., 1997. Intratracheal inhalation vs intratracheal instillation: differences in particle effects. *Fundamental and applied toxicology : official journal of the Society of Toxicology*, 40(2), pp.220–7.

Parati, G. et al., 1995. Spectral analysis of blood pressure and heart rate variability in evaluating cardiovascular regulation. A critical appraisal. *Hypertension*, 25(6), pp.1276–86.

Park, J.-H. et al., 2006. Polycyclic aromatic hydrocarbon (PAH) o-quinones produced by the aldo-keto-reductases (AKRs) generate abasic sites, oxidized pyrimidines, and 8-oxo-dGuo via reactive oxygen species. *Chemical research in toxicology*, 19(5), pp.719–28.

Park, S.K. et al., 2010. Particulate air pollution, metabolic syndrome, and heart rate variability: the multi-ethnic study of atherosclerosis (MESA). *Environmental health perspectives*, 118(10), pp.1406–11.

Paton, J.F. & Nolan, P.J., 2000. Similarities in reflex control of laryngeal and cardiac vagal motor neurones. *Respiration physiology*, 119(2-3), pp.101–11.

Pelter, M.M. & Adams, M.G., 2003. Non-conducted P waves. *American journal of critical care : an official publication, American Association of Critical-Care Nurses*, 12(6), pp.567–8.

Peretz, A. et al., 2008. Effects of diesel exhaust inhalation on heart rate variability in human volunteers. *Environmental research*, 107(2), pp.178–84.

Perez, C.M., Hazari, M.S. & Farraj, A.K., 2015. Role of autonomic reflex arcs in cardiovascular responses to air pollution exposure. *Cardiovascular toxicology*, 15(1), pp.69–78.

Perini, R. & Veicsteinas, A., 2003. Heart rate variability and autonomic activity at rest and during exercise in various physiological conditions. *European journal of applied physiology*, 90(3-4), pp.317–25.

Peters, A. et al., 2000. Air pollution and incidence of cardiac arrhythmia. *Epidemiology Cambridge Mass*, 11(1), pp.11–17.

Peters, A., 2005. Particulate matter and heart disease: evidence from epidemiological studies. *Toxicology and applied pharmacology*, 207(2 Suppl), pp.477–82.

Pham, H. et al., 2009. Central neuroplasticity and decreased heart rate variability after particulate matter exposure in mice. *Environmental health perspectives*, 117(9), pp.1448–53.

Piccirillo, G. et al., 2009. Power spectral analysis of heart rate variability and autonomic nervous system activity measured directly in healthy dogs and dogs with tachycardia-induced heart failure. *Heart rhythm : the official journal of the Heart Rhythm Society*, 6(4), pp.546–52.

Pincus, S.M., 1994. Greater signal regularity may indicate increased system isolation. *Mathematical biosciences*, 122(2), pp.161–81.

Pitts, J.N. et al., 1980. "Atmospheric" Epoxidation of Benzo[a]pyrene by Ozone: Formation of the Metabolite Benzo[a]pyrene-4,5-Oxide. *Science (New York, N.Y.)*, 210(4476), pp.1347–9.

Ponikowski, P. et al., 1997. Depressed heart rate variability as an independent predictor of death in chronic congestive heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. *The American journal of cardiology*, 79(12), pp.1645–50.

Pope, C.A. et al., 2004. Cardiovascular mortality and long-term exposure to particulate air pollution: epidemiological evidence of general pathophysiological pathways of disease. *Circulation*, 109(1), pp.71–77.

Pope, C.A. et al., 1999. Heart rate variability associated with particulate air pollution. *American heart journal*, 138(5 Pt 1), pp.890–9.

Potts, J.T., Neural circuits controlling cardiorespiratory responses: baroreceptor and somatic afferents in the nucleus tractus solitarius. *Clinical and experimental pharmacology & physiology*, 29(1-2), pp.103–11.

Pozsgai, G. et al., 2010. Evidence for the pathophysiological relevance of TRPA1 receptors in the cardiovascular system in vivo. *Cardiovascular research*, 87(4), pp.760–8.

Provost, E.B. et al., 2015. Carotid intima-media thickness, a marker of subclinical atherosclerosis, and particulate air pollution exposure: the meta-analytical evidence. *PloS one*, 10(5), p.e0127014.

Purves, D. et al., 2001. Neuroscience. 2nd edition. *Sunderland (MA): Sinauer Associates; 2001.*, pp.109–12.

Quade R . Stahl, 1969. *Air Pollution Aspects of Aldehydes*, Bethseda, Maryland.

Rahman, F. et al., 2011. Low frequency power of heart rate variability reflects baroreflex function, not cardiac sympathetic innervation. *Clinical autonomic research : official journal of the Clinical Autonomic Research Society*, 21(3), pp.133–141.

Rahman, I. et al., 2002. 4-Hydroxy-2-nonenal, a specific lipid peroxidation product, is elevated in lungs of patients with chronic obstructive pulmonary disease. *American journal of respiratory and critical care medicine*, 166(4), pp.490–5.

Reyes del Paso, G.A. et al., 2013. The utility of low frequency heart rate variability as an index of sympathetic cardiac tone: a review with emphasis on a reanalysis of previous studies. *Psychophysiology*, 50(5), pp.477–87.

Riley, M.R. et al., 2003. Effects of metals Cu, Fe, Ni, V, and Zn on rat lung epithelial cells. *Toxicology*, 190(3), pp.171–84.

Ris, C., 2007. U.S. EPA health assessment for diesel engine exhaust: a review. *Inhalation toxicology*, 19 Suppl 1, pp.229–239.

Routledge, H.C., Ayres, J.G. & Townend, J.N., 2003. Why cardiologists should be interested in air pollution. *Heart (British Cardiac Society)*, 89(12), pp.1383–8.

Rowan, W.H. et al., 2007. Heart rate variability in rodents: uses and caveats in toxicological studies. *Cardiovascular toxicology*, 7(1), pp.28–51.

Salas, M.M., Hargreaves, K.M. & Akopian, A.N., 2009. TRPA1-mediated responses in

trigeminal sensory neurons: Interaction between TRPA1 and TRPV1. *European Journal of Neuroscience*, 29(8), pp.1568–1578.

Samet, J.M. et al., 2009. Concentrated ambient ultrafine particle exposure induces cardiac changes in young healthy volunteers. *American Journal of Respiratory and Critical Care Medicine*, 179(11), pp.1034–1042.

Saria, A. et al., 1988. Release of multiple tachykinins from capsaicin-sensitive sensory nerves in the lung by bradykinin, histamine, dimethylphenyl piperazinium, and vagal nerve stimulation. *The American review of respiratory disease*, 137(6), pp.1330–5.

Sayers, B.M., 1973. Analysis of heart rate variability. *Ergonomics*, 16(1), pp.17–32.

Schneider, A. et al., 2010. Changes in deceleration capacity of heart rate and heart rate variability induced by ambient air pollution in individuals with coronary artery disease. *Particle and fibre toxicology*, 7, p.29.

Schubert, C. et al., 2009. Effects of stress on heart rate complexity—A comparison between short-term and chronic stress. *Biological Psychology*, 80(3), pp.325–332.

Schwartz, J., Bellinger, D. & Glass, T., 2011. Exploring potential sources of differential vulnerability and susceptibility in risk from environmental hazards to expand the scope of risk assessment. *American journal of public health*, 101 Suppl , pp.S94–101.

Semelka, M., Gera, J. & Usman, S., 2013. Sick sinus syndrome: a review. *American family physician*, 87(10), pp.691–6.

Shapiro, D. et al., 2013. Activation of transient receptor potential ankyrin-1 (TRPA1) in lung cells by wood smoke particulate material. *Chemical research in toxicology*, 26(5), pp.750–8.

Shetty, I. & Scott, W., 2015. *Clinical Cardiac Electrophysiology in the Young* 197th–208th ed. M. Dick, II, ed., New York, NY: Springer New York.

Shields, K.N. et al., 2013. Traffic-related air pollution exposures and changes in heart rate variability in Mexico City: a panel study. *Environmental health : a global access science source*, 12, p.7.

Shusterman, D., 2012. The Effects of Air Pollutants and Irritants on the Upper Airway. *Proceedings of the American Thoracic Society*.

Sim, V.M. & Pattle, R.E., 1957. Effect of possible smog irritants on human subjects. *Journal of the American Medical Association*, 165(15), pp.1908–13.

Simkhovich, B.Z., Kleinman, M.T. & Kloner, R. a, 2008. Air pollution and cardiovascular injury epidemiology, toxicology, and mechanisms. *Journal of the American College of Cardiology*, 52(9), pp.719–726.

Simms, A.E., Paton, J.F.R. & Pickering, A.E., 2006. Disinhibition of the cardiac limb of the arterial baroreflex in rat: a role for metabotropic glutamate receptors in the nucleus tractus solitarii. *The Journal of physiology*, 575(Pt 3), pp.727–38.

Sithu, S.D. et al., 2010. Exposure to acrolein by inhalation causes platelet activation. *Toxicology and applied pharmacology*, 248(2), pp.100–10.

Sivagangabalan, G. et al., 2011. The effect of air pollution on spatial dispersion of myocardial repolarization in healthy human volunteers. *Journal of the American College of Cardiology*, 57(2), pp.198–206.

Staruschenko, A., Jeske, N.A. & Akopian, A.N., 2010. Contribution of TRPV1-TRPA1 interaction to the single channel properties of the TRPA1 channel. *The Journal of biological chemistry*, 285(20), pp.15167–77.

Stein, P.K. et al., 1994. Heart rate variability: a measure of cardiac autonomic tone. *American heart journal*, 127(5), pp.1376–81.

Stein, P.K. et al., 2005. Sometimes higher heart rate variability is not better heart rate variability: results of graphical and nonlinear analyses. *Journal of cardiovascular electrophysiology*, 16(9), pp.954–9.

Story, G.M. et al., 2003. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell*, 112(6), pp.819–29.

Story, G.M., 2006. The emerging role of TRP channels in mechanisms of temperature and pain sensation. *Current neuropharmacology*, 4(3), pp.183–96.

Takenaka, S. et al., 2001. Pulmonary and systemic distribution of inhaled ultrafine silver particles in rats. *Environmental health perspectives*, 109 Suppl , pp.547–51.

Tani, M. et al., 2015. Long-lasting facilitation of respiratory rhythm by treatment with TRPA1 agonist, cinnamaldehyde. *Journal of neurophysiology*, 114(2), pp.989–98.

Tankersley, C.G. et al., 2010. Variation in echocardiographic and cardiac hemodynamic effects of PM and ozone inhalation exposure in strains related to Nppa and Npr1 gene knock-out mice. *Inhalation toxicology*, 22(8), pp.695–707.

Taylor-Clark, T.E. & Undem, B.J., 2010. Ozone activates airway nerves via the selective stimulation of TRPA1 ion channels. *The Journal of physiology*, 588(Pt 3), pp.423–433.

Thayer, J.F., Yamamoto, S.S. & Brosschot, J.F., 2010. The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors. *International journal of cardiology*, 141(2), pp.122–31.

Thireau, J. et al., 2008. Heart rate variability in mice: a theoretical and practical guide. *Experimental physiology*, 93(1), pp.83–94.

Thomson, E. et al., 2005. Differential regulation of the lung endothelin system by urban particulate matter and ozone. *Toxicological sciences : an official journal of the Society of Toxicology*, 88(1), pp.103–13.

Tong, H. et al., 2010. Differential cardiopulmonary effects of size-fractionated ambient particulate matter in mice. *Cardiovascular toxicology*, 10(4), pp.259–67.

Tong, H. et al., 2012. Omega-3 fatty acid supplementation appears to attenuate particulate air pollution-induced cardiac effects and lipid changes in healthy middle-aged adults. *Environmental health perspectives*, 120(7), pp.952–7.

Toraason, M. et al., 1989. Comparative toxicity of allylamine and acrolein in cultured myocytes and fibroblasts from neonatal rat heart. *Toxicology*, 56(1), pp.107–17.

Tsuji, H. et al., 1996. Impact of Reduced Heart Rate Variability on Risk for Cardiac Events: The Framingham Heart Study. *Circulation*, 94(11), pp.2850–2855.

Tsuji, H. et al., 1994. Reduced heart rate variability and mortality risk in an elderly cohort. The Framingham Heart Study. *Circulation*, 90(2), pp.878–83.

U.S. Congress, 1955. *An Act to provide research and technical assistance relating to air pollution control*,

U.S. EPA, Air Quality Trends. Available at: http://www3.epa.gov/airtrends/aqtrends.html.

Urch, B. et al., 2005. Acute blood pressure responses in healthy adults during controlled air pollution exposures. *Environmental health perspectives*, 113(8), pp.1052–5.

US EPA, 2016a. Clean Air Act Requirements and History. Available at: https://www.epa.gov/clean-air-act-overview/clean-air-act-requirements-and-history.

US EPA, 2016b. National Ambient Air Quality Standards (NAAQS). Available at: http://www3.epa.gov/ttn/naaqs/criteria.html.

US EPA, Hazardous Air Pollutants. Available at: http://www.epa.gov/haps

Valavanidis, A. et al., 2009. Influence of ozone on traffic-related particulate matter on the generation of hydroxyl radicals through a heterogeneous synergistic effect. *Journal of hazardous materials*, 162(2-3), pp.886–92.

Valkama, J.O. et al., 1995. Relation between heart rate variability and spontaneous and induced ventricular arrhythmias in patients with coronary artery disease. *Journal of the American College of Cardiology*, 25(2), pp.437–43.

Verrier, R.L., Mittleman, M.A. & Stone, P.H., 2002. Air pollution: an insidious and pervasive component of cardiac risk. *Circulation*, 106(8), pp.890–2.

Vijayaraghavan, R. et al., 1993. Characteristic modifications of the breathing pattern of mice

to evaluate the effects of airborne chemicals on the respiratory tract. *Archives of toxicology*, 67(7), pp.478–90.

Vincent, R. et al., 1997. Acute pulmonary toxicity of urban particulate matter and ozone. *The American journal of pathology*, 151(6), pp.1563–70.

W.H.O., 2012. *Burden of disease from Ambient Air Pollution for 2012*.

Wagner, J.G. et al., 2014. Cardiovascular depression in rats exposed to inhaled particulate matter and ozone: effects of diet-induced metabolic syndrome. *Environmental health perspectives*, 122(1), pp.27–33.

Wagner, J.G. et al., 2013. Cardiovascular Depression in Rats Exposed to Inhaled Particulate Matter and Ozone: Effects of Diet-Induced Metabolic Syndrome. *Environmental health perspectives*.

Walsh, L.C., 2008. Research and Development at EPA • 1,950. , pp.1–20.

Wang, G. et al., 2013. Effects of ozone and fine particulate matter (PM(2.5)) on rat system inflammation and cardiac function. *Toxicology letters*, 217(1), pp.23–33.

Wang, Y. et al., 2000. Effect of pulmonary C-fibre afferent stimulation on cardiac vagal neurones in the nucleus ambiguus in anaesthetized cats. *The Journal of physiology*, 526 Pt 1, pp.157–65.

Watkinson, W.P. et al., 2001. Cardiovascular and systemic responses to inhaled pollutants in rodents: effects of ozone and particulate matter. *Environmental health perspectives*, 109 Suppl , pp.539–546.

Wetsel, W.C., 2011. Sensing hot and cold with TRP channels. *International journal of hyperthermia : the official journal of European Society for Hyperthermic Oncology, North American Hyperthermia Group*, 27(4), pp.388–98.

Whitsel, E.A. et al., 2009. Heart rate variability, ambient particulate matter air pollution, and glucose homeostasis: the environmental epidemiology of arrhythmogenesis in the women's health initiative. *American journal of epidemiology*, 169(6), pp.693–703.

Widdicombe, J., 2001. Airway receptors. *Respiration physiology*, 125(1-2), pp.3–15.

Woodruff, T.J. et al., 2007. Estimating risk from ambient concentrations of acrolein across the United States. *Environmental health perspectives*, 115(3), pp.410–5.

Wu, C. et al., 2010. Effects of personal exposure to particulate matter and ozone on arterial stiffness and heart rate variability in healthy adults. *American journal of epidemiology*, 171(12), pp.1299–309.

Yang, I.A. et al., 2009. Genetic susceptibility to the respiratory effects of air pollution. *Postgraduate medical journal*, 85(1006), pp.428–36.

Zareba, W. et al., 2009. ECG parameters and exposure to carbon ultrafine particles in young healthy subjects. *Inhalation toxicology*, 21(3), pp.223–233.

Zholos, A. V, 2015. TRP Channels in Respiratory Pathophysiology: the Role of Oxidative, Chemical Irritant and Temperature Stimuli. *Current neuropharmacology*, 13(2), pp.279–91.