Cardiovascular Effects Evoked by Airway Nociceptive Reflexes in Healthy and Cardiovascular Diseased Rats

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Cardiovascular Effects Evoked by Airway Nociceptive Reflexes in Healthy and Cardiovascular Diseased Rats

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences Department of Molecular Pharmacology and Physiology College of Medicine University of South Florida

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ABSTRACT

Acute inhalation of airborne pollutants alters cardiovascular function and has been shown to have its greatest affects on individuals with pre-existing cardiovascular disease. Evidence suggests that pollutant-induced activation of airway sensory nerves via the gating of ion channels is critical to these systemic responses. Here, we have investigated the cardiovascular responses evoked by inhalation of AITC (TRPA1 agonist) and capsaicin (TRPV1 agonist) in healthy Sprague Dawley (SD) and Wistar Kyoto (WKY) rats, and cardiovascular diseased Spontaneously Hypertensive (SH) rats. Inhalation of the agonists by healthy SD and WKY rats caused significant bradycardia, atrio-ventricular (AV) block and prolonged PR-Intervals. Inhalation of TRP agonists caused differential cardiovascular responses in the cardiovascular diseased SH rats, such that the TRP agonists evoked brady-tachy with AV block and premature ventricular contractions (PVCs). Bradycardic responses to AITC were inhibited by the TRP channel blocker ruthenium red and the muscarinic antagonist atropine, but atropine did not prevent the tachycardic responses seen in the SH rats. Adrenergic inhibition with atenolol prevented the tachycardic responses, but did not prevent the bradycardic responses evoked by AITC in the SH rats. In healthy rats, AITC inhalation also caused a biphasic blood pressure response: a brief hypertensive phase followed by a hypotensive phase, while evoking hypertension in the SH rats. Atropine accentuated the hypertensive phase in all animals, while preventing the hypotension in the healthy animals. In all animals, AITC-evoked heart rate responses were not abolished by
terazosin, the $\alpha_1$ adrenoceptor inhibitor, which prevented the hypertensive responses. Anesthetics had profound effects on AITC-evoked bradycardia and AV block, which was abolished by urethane, ketamine and isoflurane. Nevertheless, AITC inhalation caused bradycardia and AV block in paralyzed and ventilated rats following pre-collicular decerebration. In conclusion, we provide evidence that activation of TRP channels expressed on nociceptive airway sensory nerves causes significant cardiovascular effects in healthy rats via reflex modulation of the autonomic nervous system (ANS), and that these effects are exacerbated in cardiovascular diseased rats.
CHAPTER 1
Introduction

Significance

Epidemiological and clinical evidence indicates that short-term elevations in daily particulate matter (PM) levels causes a greater absolute risk for cardiovascular disease-related mortality than for all other causes (Pope, 2000). These elevations are associated with the early mortality of tens of thousands of individuals per year in the United States alone (Brook et al., 2010). PM is an established trigger of cardiovascular events and has been shown to have its greatest effects on individuals with pre-existing cardiovascular conditions such as hypertension, heart failure and myocardial ischemia (Pope & Dockery, 2006; Simkhovich et al., 2008; Brook et al., 2010). The mechanisms through which inhalation of PM leads to increases in cardiovascular morbidity and mortality are separated into two main pathways: chronic systemic inflammation (humeral response to PM uptake) and aberrant autonomic nervous system balance resulting from activation of airway sensory nerve reflexes. Cardiovascular mortality is linearly associated with day-to-day changes in PM levels best explained through acute pathophysiological responses occurring minutes to hours after PM inhalation. Such effects are likely mediated by the ANS. Of our particular interest, is the pathway that involves ANS imbalance through activation of pulmonary afferent fibers as evidenced by
alterations in control of mean heart rate (HR (Elder et al., 2007)), heart rate variability (HRV (Pope et al., 1999)), and overall heart rhythm (Brook et al., 2010). The manner in which these alterations occur is not clearly known, as studies have had confounding results in regards to increases and decreases of the cardiovascular parameters in healthy vs. diseased individuals, suggesting a gap in our current understanding. Unfortunately, no approach to date has been effective in elucidating the mechanism of action by which PM inhalation leads to ANS imbalance resulting in arrhythmias and exacerbation of existing cardiovascular disease. In particular, numerous previous studies (Brook et al., 2010) have used PM and a multitude of other pollutants to evoke the desired responses in various models. However, due to the many actions (inflammatory and direct binding) of these particles on various systems, the specific role of the pulmonary cardiac reflex has not been identified.

**Background**

Studies have shown that TRPA1 channels expressed on pulmonary afferent C-fibers known as “nociceptors” are activated by various forms of PM including coal fly ash (CFA), diesel exhaust (DE), wood smoke and residual oil fly ash (ROFA) (Bautista et al., 2006; Deering-Rice et al., 2011; Fariss et al., 2013). Additionally, PM-induced electrocardiogram (ECG) changes were found to be prevented by TRPA1 inhibition in rats (Ghelfi et al., 2008), suggesting that arrhythmia evoked by PM inhalation occur through TRP channel-mediated autonomic reflexes in the lung. In healthy animals, the only nerve fibers innervating the airways found to express TRPA1 channels are C-fibers. Evidence for this has been shown by previous extracellular single nerve fiber recordings studies performed in our lab. We found that the majority of slowly-conducting C-fibers
innervating the rat lung were stimulated by the selective TRPA1 agonist AITC, while mechanically-sensitive A-fibers did not respond to AITC (Hooper et al., 2015). Activation of airway C-fibers, along with rapidly adapting receptor (RAR) A-fibers, initiate parasympathetic reflexes (Coleridge & Coleridge, 1984; Lee & Pisarri, 2001). In addition to RAR A-fibers, the airways are also innervated by slowly adapting receptor (SAR) A-fibers whose activation initiates sympathetic reflexes (Coleridge et al., 1989; Schelegle, 2003). Because TRPA1 channels are exclusively expressed on C-fibers in the airways, the use of a selective TRPA1 agonist allyl isothiocyanate (AITC) as opposed to a PM variant allows for the specific activation of PM neural pathways/reflexes without the confounding effects of PMs other biological actions (Deering-Rice et al., 2011). Using this approach allows for the determination of the mechanisms through which selective activation of the PM/TRPA1 neural pathways evokes differential arrhythmic profiles in cardiovascular disease models (compared to healthy animals). Elucidation of this critical pathway will yield therapeutic targets needed for preventing these effects and as such, greatly diminish the enormous public health burden derived from the ubiquitous risk associated with PM by decreasing morbidity, mortality, and hospitalization.

**Afferent Airway Innervation**

The airways are abundantly innervated by a heterogeneous population of sensory nerves. This innervation plays a critical role in host defense and in the maintenance of physiological homeostasis. Determination as to which nerves primarily contribute to host defense or homeostasis is dependent upon the nerve subtype. In general, the role of defense is played by the chemically sensitive nodose and jugular nociceptive C-fibers, which respond to harmful stimuli, with contributions also coming
from tracheal Aδ fibers that evoke cough in response to punctate mechanical stimuli and acid (Ricco et al., 1996; Kollarik & Undem, 2002; Canning et al., 2004). There is also evidence for the involvement of RARs in defense as bronchoconstriction evoked by histamine has been shown to activate these fibers, however, it is not known if histamine directly activates RARs leading to bronchoconstriction, or if RAR activation occurs secondary to the bronchoconstriction (Widdicombe, 1954; Barnes et al., 1984; Sano et al., 1992; Widdicombe, 2003). The homeostatic roles are primarily played by the SAR and RAR fibers involved in eupneic breathing. Classification of the various afferent nerves innervating the airways is currently done by identifying phenotypic differences in fiber conduction velocity, neuronal size, ganglion origin of cell body, nature of the stimulus to which they respond, rates of adaptation to maintained suprathreshold stimuli and their neurochemistry (Carr & Undem, 2003a). Due to our current inability to classify the airway afferents by a single phenotypic trait, it is best to broadly functionally classify these nerves as either primarily mechanically sensitive (low threshold mechanosensors) or primarily chemically sensitive (chemosensors/nociceptors). The low threshold mechanoreceptors respond to mechanical stimuli such as lung inflation and bronchospasm, with minimal responsiveness to chemical stimuli (Canning et al., 2001; Widdicombe, 2001). In contrast to the mechanosensors, nociceptors are activated directly by a wide range of chemicals such as capsaicin, bradykinin, adenosine and AITC, but are minimally activated during eupneic breathing (Jammes et al., 1982; Ho et al., 2001; Lee & Pisarri, 2001). Regardless of the fiber type, the majority of afferent nerves innervating the airways originate in the vagal sensory ganglia (nodose or jugular)
(Ricco et al., 1996; Mazzone & Canning, 2002), with some innervation in the dorsal root ganglia (DRG) (Oh et al., 2006) (Fig. 1.1) (Grace et al., 2013).

The low threshold mechanosensors are split into two classic types known as the RARs and SARs. In general, the phenotypic profiles of RARs and SARs are nearly indistinguishable as they are both myelinated A-fibers with conduction velocities in the Aβ range (10-20 m/s), originate in the nodose ganglia, terminate in the intrapulmonary airways and lung parenchyma, and respond to mechanical stimuli (i.e., changes in lung volume and airway smooth muscle contraction) (Widdicombe, 2003; Yu et al., 2003; Canning et al., 2004). However, RAR and SAR fibers exhibit differences in their projections into the nucleus tractus solitarius (NTS) (Davies & Kubin, 1986; Davies et al., 1987), mechanical activation profiles, adaptation properties and reflexes evoked by their activation. RARs can be activated by either inflation or deflation of the lungs and rapidly adapt (reduce action potential firing) to sustained lung inflation (Ho et al., 2001; Schelegle & Green, 2001; Widdicombe, 2003). Their
activation leads to increases in inspiratory effort, rate of respiration and parasympathetic output (Coleridge et al., 1989; Schelegle, 2003). In contrast, SARs only respond to inflation of the lungs and adapt slowly to sustained lung inflation (Ho et al., 2001; Schelegle & Green, 2001; Widdicombe, 2003). Activation of SARs causes relaxation of airway smooth muscle and tachycardia by inhibiting inspiration and parasympathetic activity (Coleridge et al., 1989; Schelegle, 2003).

The nociceptors are unmyelinated airway afferent fibers with free nerve endings found throughout the airways and originate from both the nodose and jugular ganglia (Paintal, 1973; Widdicombe, 1982). These fibers, known as bronchopulmonary C-fibers, make up approximately 80% of the afferent fibers innervating the airways (Agostoni et al., 1957; Jammes et al., 1982) and are only minimally active in the normal airways. As their classification implies, bronchopulmonary C-fibers are activated by a variety of endogenous (circulating autacoids) and exogenous (inhaled irritants) chemicals that bind directly to channels expressed on their nerve terminals (Carr & Undem, 2001; Undem & Carr, 2001; Chuaychoo et al., 2005; Kwong & Lee, 2005). Activation of these fibers generates action potentials conducted at 0.3-2 m/sec through the vagus nerves to the NTS resulting in parasympathetically-derived apnea, bradycardia, hypotension and reflex bronchoconstriction (Coleridge & Coleridge, 1984; Green et al., 1984; Lee & Pisarri, 2001). They are classified as nociceptors due to their activation by noxious stimuli and consequent evocation of defensive reflexes employed to prevent airway epithelium and alveolar membrane damage. Activation of these nerves by noxious stimuli occurs through the activation of channels expressed on these nerves, such as TRPV1 and TRPA1 (Kollarik & Undem, 2004; Nassenstein et al., 2008).
In addition to the nodose and jugular bronchopulmonary C-fibers, the extrapulmonary airways are innervated by a third nociceptive fiber type known as the nodose Aδ-fibers. These fibers have myelinated afferents with conduction velocities of approximately 5 m/s, originate from the nodose ganglion and respond to both punctate mechanical stimuli and acid (Ricco et al., 1996; Kollarik & Undem, 2002). Signals initiated by these fibers are carried by the recurrent laryngeal nerve (RLN) branch of the vagus to the CNS resulting in evocation of the cough reflex (Canning et al., 2004; Mazzone et al., 2009). Unlike bronchopulmonary C-fibers, the Aδ-fibers do not express TRPV1 (Kollarik & Undem, 2002) and current evidence suggests that these fibers respond to acid through the activation of acid-sensing ion channels (ASICs), while responses to punctate stimuli are believed to occur through the activation of TRPV4, TRPA1 and Piezo channels expressed on these nerves (Carr et al., 2001; Kwan et al., 2006; Taylor-Clark & Undem, 2006; Coste et al., 2010; Dusenkova et al., 2014).

**TRP Channels**

Transient receptor potential (TRP) channels are identified by a conserved 23-25 amino acid ‘TRP domain’ from which 28 mammalian TRP subunits have been discovered. Categorizing the 28 subunits by their sequence homology separates the TRP channel family into six related protein subfamilies (TRPC, TRPV, TRPM, TRPA, TRPP and TRPML) (Clapham, 2003). As mentioned earlier, TRP ion channels can respond to an extensive range of endogenous and exogenous stimuli with their expression found throughout the body. The diversity in activation mechanisms and selectivities is unique and greater than any other group of ion channels (Venkatachalam & Montell, 2007). Their pervasive expression and broad activation profiles enables TRP
proteins to play critical roles in sensory physiology including vision, taste, olfaction, hearing, touch, and thermo- and osmosensation. The superfamily of TRP channels are comprised of cation-selective proteins that exhibit a preference for calcium ions. Each subunit is comprised of six transmembrane domains with the channel pore forming between domains 5 and 6, N-terminal ankyrin repeats and intracellular C and N termini (Caterina et al., 1997; Ramsey et al., 2006). Studies to date have provided evidence that functioning TRP channels are formed by four subunits assembled as homo- or hetero-tetramers (Latorre et al., 2009). Of the subfamilies of TRP channels, only TRPM8, TRPV1 and TRPA1 activation has been shown to directly activate bronchopulmonary C-fibers leading to defensive reflexes, with TRPV1 and TRPA1 being solely responsible for initiating the cough reflex (Coleridge & Coleridge, 1977; Kollarik et al., 2003; Nassenstein et al., 2008). Therefore, I have chosen to focus my investigation and further discussion on these channels.

**TRPV1**

Capsaicin and bradykinin have been shown to evoke cough for many years (Lalloo et al., 1995), but it wasn’t until 1997 that the transient receptor potential vanilloid-1 (TRPV1) channel was first cloned and characterized leading to its identification as a mediator in the cough reflex (Caterina et al., 1997). The expression of TRPV1 is a defining feature of bronchopulmonary nociceptors enabling them to respond to potentially harmful exogenous and endogenous disparate signals (Caterina et al., 1997; Jordt et al., 2000; Kollarik & Undem, 2004). Exogenous stimuli such as capsaicin (active ingredient in chili pepper), resiniferatoxin and PM, along with endogenous mediators including extracellular acid and anandamide bind directly to TRPV1 causing
activation (Caterina et al., 1997; Zygmunt et al., 1999; Jordt et al., 2000; Deering-Rice et al., 2012). Studies on the structure and function of TRPV1 have shown this binding to occur at the vanilloid binding domain located in transmembrane domains 3-4 (Jordt & Julius, 2002; Johnson et al., 2006). TRPV1 has also been shown to be indirectly activated by endogenous autacoids that bind G-protein coupled receptors (GPCR) initiating intracellular signaling cascades that lead to TRP activation. These indirect agonists include bradykinin (Chuang et al., 2001; Carr et al., 2003; Kollarik & Undem, 2004), prostaglandin E2 (PGE2) (Moriyama et al., 2005; Grace et al., 2012), nerve growth factor (Chuang et al., 2001) and histamine (Kajihara et al., 2010). Studies have provided evidence that some TRPV1 agonists not only activate the channel, but may also sensitize TRPV1 lowering the activation threshold for another stimulus (Grace et al., 2014). Low pH, for instance, can activate TRPV1, or lower the activation threshold sensitizing the channel for activation by a second stimulus (Caterina et al., 1997; Tominaga et al., 1998; Trevisani et al., 2002). Inactivation of TRPV1 has been shown to occur through its binding to PIP2 and is released by PLC-mediated PIP2 hydrolysis (Prescott & Julius, 2003). Nerve fibers innervating the respiratory tract that express TRPV1 are found in the nose, larynx, trachea, lung parenchyma, alveoli, smooth muscle and blood vessels (Watanabe et al., 2006). Interestingly, the expression of TRPV1 in the airways has been shown to be increased in rats after exposure to inhaled pollutants and in patients with emphysema compared to healthy individuals (Costa et al., 2010; Baxter M, 2012). Although the activation profile of TRPV1 is remarkably diverse, there is evidence that it is not the only TRP channel responsible for protecting the airways from inhaled irritants. For example, TRPV1-knock out mice showed normal respiratory
sensitivity to electrophilic agents such as acrolein and styrene by exhibiting significant respiratory depression upon their exposure (Symanowicz et al., 2004).

**TRPA1**

Working synergistically with TRPV1 in defending the airways from potentially harmful irritants is the TRP channel TRPA1 (Transient Receptor Potential Ankyrin 1). Like TRPV1, TRPA1 has a very diverse activation profile responding to mustard oil, garlic, cinnamon, wasabi (Bandell et al., 2004; Jordt et al., 2004; Macpherson et al., 2005), PM, vehicle exhaust, cigarette smoke (isothiocyanates and acrolein) (Bautista et al., 2006; Facchinetti et al., 2007; Andre et al., 2008; Brone et al., 2008), products of oxidation (4-oxynonenal and 4-hydroxynonenal) (Trevisani et al., 2007; Taylor-Clark et al., 2008) and volatile anesthetics (desflurane and isoflurane) (Mutoh et al., 2013; Kichko et al., 2015). TRPA1 also shows similarity to TRPV1 in that it is both activated and sensitized by the GPCR autacoids bradykinin (Bandell et al., 2004; Grace et al., 2012) and PGE$_2$ (Bang et al., 2007; Maher et al., 2009). Studies have found that TRPA1, in opposition to TRPV1, is activated by extreme cold temperatures (Story et al., 2003; Zurborg et al., 2007) however, this finding is currently a subject of debate (Zhou et al., 2011). Similarities between these two channels are additionally found in their expression profiles as TRPA1 has been shown to be co-expressed with TRPV1 in nociceptive vagal (Bautista et al., 2005; Peeters et al., 2006; Nassenstein et al., 2008) and trigeminal (Kobayashi et al., 2005) airway afferent neurons. The expression of TRPA1 is found only in nociceptive nerves (Story et al., 2003; Nassenstein et al., 2008), with some evidence of expression in non-neuronal tissues (Stokes et al., 2006). Living up to its role in protecting the airways, activation of TRPA1 evokes action potential
discharge from bronchopulmonary C-fibers and consequent defensive reflexes such as cough (Birrell et al., 2009). TRPA1s mechanism of activation by electrophiles is complex with current evidence suggesting a role for an essential cluster of cysteine and lysine residues in its cytosolic N-terminus. The residues C619, C639, C663 and K708 were found to be covalently modified by electrophilic agonists leading to gating of TRPA1 in a reactivity-based, reversible manner (Hinman et al., 2006; Macpherson et al., 2007). This is not, however, TRPA1s only mechanism of activation as mutation of these residues prevented responses to cysteine-reactive agonists, but did not prevent responses to receptor-mediated PLC activation (Hinman et al., 2006). The receptor-mediated PLC activation of TRPA1 is believed to involve a region of the protein containing 18 ankyrin repeat domains from which it is named. Within this region is a canonical calcium-binding EF-hand domain responsible for sensing the PLC-evoked rise in intracellular calcium levels leading to activation of the channel in a manner that is currently not well understood (Jordt et al., 2004; Hinman et al., 2006). Of particular interest to this project is TRPA1s ability to respond to harmful environmental irritants and industrial pollutants (Deering-Rice et al., 2011; Shapiro et al., 2013). These include PM, vehicle exhaust and cigarette smoke along with wood smoke (Shapiro et al., 2013), chlorine (Bessac et al., 2008) and formaldehyde (McNamara et al., 2007). Activation of TRPA1 by these exogenous irritants leads to asthma-like symptoms such as cough, wheezing, dyspnea and subsequent hypersensitivity to chemical and physical stimuli (Preti et al., 2012) that has been found to be exacerbated in disease states (Petrus et al., 2007). It is now apparent that protection of the airways involves significant contributions from TRPA1 and TRPV1 in order to initiate physiological nocifensive responses.
Pulmonary-Cardiac Reflex

Complex interactions between the respiratory and cardiovascular systems occur constitutively and are absolutely essential for survival in mammals (Paintal, 1953; Aviado & Schmidt, 1959; Coleridge & Kidd, 1963; Thoren et al., 1976). Working in concert, these two systems engage in reflex regulation of blood supply ensuring that appropriate amounts of oxygenated blood are being supplied to all tissues in the body at all times. To accomplish this, the body must maintain an appropriate balance between the parasympathetic and sympathetic nervous systems affecting the heart, lungs and vasculature. By utilizing sensory receptors capable of sensing changes in arterial pressure and levels of oxygen and carbon dioxide in the blood, the body is able to establish the necessary autonomic nervous system balance (Simon et al., 1977; Marshall, 1994). The information from the receptors is relayed via vagal or glossopharyngeal afferent nerves to the nucleus of the solitary tract (NTS) region of the CNS for processing and consequent efferent signal generation (Marshall, 1994). The receptors primarily involved in this reflex are mechanoreceptors (or baroreceptors) located in the heart and major blood vessels, and chemoreceptors located in the carotid bodies with additional contributions coming from nerves innervating the airways (Paintal, 1973; Marshall, 1994; Lee & Pisarri, 2001).

The role of sensing changes in blood pressure is played by the baroreceptors. Baroreceptors respond to changes in vessel wall distention increasing their firing rate to increases in vessel wall distention caused by an elevation in blood pressure or decreasing firing rate when a drop in blood pressure occurs (Fahim, 2003). Activation of baroreceptors by increased blood pressure leads to inhibition of sympathetic
preganglionic neurons in the spinal cord and simultaneous activation of parasympathetic preganglionic neurons in the nucleus retro-ambiguus and nucleus ambiguus. The resulting shift in balance between the two branches of the ANS reduces the stimulatory noradrenergic effects of postganglionic sympathetic innervation on the heart and vessels while increasing the inhibitory cholinergic effects of postganglionic parasympathetic innervation on the heart. The parasympathetic nerves on the heart release acetylcholine onto cardiac pacemaker cells in the sinoatrial (SA) node and cardiac muscle cells, thus decreasing firing rate of the SA node and slowing conduction through the ventricles. The end result is a decrease in heart rate and contractility along with vasodilation, which together decrease blood pressure. In contrast, a drop in blood pressure decreases baroreceptor-firing rate leading to an increase in sympathetic outflow and inhibition of parasympathetic outflow. Increases in norepinephrine released by sympathetic innervation combined with a decrease in acetylcholine released by parasympathetic innervation results in increased HR, increased cardiac contractility, peripheral vasoconstriction and a concomitant increase in blood pressure (Chapleau et al., 1989; Korner, 1989; Head, 1995; Fahim, 2003).

In order for the body to maintain the proper balance of oxygen and carbon dioxide in the blood, it requires input from peripheral chemoreceptors located in the aortic and carotid bodies (Marshall, 1994) and central chemoreceptors located in the NTS (Dean et al., 1989). Reflexes initiated by these receptors differ from baroreceptors in that they primarily work through respiratory regulation as opposed to cardiovascular regulation. A decrease in blood oxygen concentration, pH, or increase in carbon dioxide causes activation of the peripheral chemoreceptors (Schultz et al., 2007), while only
increases in cerebral spinal fluid (CSF) CO₂ concentrations activate central chemoreceptors due to the inability of H⁺ to diffuse across the blood-brain barrier (Dean et al., 1989). Information from these receptors is sent to the medullar respiratory center of the CNS where an efferent motor signal is generated and sent via the phrenic nerve to the diaphragm and intercostal muscles causing them to contract. Contraction of these muscles leads to inspiration, which in turn increases pH by increasing oxygen and decreasing carbon dioxide concentrations in the blood (Taylor et al., 1999). Although chemoreceptor activation exerts its actions mainly on the respiratory system, some cardiovascular responses are initiated by these receptors. The cardiovascular responses evoked by chemoreceptor activation are not as clear cut as those evoked by baroreceptor activation due to the levels of parasympathetic and sympathetic efferent actions being tissue-dependent. For example, activation of chemoreceptors by hypoxia increases sympathetic vasoconstrictor outflow to peripheral tissues and increases cardiac sympathetic activity causing increased heart rate and contractility (Marshall, 1994; Cao & Morrison, 2001). This occurs with a simultaneous decrease in sympathetic activity to brown adipose tissue and increase in parasympathetic influence on the heart and cerebral vessels (Madden & Morrison, 2005). Although these dual responses appear to counteract one another, they actually work in concert to ensure that the necessary levels of oxygen are reaching vital organs. The increased sympathetic peripheral vasoconstriction and reduced sympathetic activity to brown adipose tissue reduces oxygen consumption of these tissues by a reduction in blood flow and body temperature. Meanwhile, the increase in parasympathetic outflow to the heart and cerebral vessels limits sympathetic vasoconstrictor, chronotropic and inotropic
responses ensuring that the heart and brain are not deprived of oxygen (Marshall, 1994; Cao & Morrison, 2001; Madden & Morrison, 2005; Schultz et al., 2007).

Although cardiovascular regulation is achieved primarily by the aforementioned baroreceptors and chemoreceptors, of our particular interest are the contributions arising from receptors expressed on bronchopulmonary afferent nerves. These receptors include SAR and RAR expressed on A-fibers and nociceptors (TRPV1 and TRPA1) expressed on C-fibers. In this section, I will only be discussing the cardiovascular responses evoked by these receptors; for information on methods of activation and signal transduction see Afferent Airway Innervation. Activation of SARs causes inhibition of inspiration and parasympathetic activity resulting in tachycardia (Widdicombe, 1982; Coleridge et al., 1989). Conversely, RAR activation leads to an increase in inspiration and parasympathetic outflow with discrepancies as to whether the increase in parasympathetic activity is associated with bradycardia (Carr & Undem, 2003a; Widdicombe, 2003). The receptors expressed on bronchopulmonary C-fibers also play a role in cardiovascular regulation. Activation of these receptors can inhibit inspiration (jugular) and increase inspiration (nodose) with both leading to increased parasympathetic outflow, thus decreasing HR and BP. The relatively pronounced cardiovascular effects of C-fiber activation in conjunction with their bronchoconstrictive and hypersecretory effects, aid in the clearance of inhaled irritants as well as prevention of their entry into the systemic circulation (Lee et al., 1987; Lee & Pisarri, 2001; Carr & Undem, 2003a). In addition to the vagal airway afferents, spinal afferents from the DRG also innervate the airways and lungs with their activation leading to alterations in respiration via their regulation of airway sympathetic adrenergic nerve activity (Kostreva
et al., 1975; Kummer et al., 1992; Plato et al., 2006). Current evidence suggests that these fibers work both in concert, and independently from parasympathetic and vagal afferents to regulate airway smooth muscle tone (Oh et al., 2006).
CHAPTER 2

Methods

Introduction

Throughout the process of addressing the aims laid out for this project, the implementation and subsequent optimization of various surgical procedures as well as data acquisition and analyses was required. We have, therefore, chosen to preface the chapters reviewing our results with a chapter detailing the methods by which they were acquired. In addition to detailing the surgical techniques and nature of agonist exposures, how the data were acquired and analyzed is also included in this chapter. We have also included in this chapter what the presented data is a representation of and how the data were calculated. The animals used for completion of this project were 15-week-old, male Sprague-Dawley (SD), Spontaneously Hypertensive (SH) and Wistar-Kyoto (WKY) rats. All surgical procedures, experimental protocols and data acquisition were performed in the same manner regardless of strain. The purpose of this chapter is to detail the methods used, as well as any modifications made for their optimization. As such, information regarding strains used for a particular method is outside the scope of this chapter and will be addressed in their respective introductory sections. All experiments were approved by the University of South Florida Institutional Animal Care and Use Committee.
Surgical Procedures

Telemetry Implant

When we began this project, our primary goal was to see what cardiovascular effects, if any, the inhalation of TRP channel agonists would have in a conscious, freely-moving rat. A review of relevant literature led us to conclude that this could effectively be done by measuring the electrical activity of the heart during the controlled exposures. In order to do this, the rats had to be implanted with a radiotelemetric device capable of recording their electrocardiogram (ECG) for the duration of an experiment. On the day of implantation, the rats were brought into the surgery room and left in their cages placed on top of a heating pad for an acclimation period of at least two hours. Following the acclimation period, the rats were placed in a chamber for induction into anesthesia by 5% isoflurane. After confirming anesthesia by lack of response to inter-digital pinch, the rats were placed onto a nosecone supplying 3% isoflurane. The rats were given 3% isoflurane via a nosecone for the remainder of the procedure with the level of anesthesia being confirmed by lack of response to inter-digital pinch every 15 minutes. Using a set of electric hair trimmers, the ventral surface of the rats was shaved in order to more easily access the incision sites as well as minimize infection. The rats were then moved to a sterile field and placed in the supine position onto a temperature controlled heating pad. A rectal probe was then coated in lubricating jelly, inserted into the rectum and secured to the tail with tape enabling the heating pad to sense the animals core temperature and keep it maintained at 37°C. Next, the antiseptic chlorhexidine was applied to the shaved area and then removed using sterile gauze soaked in sterile saline. This was followed by application of the antiseptic iodine and removed by the
same process. A sterile drape was then placed over the rats and a hole was cut exposing only the shaved area of the rats. Prior to making any incisions, a dose of the analgesic carprofen (5 mg/kg) was given via an i.p. injection. A two-inch-long midline incision through the dermal layers was then made beginning at the distal end of the xiphoid process proceeding distally. An identical incision was then made through the abdominal wall allowing access to the peritoneal cavity. The radiotelemetric device (4ET, Data Sciences International) containing a battery, sensing module and four sets of biopotential leads (two sets were left coiled and the remaining two were uncoiled for later placement) was then placed into the lateral sides of the cavity to help prevent interfering with the intestines. Next, the abdominal wall was retracted using four retractors held in place by a halo. A right angle 18 gauge needle was then superficially passed through the inferior side of the diaphragm and the negative electromyography (EMG) lead was fed into the lumen of the needle. Implantation of the lead into the diaphragm was done by backing the needle out and then applying Vetbond tissue adhesive to the exposed end of the lead. The positive EMG lead was then implanted approximately 2 cm inferior and 1 cm lateral of the negative lead using the same technique. The tissue adhesive was given 3-5 minutes to set before removing the abdominal wall retractors. An inch long midline incision was next made through the dermal layers over the sternum. Blunt dissection of the fascia with scissors was performed from the incision site to the right pectoral muscle and to the lower left flank of the rats. A trocar was then run between the dermal and muscle layers from the superior incision to the inferior incision. The set of electrocardiograph (ECG) leads was then fed into the lumen of the trocar and the trocar was backed out leaving the ends of the leads
exposed at the superior incision site for implantation into the lead II position. A right angle 18 gauge needle was then passed through the right pectoral muscle and the negative lead was fed into the lumen of the needle. The needle was backed out and a screw cap was placed over the exposed end of the lead. The positive lead was implanted into the muscle layers of the lower left flank using the same technique. Closure of the abdominal wall was then performed using absorbable suture. A thin layer of the antibiotic nitrofurazone was then applied onto the muscle layers lying beneath the two dermal incisions followed by their closure with monofilament suture. At this point the isoflurane was reduced to 1%, the sterile drape was removed and a final cleaning of the incision sites was performed using sterile saline. The temperature probe was then removed and the nails of the rats were trimmed in order to prevent removal of the sutures. The rats were monitored for a period of two hours and then given an additional dose of carprofen (5 mg/kg, i.p.). The rats were given a final dose of carprofen (5 mg/kg, i.p.) 24 hours following surgery and then monitored on a daily basis by vivarium staff until they were needed for experimentation 7-10 days post-surgery.

As is to be expected, troubleshooting issues that arose over the progression of this project led to a few modifications of the telemetry implant procedure. After analyzing ECG traces from the first few animals, it became apparent that one set of leads in the lead II position wasn’t going to be sufficient for resolving all phases of the cardiac cycle. Movement of the animals during recordings was causing interference in the ECG signal leaving only the QRS complex and T wave discernable (Fig. 2.1, see page 21). This wouldn’t be an issue if we were only trying to determine effects on overall heart rate, however we were also interested in identifying any effects that could be occurring on the
electrical conduction of the heart. In order to identify effects as such, it is essential that all phases of the cardiac cycle are resolved. To address this, we implanted an additional set of ECG leads in the Lewis lead position. This requires placement of the negative lead into the muscle above the superior end of the sternum and the positive lead into the muscle above the inferior end of the sternum. Placement of an additional set of ECG leads in the Lewis lead position allowed for better resolution of the electrical conduction through the atria (P wave), thus enabling us to acquire data on all phases of the cardiac cycle, as shown in figure 2.2 (see page 22).

In addition to adding an extra set of ECG leads, modifications were also made to address issues occurring during the recovery period. The rats began to pull their sutures due to irritation caused by fluid build-up in the pockets formed under the dermal incision sites. This not only increased the probability of infection by creating an open wound, but also caused some of the leads to become displaced. The first change we made was to

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**Figure 2.1: Noisy ECG trace.** Example of an early ECG trace displaying indiscernible p waves.
close the dermal incisions with wound clips instead of monofilament suture making it much more difficult for the rats to open the wounds. Confident that our sterile technique was sound and that the rats would not be able to open their wounds, we no longer saw a need for the application of nitrofurazone. We found that leaving out the antibiotic application greatly minimized fluid build-up in the pockets, which led to a decrease in irritation levels without increasing the probability of infection. However, these changes were not enough to prevent the leads from occasionally becoming displaced. We believed that by running the leads subdermally we were allowing for too much range of motion of the leads as well as making it too easy for the rats to pull on them. To address this, we chose to replace the large 1-inch superior dermal incision with smaller 1-centimeter incisions made directly above where the ECG leads were to be implanted. After making each incision, a 1-centimeter blunt dissection of the fascia was performed under the dermis above the incision site. A trocar was then run between the muscle

Figure 2.2: Improved ECG trace. Example of an improved ECG trace displaying discernible p waves.
layers, as opposed to subdermally done previously, from the bottom of the incision sites through the abdominal wall to the peritoneal cavity. The respective lead was fed into the lumen of the trocar and then the trocar was backed out leaving the end of the lead at its implantation site. The end of the lead was capped and further secured by suturing it in place with a single monofilament suture. By implementing these changes, we were able to minimize time in surgery, recovery time, levels of discomfort and irritation, while also preventing the leads from becoming displaced. Therefore, we recommend that ECG telemetry implantation be done according to the protocol described.

**Blood Pressure Acquisition**

Invasive blood pressure recording was done by cannulation of the carotid artery. On the day of implantation, the rats were brought into the surgery room and left in their cages placed on top of a heating pad for an acclimation period of at least two hours. Following the acclimation period, the rats were placed in a chamber for induction into anesthesia by 5% isoflurane. After confirming anesthesia by lack of response to inter-digital pinch, the rats were placed onto a nosecone supplying 3% isoflurane. The rats were given 3% isoflurane via a nosecone for the remainder of the procedure with the level of anesthesia being confirmed by lack of response to inter-digital pinch every 15 minutes. Using a set of electric hair trimmers, the chest, neck and dorsal areas from the caudal end of the skull to the shoulder blades of the rats was shaved in order to more easily access the incision sites as well as minimize infection. The rats were then moved to a sterile field and placed in the prone position onto a temperature controlled heating pad. A rectal probe was then coated in lubricating jelly, inserted into the rectum and secured to the tail with tape enabling the heating pad to sense the animals core
temperature and keep it maintained at 37°C. Next, the antiseptic chlorhexidine was applied to the shaved area and then removed using sterile gauze soaked in sterile saline. This was followed by application of the antiseptic iodine and removed by the same process. These steps were then repeated on the shaved ventral areas. A sterile drape was then placed over the rats and a hole was cut exposing only the shaved area of the rats. Prior to making any incisions, a dose of the analgesic carprofen (5 mg/kg) was given via an i.p. injection. A 1-inch midline incision beginning at the caudal end of the skull was then made through the dermal layers. Subdermal blunt dissection of a 1-inch by 1-inch area to the right of the incision was then performed. Saline soaked gauze was then placed over the incision and the rats were rotated to the supine position. Next, a 2 cm midline incision was made in the dermal layers of the neck. An identical incision of the platysma muscles surrounding the trachea was then made and the muscles were retracted revealing the trachea, carotid arteries and vagus nerves. The right carotid artery was then separated from the adjacent connective tissue and cleaned with a blunt needle taking care to not damage the vagus nerve. The inferior (cardiac) end of the carotid artery was then clamped with a bulldog clamp and a braided suture was used to tie off the artery 0.5 inches superior to the clamp. A lateral incision across the top of the artery was then made at the midpoint of the segment between the clamp and suture. A custom made 18-inch sterile polyethylene catheter with a small bubble on one end (Fig. 2.3: Blood pressure cannula. Cannula implanted for BP measurements.)
2.3) was then pre-filled with heparinized normal saline (0.5 IU/ml) and used to cannulate the vessel by inserting the end containing the bubble into the vessel. A braided suture was then tied around the vessel just below and above the bubble without obstructing blood flow in the cannula. The free end of the catheter was sealed using a flame and the bulldog clamp was released slowly to ensure that there was no bleeding at the cannulation site. Blunt dissection under the dermis was then performed from the right side of the incision moving dorsally until the pocket formed earlier is breached. The free end of the catheter was then fed through the blunt dissected area into the dorsal pocket where the extra length was coiled and laid flat. At this point, a single absorbable suture was used to close the incision on the platysma muscles and the ventral incision of the dermal layers was closed using wound clips. The dorsal incision was next closed using wound clips leaving the free end of the catheter exteriorized and secured with a wound clip in a manner that did not occlude the catheter. To ensure that the vessel was properly cannulated, the catheter was pulled out approximately 2-3 inches and the sealed end was cut while being loosely held with rubber clamps. The catheter was clamped after movement of solution out of the catheter confirmed successful cannulation of the vessel. Using a syringe, approximately 1 ml of heparinized normal saline (0.5 IU/ml) was then injected into the catheter to prevent clotting of blood in the catheter. The catheter was then re-clamped and the end was sealed using a flame before pushing the extra length back into the pocket. The rats were then taken off anesthesia and monitored for two hours after which an additional dose of carprofen (5 mg/kg, i.p.) was given. The rats were given a recovery period of 48 hours before being exposed to the TRP agonists.
Bilateral Vagotomy

Satisfactory identification of cardiovascular responses evoked by TRP channel activation can be accomplished by recording HR and BP while selectively stimulating the TRP channels. However, in order to characterize these responses, determination of the pathways involved must also be performed. This is most commonly done through pharmacological or surgical intervention. A method typically used for surgical intervention in studies involving the activity of nerves is that of nerve sectioning. This involves severing of the nerves believed to be involved in the evoked reflexes of interest, thus resulting in inhibition of the reflexes. Based on a review of literature and some of our initial studies, we believed that the activation of TRP channels was resulting in an increase of parasympathetic outflow to the heart as a result of increased parasympathetic afferent activity from bronchopulmonary C-fiber activation. Parasympathetic innervation of the heart occurs through the right and left vagus nerves, which innervate the SA and AV nodes, respectively. To test whether the TRP agonist evoked cardiovascular responses occurred through activation of TRP channels expressed on airway nerves as opposed to TRP channels expressed on the heart, we chose to perform exposures after cervical bilateral vagotomy.

On the day of surgery, the rats were brought into the surgery room and left in their cages placed on top of a heating pad for an acclimation period of at least two hours. The rats were then given urethane (1.2 g/kg, i.p.) and onset of the anesthetic was confirmed through absence of the hind limb withdrawal reflex to inter-digital pinch. The entire ventral surface of the rats was then shaved with electric hair trimmers and the rats were placed on a heating pad. Because these were non-survival surgeries,
sterilization of the surfaces was not necessary. Two minor incisions (2 cm) were then made exposing the right pectoral and left oblique muscle layers. The negative and positive ECG leads were then fixed onto the pectoral and oblique muscle layers, respectively, by the application of Vetbond tissue adhesive. ECG acquisition was started and continued for the duration of the experiment. Next, a 2 cm midline incision was made in the dermal layers of the neck. An identical incision of the platysma muscles surrounding the trachea was then made and the muscles were retracted revealing the trachea, carotid arteries and vagus nerves. The right vagus nerve was then separated from the adjacent connective tissue and cleaned with a blunt needle before being cut with a pair of springbow scissors. A period of 5 minutes was then given prior to repeating the previous step on the left vagus nerve. The rats were then exposed to the TRP agonists and subsequently sacrificed by CO$_2$ asphyxiation.

*Pre-Collicular Decerebration*

As mentioned previously, the expression of TRP channels is not limited to nerves innervating the lower airways. Their expression is also found in trigeminal nerves innervating the upper airways. Therefore, the relative contributions of C-fibers innervating the upper and lower airways to the cardiovascular responses seen after inhalation of nebulized TRP agonists in freely-breathing rats could not be determined. To address this issue, we chose to expose the rats to nebulized TRP agonists by way of a tracheal cannula after pre-collicular decerebration. Additionally, by performing TRP agonist exposures on decerebrate rats, we were able to assess the TRP agonist-induced responses without the complicating factor of anesthesia. Although this
preparation has the added benefit of not requiring anesthesia, it is not identical to the normal condition due to the necessary removal of input from higher order brain regions.

On the day of surgery, the rats were brought into the surgery room and left in their cages placed on top of a heating pad for an acclimation period of at least two hours. Following the acclimation period, the rats were placed in a chamber for induction into anesthesia by 5% isoflurane. After confirming anesthesia by lack of response to inter-digital pinch, the rats were placed onto a nosecone supplying 3% isoflurane. The rats were given 3% isoflurane via a nosecone for the remainder of the procedure with the level of anesthesia being confirmed by lack of response to inter-digital pinch every 15 minutes. Using a set of electric hair trimmers, the ventral surface and dorsal area of the skull to the shoulders was shaved in order to more easily access the incision sites. The rats were then placed in the supine position onto a temperature controlled heating pad. A rectal probe was then coated in lubricating jelly, inserted into the rectum and secured to the tail with tape enabling the heating pad to sense the animals core temperature and keep it maintained at 37°C. As these were non-survival surgeries, sterile technique to prevent infection was not necessary. Two minor incisions (2 cm) were then made exposing the right pectoral and left oblique muscle layers. The negative and positive ECG leads were then fixed onto the pectoral and oblique muscle layers, respectively, by the application of Vetbond tissue adhesive. ECG acquisition was started and continued for the duration of the experiment. Next, a 2 cm midline incision was made in the dermal layers of the neck. An identical incision of the platysma muscles surrounding the trachea was then made and the muscles were retracted revealing the trachea, carotid arteries and vagus nerves. The carotid arteries and vagus nerves were
then carefully separated from the trachea using a blunt needle. The right carotid artery was then further separated from the right vagus nerve and a clamp was placed on the artery to minimize cerebral hemorrhage. A 3-inch length of braided suture was then fed around the trachea proximal to the right and left carotid arteries and vagi at a point between two of the cartilaginous rings. Leaving the suture untied, the wound was covered with saline soaked gauze and the rats were placed into a stereotaxic apparatus. A midline incision through the dermal layers over the dorsal surface of the skull was then made and the dermis was retracted. A cautery unit was then used to reflect any remaining tissue covering the dorsal and parietal surfaces of the skull. A bilateral craniotomy was then performed using a set of rongeurs to burr holes into the parietal skull. After the right and left parietal sections of the skull were removed, a suture was placed inferior to the central sagittal sinus and the portion of bone superior to the central sagittal sinus was removed. The dura mater was then breached and reflected. Next, the cerebral cortex was gently aspirated until the superior and inferior colliculi could be visualized. Using a blunt instrument, the brain was then sectioned directly rostral of the superior colliculus (and caudal of the hippocampus/thalamus) and the transected portions of the forebrain were aspirated. Having completed the decerebration, the rats were then taken off of isoflurane. Small pieces of oxidized regenerated cellulose were then placed on the exposed surfaces of the brain and mineral oil was used to fill the remaining cavity in order to prevent the tissue from desiccating. A piece of saline soaked gauze was then placed over the wound and the dermal layers were closed over the gauze using monofilament suture. The rats were removed from the stereotaxic apparatus and laid in the supine position.
tracheotomy incision was then made and an endotracheal cannula was inserted into the trachea. The braided suture placed earlier was then gently tied around the cannulated portion of the trachea without obstructing airflow through the cannula. The rats were rotated into the prone position and an initial bolus injection of the paralytic vecuronium (0.3 mg/kg, i.p.) was then given and maintained (0.15 mg/kg, i.p. every 30 min.) to prevent spontaneous skeletal muscle activity. The rats were then put on a dual mode ventilator (Kent Scientific) with respiration rate set to 70-80 bpm, inspiration % of 35 and the ETCO$_2$ was confirmed to be between 30-45 mmHg using a capnograph (MDS Matrix). The rats were then given a period of 1 hour before experimentation in order to allow for the effects of isoflurane anesthesia to be eliminated from the preparation.

**Nebulized Agonist Exposure Protocol**

In order to expose the rats (and only the rats) to the nebulized TRP agonists, we performed the exposures in a sealed plexiglass chamber (4.5 X 11 inches) complete with inflow and outflow hook-ups on opposite ends of the chamber. A Trek S (PARI Respiratory Equipment) nebulizer (4 L/min) was hooked onto the inflow introducing the agonists to the chamber in 1-5 µm particles. Tubing was run from the outflow of the chamber to a central vacuum line for passive removal of the particles from the chamber. The complete set-up is shown in figure 2.4 (see page 31). The rats were exposed (in sequential 10-minute intervals) to ambient air, nebulized vehicle (4% ethanol in PBS, 10% ethanol in 3 mM capsaicin studies), and either capsaicin, AITC, or ATP made up in 4% ethanol and PBS (10% ethanol in 3 mM capsaicin studies). Exposures for all studies were performed using this set-up apart from the decerebration studies. For the decerebration studies the sequence of exposures was identical to that described,
Figure 2.4: Exposure chamber diagram. Illustration of the set-up by which the rats were contained and exposed to nebulizer agonist during which ECG was recorded. Nebulized agonist or vehicle entered into the chamber via an inflow tube and was then passively removed through an exhaust tube attached to a central vacuum line preventing areas outside of the chamber from exposures.

however exposures could not be performed in the chamber due to the requirement of the rats to be mechanically ventilated. For these studies, the nebulized agonist was introduced into the “post-ventilator” inspiratory cannula using an Aeroneb Pro-X nebulizer (Fig. 2.5, see page 32). The Aeroneb nebulizer also produces particles in the 1-5 µm range, however its production is passive as opposed to the active pumping of the Trek S nebulizer. By using this nebulizer, we were able to expose the airways below the tracheal bifurcation without exposing the upper airways and without causing an increase in inspiration pressure. As such, we were able to determine the contributions of the airways below the trachea in agonist-induced responses, as well as any effect that changes in respiration rate could potentially have on the responses.
Data Analysis

ECG

Identification and characterization of the cardiovascular responses evoked by inhalation of TRP agonists required detailed analysis of acquired ECG traces. For acquisition of ECG data, the sensing module (implanted as described earlier) was first turned on by lightly placing a magnet over the abdominal area of the rats. A light on the DSI receiver (RPC-1) placed under the exposure chamber confirmed that the module was on and that a signal was being received. The receiver was connected to a computer running Ponemah software via an A/D converter. Data were recorded at 1000 Hz and the cardiac cycle, including P, QRS and T waves was resolved. Using the P3Plus analysis software, the relevant “windows” for each phase of the cardiac cycle (Fig. 2.6, see page 33) were adjusted so that demarcation of each phase was optimal. However, due to movement of the animals during experimentation, it was impossible for the software to correctly mark every cardiac cycle.

Figure 2.5: Decerebration exposure diagram. Diagram of the set-up used for exposing nebulized agonists to decerebrate rats. The circuit displayed was a closed circuit between the ventilator, nebulizer and distal airways of the rats.
Therefore, we went through all analyzed ECG data and ensured that every cycle was properly marked. In doing so, we were able to get relevant data on electrical parameters including RR-I and PR-I. Data presented throughout this project represents the first 6 minutes of each 10-minute exposure (ambient, vehicle, agonist). Bar graph data displays the populational average of all data points taken during each 6-minute period, while each point in the consecutive average figures represents a bin of 10 consecutive data points averaged across the group. We must also mention that the data analyzed during the first 6 minutes was not continuous, but rather a representation of resolvable data within the first 6 minutes of each exposure. This is a particularly important note in regards to the consecutive average figures, as the amount of resolvable data varied between exposures and individuals. Thus, because each data point is a bin of 10 consecutive data points averaged across the group, the amount of bins shown for each exposure was determined by the individual with the least amount of resolvable data for that exposure. As such, data shown in the consecutive average figures does not always appear to be in agreement with the bar graph figures showing the combined populational averages of all resolved data.
As mentioned earlier, two of the ECG parameters analyzed were the RR-I and PR-I. The RR-I is the time (ms) between two consecutive R waves as shown in figure 2.7. RR-I data gives you information on overall HR, thus enabling you to determine if any bradycardic or tachycardic effects have occurred.

The PR-I is the time (ms) from the beginning of the P wave to the beginning of the QRS complex within a single cardiac cycle as shown in figure 2.7. Because the P wave is a measurement of atrial conduction and the R wave is a measurement of ventricular conduction, the PR-I can be looked at as a measurement of the time it takes for the electrical signal generated at the SA node to reach the ventricles. For this reason, the PR-I is used for the classification of conduction arrhythmias such as atrioventricular (AV) block. Although analyzing PR-I data can aid in classifying arrhythmia types, verification that an arrhythmic event has occurred is best done by simply looking at the ECG trace. Cardiac arrhythmia display characteristic ECG deformations that are manifestations of underlying physiological issues. Analysis of ECG data throughout this project led to the identification of two such cardiac events: Second-degree AV block and premature ventricular contractions (PVC). Second-degree AV block occurs when electrical conduction through the AV node is partially blocked resulting in prolongation of the PR-I eventually leading to a “dropped” or “skipped” beat wherein a P wave is conducted without being followed by a QRS complex. For the
purpose of this project, an AV block event was counted when a dropped beat occurred as shown in figure 2.8. In some animals, the p wave was indiscernible during agonist exposures. For these animals, a dropped beat was taken as an RR-I > 2 X the vehicle RR-I average of that individual. A PVC occurs when an ectopic beat arises from the ventricles without having first received a signal from the atria. Although there is no standard method for identifying a PVC, the general presentation is a premature and bizarrely shaped QRS complex that is not preceded by a P wave with a large T wave projecting in the opposite direction of the QRS deflection as shown in figure 2.8. The AV block and PVC data shown represents the combined average number of events counted during the initial 6 minutes of analyzed data.

Apart from simply recording and averaging the raw RR-I and PR-I data, we wanted to put the data into a more clinically relevant context. Therefore, we performed additional RR-I analysis similar to that done in the clinic by generating a type of scatter plot known as a Poincaré plot. A Poincaré plot is traditionally used as a way to investigate heart rate variability along with heart functionality by enabling one to
visualize oscillations in the periodicity of the heart rhythm. To create a Poincaré plot, we simply took a sequence of RR-Intervals and plotted each RR-I (x-axis) against the succeeding RR-I (y-axis). Implementation of these ECG analyses was essential for interpretation of the ECG data acquired.

**Blood Pressure**

The blood pressure data were acquired using the PowerLab 4/35 with a Quad Bridge Amp (AD Instruments) and analysis was performed using the LabChart 7 (AD Instruments) analysis software. Prior to acquisition, the wound clip securing the catheter (implanted as described previously) was removed and the length of coiled catheter was pulled out of the pocket until no slack remained. The free end of the catheter was cut and flow was permitted until a drop of blood exited the catheter at which point the catheter was clamped. A syringe containing sterile physiologic saline was then inserted into the catheter, the clamp was removed and the blood remaining in the catheter was returned by administration of saline from the syringe. The catheter was then re-clamped and the free end was sealed by quickly passing it over a flame. The clamp was then released and the catheter was fed through a thin plastic tube to prevent access from the rats. The free end of the catheter was then fed from the inside of the chamber into a small opening below the inflow while simultaneously placing the rats into the chamber. The free end of the catheter was then pulled from the outside of the chamber leaving enough length inside of the chamber as to not restrict the movement of the rats. The catheter was then lightly taped onto the outside of the chamber taking care to not occlude the catheter. The catheter was clamped about an inch from the free end and then the end was cut. Next, a syringe loaded with sterile physiologic saline and hooked
to a pressure transducer was inserted into the catheter and the system was primed with approximately 100 µL by depressing the syringe before beginning acquisition. Instantaneous BP data was acquired throughout the remainder of the experiments.

The acquired instantaneous BP data were then used to attain information regarding effects of TRP agonist inhalation on mean arterial blood pressure (MABP) and pulse interval (PI). Using LabChart 7, two additional channels (2 and 3) were opened apart from the instantaneous BP (channel 1). Channel 2 was used for acquisition of MABP by setting it to derive the mean from channel 1 with smoothing and minimum peak height set to 0.05. Channel 3 was then set to display and acquire PI by setting it to derive the “period” measurement from channel 1 also with smoothing and minimum peak height set to 0.05. Proper set-up of these channels resulted in traces displayed as shown in figure 2.9.

MABP and PI data was then taken from channels 2 and 3 for a period of 6 min during each exposure at a sampling rate of 100 ms for further analysis. Responses to inhalation of nociceptive stimuli were subdivided into

![Image showing proper LabChart channel set-up. The first channel displays instantaneous BP, the second channel shows MABP and the third channel shows PI.](image-url)
“early phase” (30s period beginning 10s into the exposure) and “late phase” (30s period beginning 2 min and 10s into the exposure).

Respiration Rate

As described previously, one of the major effects caused by activation of TRP channels expressed in the airways is apnea followed by tachypnea. Because rate of respiration can have profound effects on HR, we wanted to investigate whether HR responses to TRP agonist inhalation were secondary to effects on rate of respiration. In order to do this, video was recorded of the rats during the exposures discussed previously and synced with the ECG acquisition. The video was then reviewed and every breath was counted during the periods of analyzed ECG acquisition for representation in breaths per minute (BPM).

Statistics

Data were analyzed using GraphPad software. Where appropriate, paired or unpaired Student’s t-tests were used. A $P$ value less than 0.05 was taken as significant. A $P$ value greater than 0.05 was considered not significant (n.s.). All data were expressed as mean ± S.E.M. unless otherwise noted.

Chemicals

AITC, ATP disodium salt hydrate, atropine (free base), atenolol, prazosin hydrochloride, capsaicin and ruthenium red were purchased from Sigma. $\alpha,\beta$ methylene ATP trisodium salt and terazosin hydrochloride were purchased from Tocris.
CHAPTER 3
Identification of Arrhythmias Evoked by Inhalation of Selective TRP Channel Agonists in Healthy Sprague Dawley Rats

Introduction

Inhalation of noxious irritants and pollutants have been shown to have profound acute cardiovascular effects (Mills et al., 2009; Brook et al., 2010). Common air pollutants derived from industrial processes, combustion engines and tobacco smoking include PM, ozone, isocyanates, aldehydes and acrolein. In particular, it has become increasingly evident that a rise in PM levels positively correlates with rises in cardiovascular morbidity and mortality (Brook et al., 2010). The deleterious effects of pollutant inhalation have been shown to occur in both chronic and acute exposures. It is believed that effects caused by chronic exposures may occur through inflammatory pathways, while the effects seen after acute exposures are less well understood. This gap in current knowledge is, in part, due to the many actions PM has on the body making elucidation of specific pathways difficult. However, recent studies have identified the importance of two sensory nerve ion channels, TRPA1 and TRPV1, in the cardiovascular effects of PM (Agopyan et al., 2004; Deering-Rice et al., 2011; Hazari et al., 2011; Deering-Rice et al., 2012). The high expression levels of these channels on
nerves innervating the airways suggests that cardiovascular reflexes evoked by acute inhalation of PM are initiated from the airways.

As mentioned previously, 80% of the vagal sensory (afferent) nerves innervating the airways are unmyelinated nociceptive C-fibers that respond to noxious stimuli (Coleridge et al., 1965; Paintal, 1969; Ho et al., 2001; Undem et al., 2004). The ability of these nerves to respond to a plethora of noxious stimuli is due to their expression of the polymodal TRPV1 and TRPA1 ion channels (Nassenstein et al., 2008). Activation of these channels by noxious stimuli (e.g. capsaicin, heat, pH, cinnamaldehyde, AITC, and acrolein) (Undem et al., 2004; Ruan et al., 2005; Nassenstein et al., 2008; Nassenstein et al., 2010; Zhou et al., 2011; Lin et al., 2015) initiates defensive mechanisms such as cough, hypersecretion and bronchospasm (Carr & Undem, 2003b; Taylor-Clark, 2015). Their polymodal irritant activation and resulting initiation of protective reflexes led to the belief that they could potentially have a role in acute responses to PM. Indeed, acute treatment of PM was shown to activate nociceptive sensory nerves in a manner that was substantially reduced by knockout or inhibition of TRPA1 and TRPV1 (Agopyan et al., 2003a; Agopyan et al., 2003b; Agopyan et al., 2004; Deering-Rice et al., 2011; Deering-Rice et al., 2012). Furthermore, PM inhalation has been shown to evoke bradycardia and increased heart rate variability (HRV) in healthy animals (Watkinson et al., 1998; Ghelfi et al., 2008), suggesting that PM evoked reflex-mediated increases in parasympathetic activity similar to those seen upon activation of airway C-fibers (Davis et al., 1982; Kaufman et al., 1982; Palecek et al., 1989). In fact, the PM-induced ECG changes were shown to be inhibited by TRPV1 (Ghelfi et al., 2008) and TRPA1 (Hazari et al., 2011) inhibition, suggesting that these autonomic reflexes occur through
activation of TRP channel-expressing afferents in the lung. Nevertheless, the multiple cellular effects of PM and other pollutants (Lippmann & Chen, 2009) impair our ability to directly correlate pulmonary sensory nerve activation through TRP channel gating with modulation of cardiac function. In this chapter, we provide evidence of the powerful effect airway nerve TRP channel activation has on cardiovascular function by recording ECG changes in healthy SD rats during exposures to selective TRP channel agonists.

**Results**

Given that pulmonary C-fiber activation by capsaicin has been shown to cause bradycardia (Kaufman et al., 1982; Palecek et al., 1989), we chose to perform an initial pilot study aiming to replicate these results by recording ECG from conscious SD rats during exposure to nebulized capsaicin (3 mM). We hypothesized that activation of vagal nociceptors through inhalation of the specific TRPV1 agonist would evoke bradycardia similar to that shown in previous studies and therefore, establish the ability of our experimental design to accurately measure ECG responses to TRP agonist inhalation in conscious rats. As expected, exposure to capsaicin (3 mM) caused substantial bradycardia with significant increases in RR-I (234 ± 25 ms compared to vehicle, 169 ± 12, p<0.05) (Fig. 3.1, see page 42). Furthermore, by plotting the relationships of consecutive RR-Is (Fig. 3.2, see page 43), it was clear that the bradycardia was unstable, with slow beats following faster beats in no particular rhythm. The lack of consistency or rhythmicity seen in the RR-I responses precluded analysis by conventional heart rate variability (HRV) methods (Rowan et al., 2007). In addition to the lack of consistency, there were two other limitations of these studies in regards to the feasibility of HRV analysis to be performed on the acquired data. The first limitation
is that the 6-minute period is not long enough for HRV analysis, which typically requires 24 hours of recorded data to be analyzed in order for conclusions from the analysis to be of any value (Malliani et al., 1994). The second limitation is that HRV analysis can only be performed on consecutive normal beats (NN), and there were virtually no NN
beats during exposure to the agonists. The severity of bradycardia seen upon exposure to capsaicin was found to be capable of evoking second-degree AV block going from no such events during vehicle exposures, to $25 \pm 10$ events during capsaicin exposures. In agreement with second-degree AV block identification, we found that PR-I increased upon exposure to capsaicin ($57 \pm 1$ ms compared to vehicle, $46 \pm 0.4$ ms, p<0.05) (Fig. 3.3, see page 44). While exposing the rats to reduced capsaicin concentrations of 300 µM (n=22) and 600 µM (n=7) evoked minimal RR-I prolongation ($164 \pm 2$ ms compared to vehicle, $162 \pm 3$, n.s.; $156 \pm 6$ ms compared to vehicle, $150 \pm 3$, n.s., respectively) with no AV block or PR-I prolongation (Fig. 3.4, see page 45).

As mentioned earlier, previous studies have suggested that, in addition to TRPV1 activation, PM most likely exhibits its effects through the activation of TRPA1. Therefore, we believed that in order to further understand responses evoked by PM inhalation, we needed to also investigate ECG responses to selective activation of TRPA1. The co-expression of TRPA1 with TRPV1 on pulmonary C-fibers led us to hypothesize that activation of vagal nociceptors through inhalation of the specific TRPA1 agonist AITC would evoke responses similar to those seen in our capsaicin studies. We found that exposure of conscious rats to AITC (30 mM) evoked pronounced bradycardia with
significant increases in RR-I (270 ± 15 ms compared to vehicle, 153 ± 3, p<0.05) (Fig. 3.5, see page 46). As with responses to capsaicin, plotting the relationships of consecutive RR-Is (Fig. 3.6, see page 47) revealed that the evoked bradycardia was also unstable. Additionally, the occurrence of AV block was found to be highest during exposure to 30 mM AITC than all other agonist exposures with an average of 112 ± 37 events, and this was associated with a significant increase in PR-I (93 ± 13 ms compared to vehicle, 44 ± 1, p<0.05) (Fig. 3.7, see page 48). Exposure to the TRP agonists also caused a decrease in p wave amplitude as shown in figure 3.8 (see page 48). Reducing the exposed concentration of AITC to 10 mM (n=3) failed to evoke AV block and PR-I prolongation, but was able to evoke a significant increase in RR-I (219 ± 30 ms compared to vehicle, 167 ± 2, p<0.05) (Fig. 3.9, see page 49).

Figure 3.3: Capsaicin evokes AV block in healthy SD rats. Left: Mean ± SEM AV block count. Right: Mean ± SEM PR-I. *denotes significant difference to vehicle response (p<0.05).
In addition to TRPV1 and TRPA1, the expression of the purinergic P2X$_{2/3}$ receptor is also found on C-fibers innervating the airways (Kwong et al., 2008). However, the expression of P2X$_{2/3}$ differs in that it is found only in nodose C-fibers with no expression seen in jugular C-fibers (Kwong et al., 2008). Therefore, by selectively activating P2X$_{2/3}$ with ATP (Burnstock, 2000; Undem & Carr, 2001), we could potentially determine whether the responses seen after TRP channel activation were a result of nodose or jugular airway C-fiber activation. Based on previous studies showing that selective activation of P2X$_{2/3}$ by ATP evokes bradycardia (Burnstock, 2000), we hypothesized that activation of vagal nociceptors through inhalation of ATP would evoke bradycardic responses like those seen in the capsaicin studies. We found that by exposing the rats to 30 mM ATP we were able to evoke a significant increase in RR-I (165 ± 3 ms compared to vehicle, 150 ± 4, p<0.05) (Fig. 3.10, see page 50). Although the RR-I increase was found to be significant, the response was minimal compared to the responses evoked by capsaicin and AITC. Therefore, it was not surprising that the

![Graph showing RR-I response to capsaicin](image)
bradycardia evoked by 30 mM ATP was associated with only a minimal increase in AV block events (1 ± 1) and PR-I (49 ± 0.3 compared to vehicle, 48 ± 1, n.s.). Minimal responses were also seen after exposing the rats to a concentration of 3 mM αβ-methylene ATP (n=2), which led to a slight increase in RR-I (160 ms compared to vehicle, 153, n.s.) with no incidence of AV block or PR-I prolongation. Similar responses

**Figure 3.5: AITC evokes bradycardia in healthy SD rats.** (A) Representative ECG recorded during ambient (Amb), vehicle (Veh) and 30mM AITC exposures. (B) Populational consecutive bin averages of RR-I during ambient, vehicle and 30 mM AITC exposures. (C) Histogram of the % of RR-I occurring during vehicle (black line) and 30 mM AITC (red line) exposures. (D) Mean ± SEM RR-I. *denotes significant difference to vehicle response (p<0.05), n=11.
were seen after exposing the rats to an identical 3 mM concentration of ATP (n=3) wherein mild increases of RR-I ($152 \pm 2.5$ ms compared to vehicle, $147 \pm 2.6$, n.s.) occurred without AV block or PR-I prolongation (Fig. 3.11, see page 51).

**Discussion**

We found that exposing conscious SD rats to nebulized capsaicin and AITC evoked immediate and substantial bradycardia accompanied by significant increases in PR-I and dropped QRS waves indicative of AV block. These responses have been shown to occur after direct stimulation of cardiac parasympathetic efferents (Levy & Zieske, 1969; Rinkema *et al.*, 1982) suggesting that activation of TRPV1 and TRPA1 channels expressed on airway nerves by these specific agonists leads to increased parasympathetic drive to the heart. The 3 mM concentration of capsaicin was found to be necessary for evoking substantial bradycardia and AV block as exposure to lower concentrations evoked minimal RR-I prolongation with no AV block or PR-I prolongation. Similarly, a reduction in the exposed concentration of AITC failed to evoke AV block and PR-I prolongation. The 3 mM capsaicin and 30 mM AITC nebulized concentrations are higher than doses typically used as 50 µM capsaicin and 10 mM AITC nebulized concentrations.
concentrations have been shown to be efficient for evoking cough in awake animals (Brozmanova et al., 2012). However, we found that with our set-up, the higher concentrations were necessary for evoking maximal responses. This is likely due to the size of our chamber being greater than those used in previous nebulized exposures, thus, the increase in chamber volume led to increased dilution of the agonist. It is interesting that the AITC concentration required to evoke a maximal response was only 10-fold greater than that of capsaicin, as capsaicin has

Figure 3.7: AITC evokes AV block. Left: Mean ± SEM AV block count. Right: Mean ± SEM PR-I. *denotes significant difference to vehicle response (p<0.05).

Figure 3.8: P wave amplitude was reduced during AITC exposure. Black arrows indicate p waves conducted during vehicle exposure and the red arrows indicate p waves conducted during AITC exposure. Dotted line denotes a time break.
been shown to be 1000 times more potent than AITC (Yu et al., 2013). The inability of these lower concentrations to evoke AV block was most likely a factor of a reduction in the concentration of the agonists reaching the airways rather than a reflection of the ability of the exposed concentration to initiate pulmonary-cardiac reflexes through activation of bronchopulmonary C-fibers. The saturating concentration of AITC has been shown to be 1 mM in single channel TRPA1 studies (Raisinghani et al., 2011), however, determining the concentration of agonist inhaled into the airways was not possible with our experimental design of exposing nebulized agonists to unrestrained, conscious, freely-breathing rats.

We were unable to determine if the level of contribution varied between activation of nodose or jugular C-fibers in the evoked pulmonary-cardiac reflexes as minimal responses were seen after exposure to 30 mM ATP. The limited responsiveness to ATP could be a consequence of a few factors. First, selective activation of nodose C-fibers might not be sufficient for evoking substantial bradycardia on its own, and that such a response also requires activation of jugular C-fibers. However, as no studies to date
have investigated cardiac responses to selective activation of nodose C-fibers, we are unable to confirm this possibility. Second, it is possible that our method of exposure was allowing for the reduction of ATP to ADP prior to inhalation. This would explain the lack of responsiveness as ADP has been shown to have no agonistic or antagonistic effects.

**Figure 3.10: ATP evokes mild bradycardia in healthy SD rats.** (A) Representative ECG recorded during ambient (Amb), vehicle (Veh) and 30mM ATP exposures. (B) Populational consecutive bin averages of RR-I during ambient, vehicle and 30mM ATP exposures. (C) Histogram of the % of RR-I occurring during vehicle (black line) and 30mM ATP (red line) exposures. (D) Mean ± SEM RR-I. *denotes significant difference to vehicle response (p<0.05), n=4.
on the P2X receptor (Spelta et al., 2003), however, we find this unlikely due to the fact that ADP is rapidly reduced to AMP which can activate these channels. A third possibility is that the minimal effects were a result of ATPs limited selectivity to the P2X$_{2/3}$ receptor as it has been shown to activate all P2X receptor subunits (Spelta et al., 2003). We attempted to address this issue by performing exposures with the ATP analogue αβ-methylene ATP, which has been shown to be a highly selective agonist of the P2X$_{2/3}$ receptor (Spelta et al., 2003). Exposing the rats to a concentration of 3 mM αβ-methylene ATP led to a slight increase in RR-I with no incidence of AV block or PR-I prolongation. Similar responses were seen after exposing the rats to an identical 3 mM concentration of ATP wherein mild increases of RR-I occurred without AV block or PR-I prolongation. The similarity in the responses between identical concentrations of αβ-methylene ATP and ATP, along with the selectivity and stability of αβ-methylene ATP, argue against ATPs evocation of minimal responses being due to issues with selectivity or reduction. However, we were unable to perform studies using a 30 mM concentration of αβ-methylene ATP necessary for making any definitive conclusions on these issues due to the prohibitive cost of using

**Figure 3.11:** 3 mM ATP and αβ-methylene ATP evoked minimal RR-I responses. Populational consecutive bin averages of RR-I during ambient, vehicle, 3 mM ATP (black, n=3), 3 mM αβ-methylene ATP (aqua, n=2) and 30 mM ATP (red, n=4) exposures.
αβ-methylene ATP at such a high concentration. The last possibility is that parasympathetic reflexes evoked by the activation of P2X receptors on bronchopulmonary C-fibers were being masked by concomitant activation of sympathetic reflexes from SAR activation. Previous studies have shown that P2X receptors are expressed on SAR fibers and that these fibers respond to ATP (Canning et al., 2004). As mentioned previously, the activation of SAR fibers leads to increased efferent sympathetic activity and could therefore be antagonizing the parasympathetic effects of bronchopulmonary C-fiber activation. Confirmation of this antagonistic interaction would require exposing rats to ATP after separate pharmacological inhibition of the parasympathetic and sympathetic nervous systems.
CHAPTER 4
Investigation into the Mechanisms by which Inhalation of AITC Evokes Arrhythmic Responses in Healthy Sprague Dawley Rats

Introduction

The specificity of the TRPV1 and TRPA1 agonists used in our initial exposure studies (Undem et al., 2004; Taylor-Clark et al., 2009; Nesuashvili et al., 2013), as well as the selective expression of these TRP channels on bronchopulmonary C-fibers (Nassenstein et al., 2008; Nassenstein et al., 2010), suggests that the pulmonary-cardiac reflexes evoked by AITC and capsaicin were initiated by bronchopulmonary C-fiber activation through direct gating of TRP channels expressed on these nerves. Additionally, the slowed pacing and electrical conduction of the heart seen during exposure to the TRP agonists are indicative of an increase in cardiac parasympathetic activity (Levy & Zieske, 1969; Rinkema et al., 1982; Palecek et al., 1989). To further investigate the potential roles of TRP channel and parasympathetic activation in the mechanisms underlying the TRP agonist evoked pulmonary-cardiac reflexes, we performed exposures after TRP channel and muscarinic inhibition. These studies would determine if TRP channel and parasympathetic activation were required for evoking the pulmonary-cardiac reflexes, however, they would not fully explain the nature of their contributions. The expression of TRPV1 and TRPA1 is found in nociceptive neurons.
throughout the body (Kunert-Keil et al., 2006; Dietrich & Gudermann, 2011) and responses initiated by their activation vary dependent upon where they are expressed (Fernandes et al., 2012; Xing et al., 2015). Furthermore, inhaled irritants can freely enter into the pulmonary circulation potentially having direct effects on the heart through their activation of TRP channels expressed on the heart. Therefore, results from these studies using whole body TRP agonist exposures cannot determine if TRP channels expressed on bronchopulmonary C-fibers are solely responsible for evoking the pulmonary-cardiac reflexes. To address this issue, we determined that exposures would need to be performed either after vagus nerve sectioning or isolated to the airways below the trachea. Such studies came with the caveat of requiring the rats to be anesthetized or decerebrate. We therefore investigated the effects of anesthesia and pre-collicular decerebration on responses induced by TRP agonist exposure. The TRPA1 agonist AITC was used in the following studies as responses to AITC were found to be greater in our initial studies thus enhancing our ability to interpret the results. Furthermore, as TRPA1 responds to more chemicals commonly found in air pollution than TRPV1 does, TRPA1 is the more important channel to study for understanding how PM inhalation evokes cardiovascular effects.

Results

In order to determine whether the pulmonary-cardiac reflexes seen upon exposure to AITC were a result of TRPA1 activation, we recorded ECG responses of rats during exposure to AITC after administering the TRP channel blocker ruthenium red. Based on the selective activation of TRPA1 by AITC, we hypothesized that ruthenium red would prevent ECG responses evoked by inhalation of AITC. We found
that ruthenium red (4 mg/kg, i.p.) decreased responses to AITC (30 mM) by 45% in the case of RR-I, 86% in PR-I and 83% of AV block compared to control (p<0.05) (Fig. 4.1).

The ECG responses to AITC inhalation were indicative of increased parasympathetic drive on the heart leading to our hypothesis that the parasympathetic branch of the ANS was required for AITC-induced reflex responses. To confirm parasympathetic involvement, we administered the selective muscarinic inhibitor atropine prior to recording ECG responses to AITC exposure. Atropine (1 mg/kg, i.p.) caused a mild tachycardia (+ atropine: 140.2 ± 4.6 ms compared to - atropine: 152.7 ± 3.2, p<0.05), consistent with the limited tonic parasympathetic control of the heart in rodents (Farraj et al., 2011). As expected, atropine reduced RR-I and PR-I prolongation to AITC (30 mM) exposure by 86 and 98%, respectively, when compared to control (p<0.05, Fig. 4.2, see page 56). Additionally, we found that atropine completely

![Figure 4.1: Ruthenium red (RuR) attenuates responses to AITC. Mean ± SEM RR-I (left), PR-I (middle) and AV block (right) upon exposure to vehicle, AITC (30 mM) without ruthenium red (control, white) and after ruthenium red (4 mg/kg, i.p., black). *denotes significant difference to vehicle response (p<0.05). # denotes significant difference between AITC responses (p<0.05), n=9.](image)
prevented AITCs ability to evoke AV block as no events were seen during exposure to AITC after the administration of atropine.

To investigate the effects of anesthesia on the AITC-induced responses, we exposed the rats to AITC after separate administrations of ketamine, urethane and isoflurane. Previous studies have shown the evocation of mild bradycardic responses after instillation of TRP agonists into the airways under chloralose, urethane and pentobarbital anesthesia (Palecek et al., 1989; Lee et al., 1992; Bootle et al., 1996; Bergren et al., 1997). We, therefore, hypothesized that exposing anesthetized rats to AITC would not prevent AITC-induced responses, but rather lead to an attenuation of the responses. Instead, we found that inhalation of AITC (30 mM) had virtually no effect on heart rate following anesthetic administration. Ketamine (100 mg/kg, i.p.) alone produced bradycardia (304.3 ± 33 ms compared to 145 ± 3, p<0.05), however no change in RR-I was seen during exposure to AITC (309.8 ± 29 ms compared to vehicle,
298.3 ± 35, n.s.). Similarly, urethane (1.2 g/kg, i.p.) alone produced bradycardia (227.5 ± 5.4 ms compared to 145 ± 3, p<0.05), but prevented the ability of AITC to evoke changes in RR-I (229 ± 10 ms compared to vehicle, 228 ± 8, n.s.) (Fig. 4.3).

Although anesthesia prevented RR-I responses, analysis of respiration rate provided evidence for some defensive reflexes being functional as the administration of anesthesia did not prevent AITC-induced bradypnea (conscious: vehicle 70 ± 2 breaths/min compared to AITC 22 ± 1, n=3, p<0.05; ketamine: vehicle 49 ± 5 compared to AITC 29 ± 2, n=3, p<0.05) (Fig. 4.4, see page 58). Furthermore, we found that urethane did not entirely prevent tonic parasympathetic control of the heart evident by a significant reduction in RR-I seen after bilateral vagotomy (173 ms compared to 211.5, n=2, p<0.05, data not shown). To study the effects of isoflurane on AITC-induced responses, we gave WKY rats 5% isoflurane via nosecone for a period of 45 minutes and then allowed the rats to breathe ambient air for a period sufficient for coming out of anesthesia (15 min) before exposing them to AITC. Despite the observation that the rats were able to walk and groom normally, exposures to AITC

![Figure 4.3: Anesthesia prevents AITC response. Mean ± SEM RR-I upon exposure to vehicle and AITC (30 mM) alone (white), after ketamine (100 mg/kg, i.p., black, n=4) and after urethane (1.2 g/kg, i.p., red, n=5). *denotes significant difference to vehicle response (p<0.05).]
evoked no changes in RR-I (157.9 ± 1 ms compared to vehicle, 155.2 ± 0.6, n.s.). However, when we allowed the rats to recover for 45 minutes after isoflurane anesthesia, we found that exposure to AITC was again able to evoke a significant increase in RR-I (215.1 ± 3 ms compared to vehicle, 145.1 ± 0.4, p<0.05) (Fig. 4.5, see page 59).

Lastly, we investigated ECG responses to AITC in decerebrate and paralyzed rats. The rats were mechanically ventilated for an hour following decerebration before nebulized AITC (30 mM) was administered directly into the lower airways via a tracheal cannula. AITC caused a significant increase in RR-I (235 ± 26 ms compared to vehicle, 170 ± 10, p<0.05) and AV block (13 ± 13 events compared to vehicle, 0.3 ± 0.3, p<0.05) (Fig. 4.6, see page 60). However, no effect on PR-I was seen (53 ± 3 ms compared to vehicle, 51± 2). Sectioning of the brainstem caudal of the medulla prevented any AITC-induced ECG changes (194 ms compared to vehicle, 192, n.s., n=2).

Figure 4.4: Ketamine did not prevent AITC-induced bradypnea. Mean ± SEM breaths per minute upon exposure to vehicle and AITC (30 mM) alone (black) and after ketamine (100 mg/kg, i.p., aqua). *denotes significant difference to vehicle response (p<0.05). # denotes significant difference to control responses (p<0.05).
Studies performed previously in our lab found that 30 mM AITC evoked substantial bradypnea when instilled into the nasal cavity of wild-type mice, but had no effect on TRPA1 -/- mice (Taylor-Clark et al., 2009), thus demonstrating the selectivity of AITC for TRPA1. Here ruthenium red, an inhibitor of both TRPA1 and TRPV1, greatly

**Figure 4.5: Isoflurane reversibly inhibits AITC response.** Populational consecutive bin averages of RR-I during ambient, vehicle and AITC (30 mM) exposures before 45 min 3% isoflurane (AITC 1) and after (AITC 2). Black data: AITC 2 exposure 15 min after anesthesia, n=2; red data: AITC 2 exposure 60 min after anesthesia, n=2.

**Discussion**

Studies performed previously in our lab found that 30 mM AITC evoked substantial bradypnea when instilled into the nasal cavity of wild-type mice, but had no effect on TRPA1 -/- mice (Taylor-Clark et al., 2009), thus demonstrating the selectivity of AITC for TRPA1. Here ruthenium red, an inhibitor of both TRPA1 and TRPV1, greatly
reduced ECG responses to AITC, suggesting the direct involvement of TRP channels in these responses. The inhibition of AV block by ruthenium red was shown to be greater
than its effects on RR-I, possibly due to the inhibitor hindering high intensity action potential discharge from airway C-fibers without affecting all C-fiber activity (Nassenstein et al., 2008). Due to the off-target effects of ruthenium red on channels such as ryanodine receptors (RyR) (Netticadan et al., 1996), we did not investigate whether responses could be completely inhibited using higher concentrations of ruthenium red. Additionally, we have provided evidence that AITC-induced ECG responses occur via an increase in parasympathetic drive to the heart as responses to AITC were decreased by 86% after administration of the muscarinic antagonist atropine. That atropine was not seen to completely prevent an AITC-induced increase in RR-I, could simply be explained by the dose not being sufficient for total muscarinic blockade. It is also possible that some parasympathetic activity remained due to contributions from non-adrenergic, non-cholinergic (NANC) nerves (Rand, 1992). Nevertheless, inhibition of AITC-induced responses by ruthenium red and atropine suggest that bradycardic responses to AITC exposure are a consequence of TRPA1 channel activation resulting in increased parasympathetic outflow to the heart.

We found that administration of anesthesia caused an expected increase in RR-I (Rojas et al., 2006) and prevented the bradycardia evoked by inhalation of AITC in freely-breathing rats. This is in agreement with studies demonstrating that bradycardia evoked by smoke inhalation was completely abolished (Nakamura & Hayashida, 1992), or attenuated (White & McRitchie, 1973) by pentobarbital and chloralose anesthesia. The inhibition of these responses by anesthesia is likely due to its effects on the ANS, as anesthesia has been shown to cause variable block of homeostatic control of cardiovascular and respiratory rhythms (Elsner et al., 1966; Cullen et al., 1987; Watkins
& Maixner, 1991; Sellgren et al., 1994). Indeed, we saw no ECG responses to tests commonly used for measuring ANS reflex function in rats under ketamine anesthesia (Baseline: 158 ms, Diving reflex: 158, Limb pinch: 154, Carotid massage: 153; n=2, n.s.). However, we were able to provide evidence that tonic parasympathetic control of the heart was maintained after urethane administration as a significant reduction in RR-I was seen after bilateral vagotomy. Furthermore, the administration of anesthesia was found not to inhibit AITC-induced bradypnea. The ability to evoke defensive reflexes from the airways in animals under anesthesia has been shown by multiple groups (Lee et al., 1992; Canning et al., 2004), however, comparisons between these and our studies are complicated by variation in the methods of agonist administration (inhalation vs. injection) employed. Nevertheless, the contrasting inhibitory effects of anesthesia on AITC-induced cardio-respiratory reflexes suggests significant differences between the central neural circuits involved with reflexes initiated by the airways.

Isoflurane anesthesia was also found to inhibit AITC-induced ECG responses with inhibition persisting even after apparent recovery from the anesthetic. This suggests that residual anesthesia has a profound effect on pulmonary-cardiac reflexes evoked by AITC. As mentioned previously, it is possible that desensitization of TRPA1 played a factor in the lack of responses seen after isoflurane inhalation, as isoflurane has been shown to activate TRPA1 (Mutoh et al., 2013; Kichko et al., 2015). Nevertheless, we found that isoflurane inhibits responses to AITC in a reversible manner as responses to AITC returned following a recovery period of 45 minutes. The results from the isoflurane studies gave us confidence that we could effectively record responses to AITC in a decerebrate rat model as long as exposures were performed at
least 45 minutes following decerebration and cessation of isoflurane administration. We found that exposing nebulized AITC into the lower airways of decerebrate rats evoked significant bradycardia associated with AV block. These responses to AITC seen after removal of the somatosensory brain regions suggest that sensation is not required for the AITC-induced bradycardia (Xing et al., 2015). Additionally, by isolating AITC exposures to the lower airways, we were able to show that AITC-induced responses occur through activation of TRPA1 channels expressed on bronchopulmonary C-fibers and do not require activation of TRPA1 expressed in the upper airways. It is possible that nebulized AITC may have entered the pulmonary circulation leading to activation of TRPA1 channels expressed on the heart, however, removal of the brainstem abolished responses to AITC suggesting that its effects were not due to a direct effect on the heart. It is also possible that the effects of AITC on respiration rate contribute to the effects seen on HR. While it is likely AITC-induced bradypnea contributes to some degree in the AITC-induced bradycardia, results from our decerebrate studies suggest that its contributions are minor as respiration rate was controlled through mechanical ventilation throughout the duration of these studies.
CHAPTER 5

Inhalation of AITC Evokes a Biphasic Blood Pressure Response that is Sensitive to Atropine and Terazosin in Healthy Sprague Dawley Rats

Introduction

To further understand pulmonary-cardiac reflexes evoked by AITC inhalation, we recorded BP responses of healthy SD rats during exposures to AITC. Although similar effects on HR are seen by activation of TRP channels expressed on upper and lower airway nerves, this is not the case in regards to their effects on BP. Activation of TRPA1-expressing upper airway nociceptive fibers innervating the nasal airways or the larynx (Kobayashi et al., 1999; Mutoh et al., 2000; Liu et al., 2013) has been shown to evoke an increase in blood pressure (Taylor-Clark et al., 2009; Mutoh et al., 2013). In contrast, activation of TRPA1-expressing lower airway nociceptive fibers leads to a drop in BP (Coleridge et al., 1964; Davis et al., 1982; Lee et al., 1987; Lee & Lundberg, 1994). Such discrepancies in BP responses could potentially aid in the determination of the roles upper and lower airway TRPA1-expressing nociceptive fibers play in the AITC-induced responses. To further characterize reflexes evoked by AITC inhalation, we recorded BP responses to AITC exposure with and without muscarinic inhibition. Additionally, arguably the most powerful reflex involved in cardiovascular regulation is that of the baroreflex (Paintal, 1973; Fahim, 2003). As mentioned previously, increases
in blood pressure can cause a decrease in HR via a baroreceptor-mediated reflex. Therefore, it is possible that the AITC-induced bradycardia is a result of increased baroreceptor firing to increases in BP evoked by activation of TRPA1 channels expressed in the upper airways. To determine whether the AITC-induced bradycardia was secondary to a hypertensive effect, we pretreated rats with a selective α1 inhibitor before recording BP responses to AITC exposure. Inhibition of peripheral α1-adrenergic receptors decreases systemic vascular tone without affecting baroreflex sensitivity (Pawar & Fahim, 2004; Sharma et al., 2004), thus allowing for any responses to be attributed to reflexes initiated in the airways and not arterial baroreceptors.

**Results**

Based on the severity of bradycardia induced by inhalation of AITC, we predicted that it would also cause hypotension, possibly secondary to the bradycardic effects. Exposing rats to AITC (30 mM) caused a significant increase in pulse interval (PI) derived from the BP recordings shown in Figure 5.1. Additionally, AITC appeared to cause dropped beats as large breaks in pulsatile oscillations were seen along with a fall in BP as shown in figure 5.2 (see page 66). However, this cannot be confirmed from such analysis. Surprisingly, AITC inhalation evoked a biphasic BP response with an initial hypertension
followed by a prolonged hypotension (Fig. 5.3, see page 67).

Administration of the muscarinic antagonist atropine (1 mg/kg, i.p.) did not affect baseline BP (+ atropine: 127.3 ± 6.6 mmHg compared to - atropine: 128.4 ± 2, n.s.), but did cause a significant reduction in PI (+ atropine: 144.3 ± 4.9 ms compared to - atropine: 164.9 ± 5.4, p<0.05). Atropine was found to prevent the AITC-induced increase in PI and late hypotensive phase, however, it potentiated the early hypertensive phase (Fig. 5.4, see page 68). To further investigate the biphasic BP response evoked by AITC, we pretreated the rats with terazosin (0.3 mg/kg, i.p.), a selective α1 inhibitor with limited CNS penetration. As expected, inhibition of sympathetic vascular tone by terazosin caused a decrease in baseline BP (+ terazosin: 100.6 ± 4 mmHg compared to - terazosin: 128.4 ± 2, p<0.05), but did not have a significant effect on baseline PI (+ terazosin: 148.9 ± 7 ms compared to - terazosin: 164.9 ± 5, p=0.07). Furthermore, terazosin prevented the AITC-induced BP responses (100.6 ± 2.4 mmHg compared to

Figure 5.2: AITC evokes prolonged pulse intervals. Raw BP traces during ambient (top), vehicle (middle) and AITC (30 mM, bottom) exposures.
vehicle, 102.5 ± 2.8, n.s.), but did not prevent the bradycardic response evoked by AITC (254.4 ± 49 ms compared to vehicle, 145 ± 7, p<0.05) (Fig. 5.5, see page 69).

Discussion

We have shown that inhalation of AITC in conscious rats yielded a biphasic blood pressure response, with an early hypertensive phase followed by a late hypotensive phase. The initial hypertension is perhaps due to activation of upper airway nasotrigeminal afferent C-fibers whose activation initiates the diving reflex resulting in bradycardia and hypertension through increased peripheral vasoconstriction (Yavari et al., 1996; Mutoh et al., 2000; Liu et al., 2013). Hypertensive responses to activation of TRPA1 channels expressed in skeletal muscle by systemic administration of AITC have also been shown (Koba et al., 2011), however, the instantaneous nature of the hypertension argues against this possibility. The late hypotensive phase is best explained by AITC-induced activation of lower airway bronchopulmonary C-fibers evoking a parasympathetic depressor effect (Coleridge et al., 1964; Rinkema et al., 1982; Lee et al., 1987). Results from our atropine studies provide evidence for an AITC-induced parasympathetic depressor effect, as AITC simply evoked a sustained hypertension with no decrease in HR in the presence of

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**Figure 5.3: AITC evokes a biphasic blood pressure response.**

Populational consecutive bin averages of MABP during ambient, vehicle and AITC (30 mM, n=6) exposures.
Figure 5.4: Atropine inhibits the hypotensive and bradycardic responses to AITC. (A) Populational consecutive bin averages of MABP during ambient, vehicle and AITC (30 mM) exposures. (B) Mean ± SEM MABP. (C) Populational consecutive averages of PI during ambient, vehicle and AITC (30 mM) exposures. (D) Mean ± SEM PI. Black data: Control animals; aqua data: Animals given atropine (1 mg/kg, i.p., n=7). * denotes significant difference to vehicle response (p<0.05). # denotes significant difference between control and atropine responses (p<0.05).
Figure 5.5: Terazosin prevents AITC-induced BP responses without preventing the AITC-induced bradycardia. (A) Populational consecutive bin averages of MABP during ambient, vehicle and AITC (30 mM) exposures. (B) Mean ± SEM MABP. (C) Populational consecutive averages of PI during ambient, vehicle and AITC (30 mM) exposures. (D) Mean ± SEM PI. Black data: Control animals; red data: Animals given terazosin (0.3 mg/kg, i.p., n=4). * denotes significant difference to vehicle response (p<0.05). # denotes significant difference between control and terazosin responses (p<0.05).
atropine. Similarly, previous studies found that atropine prevented bradycardic responses to intravenous AITC administration, but did not affect the pressor response (Pozsgai et al., 2010). It is difficult to say if such a depressor effect is exclusively due to a decrease in HR, or if a reduction in peripheral resistance is also involved. Acute increases in BP can cause reflex bradycardia secondary to increased baroreceptor firing. However, previous studies have shown that inhibition of α1-adrenergic receptors by prazosin prevented hypertensive responses of the diving reflex without affecting the bradycardic responses (Yavari et al., 1996), suggesting that the bradycardic responses occur independent of baroreceptor firing. Furthermore, previous studies investigating the extent to which baroreflex activation modulates HR found that a 10 mmHg increase in BP coincided with a 15 ms increase in PI (Head & McCarty, 1987; Ferrari et al., 1991; Oliveira et al., 1996; el-Mas & Abdel-Rahman, 1998; Miller et al., 1999). This effect is modest compared to the 145 ms increase in PI we saw to a 10 mmHg increase in BP, thus baroreflex activation cannot solely be responsible for the AITC-induced bradycardia. Additionally, we found that pretreatment with terazosin blocked the AITC-evoked hypertension through inhibition of peripheral α1 receptors, while AITC was still able to evoke a 109 ms increase in PI. We saw similar responses to AITC in rats pretreated with the α1-antagonist prazosin (0.1 mg/kg, i.p.), as AITC had no effect on BP (102 ± 8 mmHg compared to vehicle, 101 ± 8, n=4), but was able to evoke an increase in PI (181 ± 14 mmHg compared to vehicle, 148 ± 3, p<0.05, n=4). However, interpretation of the prazosin data was complicated by its apparent effects on the CNS, as the animals displayed lucid behavior with lower body paralysis (Anden et al., 1978). Therefore, these results were not shown. The ability of AITC to evoke such a large
increase in PI to a 10 mmHg increase in BP, along with its bradycardic effects persisting after peripheral α1 receptor inhibition, suggests that much of the bradycardia is a direct effect of AITC on reflex parasympathetic signaling to the heart and not secondary to baroreceptor activity (Yavari et al., 1996).
CHAPTER 6

Inhalation of Selective TRP Channel Agonists Evokes Complex Arrhythmias
Sensitive to Adrenergic Inhibition in Spontaneously Hypertensive Rats

Introduction

PM is known to disproportionately increase cardiovascular effects in individuals with pre-existing cardiovascular diseases (Pope et al., 2006). Despite the well-reported effects of PM on at-risk individuals, little is presently known of the specific mechanisms by which inhalation of pollutants/irritants modulate the cardiac cycle, particularly in the context of pre-existing cardiovascular disease (Brook et al., 2010). A marked withdrawal of the baroreceptor reflex and a concomitant increase of sympathetic drive on the heart have been well established in cardiovascular disease models (Frohlich & Pfeffer, 1975; Modolo Rda et al., 1995; Du et al., 1998; Bibevski & Dunlap, 2011). Some studies have shown that the parasympathetic arm of the baroreceptor reflex is dysfunctional at the peripheral ganglia as stimulation of the pre-ganglionic vagus nerve showed attenuated cardiac responses (Modolo Rda et al., 1995; Du et al., 1998; Nihei et al., 2005; Bibevski & Dunlap, 2011), while stimulation of the post-ganglionic vagus nerve evoked an even greater response compared to controls (Ferrari et al., 1992). It is believed that the defect at the ganglion specifically involves nicotinic receptors whose subunit composition has been found to be altered (Bibevski & Dunlap, 2011). Interestingly,
previous studies have found that a supersensitivity of the sinus and AV nodes to muscarinic cholinergic agents occurred after parasympathetic denervation (Kaseda & Zipes, 1988). In addition, multiple groups investigating models of cardiovascular disease have found acetylcholinesterase downregulation (Bibevski & Dunlap, 2011), upregulation of muscarinic receptors (Nihei et al., 2005; Olshansky et al., 2008), hypersensitivity of the Gi protein mediated muscarinic receptor adenylyl cyclase system (Fu et al., 1993), and an increase of acetylcholine concentration in the heart (Tsuboi et al., 1987). Furthermore, SH rats were found not to have an impaired bradycardic response to electrical or pharmacological parasympathetic stimul i, but a response twice that of controls (Ferrari et al., 1992). Thus, a decrease in the baroreceptor reflex has been proposed to cause augmented parasympathetic activity counteracting or compensating for the loss of vagal tone and increase in sympathetic drive associated with cardiovascular disease (Tsuboi et al., 1987; Kaseda & Zipes, 1988; Ferrari et al., 1992). As PM has been shown to activate bronchopulmonary C-fibers through activation of TRP channels expressed on these fibers (Agopyan et al., 2003a; Agopyan et al., 2003b; Agopyan et al., 2004; Undem et al., 2004; Deering-Rice et al., 2011; Deering-Rice et al., 2012), we chose to investigate the potential involvement of pulmonary afferents in the PM-induced deleterious cardiovascular effects seen in individuals with pre-existing cardiovascular diseases by recording ECG responses to a selective TRPA1 agonist (AITC) exposure in cardiovascular disease animal models. Interestingly, sidestream cigarette smoke was shown to affect sympathetic and parasympathetic components of the baroreflex in normotensive rats, while only affecting the sympathetic component in SH rats (Narkiewicz et al., 1998; Valenti et al., 2010); and heart failure-
prone rats were shown to have aberrant sympathetic dominance upon inhalation of diesel exhaust as evidenced by decreased cardiac performance and HRV (Carll et al., 2012; Carll et al., 2013a). These findings suggest that inhalation of pollutants in individuals with cardiovascular disease could potentially exert their effects through modulation of sympathetic activity known to be increased in cardiovascular disease. To investigate whether cardiovascular disease induces plasticity in the afferent arm of the pulmonary-cardiac reflex resulting in a phenotypic switch of the reflexes initiated by airway nerves, we recorded the ECG responses of cardiovascular diseased SH rats and healthy WKY rats to AITC exposure.

We chose to use the SH rat model due to its cardiac outcomes and acceptance as a model for determining susceptibility with pulmonary and cardiovascular complications. The aged SH rat is widely accepted as a model of human essential hypertension due to its high incidence of cardiovascular disease, attenuated arterial baroreflex regulation of BP, increased lung and LV weights, and lung inflammation confirmed by the presence of activated macrophages, neutrophils, and hemorrhage (Trippodo & Frohlich, 1981; Kodavanti et al., 2000). Importantly, the SH rat has been found to exhibit effects consistent with a greater susceptibility to adverse health effects upon PM inhalation such as, exacerbated pulmonary injury, attenuated antioxidant responses, acute depression in ST segment area (Kodavanti et al., 2000), ventricular arrhythmias, and ventricular fibrillation (Versailles et al., 1982). This is consistent with increased arrhythmia susceptibility seen in humans with hypertension upon exposure to PM (Brook, 2007; Brook et al., 2010; Tsai et al., 2014).
Results

To investigate the contribution of bronchopulmonary afferents in the increased risk of cardiovascular effects of individuals with pre-existing cardiovascular disease upon PM inhalation, we recorded ECG from SH and WKY rats during exposures to the selective TRPA1 agonist AITC. SH rats are bred from WKY rats, thus responses from AITC exposures in WKY rats served as the control for the SH studies. An expected increase in baseline blood pressure was seen in the SH rats compared to the WKY rats (185.9 ± 6.7 mmHg compared to 106.9 ± 2.9, p<0.05, data not shown). Comparisons between individual cardiac cycles in the WKY and SH rats revealed that the SH rats also exhibited apparent ECG abnormalities such as ST segment depression and QT interval prolongation as shown in figure 6.1. It should be noted that these abnormalities were only observed and were not quantified.

Comparisons of RR-I at rest between SH and WKY rats showed a significant decrease in SH RR-I (140.4 ± 4 ms compared to 151 ± 3, p<0.05), indicating parasympathetic withdrawal and increased sympathetic control of HR in SH rats. Given that parasympathetic activity is attenuated in cardiovascular disease leaving sympathetic activity to dominate, we hypothesized that selective activation of TRPA1 would lead to exacerbated

![Figure 6.1: SH rats exhibited apparent ST segment depression and QT interval prolongation. Examples of single cardiac cycles in the WKY (left) and SH (right) rat.](image)
cardiovascular effects in the SH rat. We found that AITC (30 mM) evoked differential ECG responses in the SH compared to the WKY rats (Fig. 6.2).

Analysis of ECG data acquired during exposures to AITC revealed a significant RR-I increase in SH rats (187 ± 3 ms compared to vehicle, 158 ± 4, p<0.05), as well as PR-I (58 ± 2.3 ms compared to vehicle, 49 ± 0.4, p<0.05) and incidence of AV block (18 ± 6 events compared to vehicle, 0.2 ± 0.2, p<0.05) (Fig. 6.3, see page 77). However, in WKY rats, the AITC-induced RR-I prolongation (239.9 ± 6 ms compared to vehicle, 169.6 ± 5, p<0.05) and evocation of AV block (41 ± 18 events compared to vehicle, 0 ± 0) were greater than that seen in the SH rat, with a similar increase seen in PR-I (52 ± 0.9 ms compared to vehicle, 45.4 ± 1, p<0.05) (Fig. 6.4, see page 78). Although AITC-induced bradycardic effects were greater in WKY rats compared to SH rats, further ECG analysis revealed that AITC also evoked PVCs in the SH rat (Fig. 6.5, see page 79).
The significant increase in PVCs evoked by AITC in SH rats (6 ± 2 events compared to vehicle, 0.2 ± 0.1, p<0.05) compared to the minimal increase seen in WKY rats (0.15 ± 0.1 events compared to vehicle, 0), suggested that exposure to AITC was causing...
increased sympathetic activity in the SH rat. Furthermore, by plotting the relationship of consecutive RR-Is from SH and WKY AITC exposures (Fig. 6.6, see pg. 80), we noticed that AITC caused an apparent increase in consecutive tachycardic beats in the SH rat.

Figure 6.4: AITC evokes greater bradycardic response in the WKY rat compared to the SH rat. (A) Populational consecutive bin averages of RR-Interval during ambient, vehicle and AITC (30 mM) exposures. (B) Mean ± SEM RR-I. (C) Mean ± SEM AV block. (D) Mean ± SEM PR-I. Black data: WKY rats, n=11; red data: SH rats, n=7. * denotes significant difference to vehicle response (p<0.05). $ denotes significant difference between WKY and SH responses (p<0.05).
To investigate a potential AITC-induced increase in tachycardic beats, we calculated the % of tachycardic RR-Is occurring in each individual exposure. An RR-I was considered tachycardic if it was less than two standard deviations below the vehicle mean RR-I of each individual. By performing this analysis, we were able to show that AITC did indeed evoke a significant increase in % of tachycardic beats in the SH rat (13 ± 4.5 % compared to vehicle, 1.4 ± 0.5, p<0.05), while a significant decrease was seen in the WKY rat (1 ± 0.4 % compared to vehicle, 4.2 ± 0.9, p<0.05) (Fig. 6.7, see page 81). The results from these studies suggest that AITC affects parasympathetic activity in both strains, while only affecting sympathetic activity in the SH rat.

To investigate the level of involvement of the parasympathetic nervous system in AITC-induced responses seen in SH and WKY rats, we exposed both strains to AITC (30 mM) after pretreating them with the muscarinic antagonist atropine (1 mg/kg, i.p.). The administration of atropine did not affect baseline RR-I in the SH rats (+ atropine: 135 ± 2 ms compared to - atropine: 138 ± 4, n.s.) nor in the WKY rats (+ atropine: 149 ±
3 ms compared to - atropine: 150 ± 4, n.s.). Based on results from the previous studies, we hypothesized that exposures to AITC after pretreatment with atropine would prevent AITC-induced bradycardic responses in the SH and WKY rats without preventing, but potentially enhancing, the tachycardic effects seen in the SH rats. As expected, atropine prevented AITC-induced RR-I prolongation in the WKY rats (149 ± 5 ms compared to vehicle, 145 ± 3, n.s.), while a significant decrease in RR-I was seen in the SH rats (123.9 ± 1.6 ms compared to vehicle, 136 ± 2, p<0.05) (Fig. 6.8, see page 82). Atropine prevented AITC-induced AV block in both strains, however, it did not affect AITCs ability to evoke PVCs in the SH rats (5 ± 1 events compared to vehicle, 0.6 ± 0.3, p<0.05) (Fig. 6.9, see page 83). Additionally, the % of tachycardic beats evoked by AITC was significantly increased in SH rats pretreated with atropine (39 ± 10.6 % compared to vehicle, 2 ± 0.8, p<0.05), and this increase was found to be significantly greater than

**Figure 6.6:** AITC caused an increase in consecutive tachycardic beats in the SH rat. Left: Poincare plot of the AITC responses in SH (red) and WKY (black) rats. Right: Zoom in of the area showing tachycardic beats.
that seen in SH rats not given atropine (39 ± 10.6 % compared to 13 ± 4.5, p<0.05) (Fig. 6.10, see page 84). The increase in AITC-induced tachycardic responses seen in SH rats pretreated with atropine further indicates that AITC not only evokes an increase in parasympathetic activity, but also an increase in sympathetic activity in SH rats.

The potential increase in sympathetic activity caused by AITC exposure in SH rats was studied by exposing SH rats to AITC (30 mM) after pretreatment with the β1-antagonist atenolol (0.5 mg/kg, i. p.). Based on results from previous studies, we hypothesized that atenolol would prevent tachycardic responses evoked by AITC and have no effect on the AITC-induced bradycardia. Indicative of tonic sympathetic activity on the heart, the administration of atenolol caused a significant increase in baseline RR-I (+ atenolol: 191 ± 0.8 ms compared to - atenolol: 141 ± 7, p<0.05). As expected, exposure to AITC resulted in a significant RR-I increase (227 ± 6 ms compared to vehicle, 193 ± 3, p<0.05) and concomitant increase in incidence of AV block (10 ± 3 events compared to vehicle, 0) (Fig. 6.11, see page 85). No significant differences were found between the relative increase in RR-I and AV block incidence evoked by AITC in SH rats pretreated with atenolol and those not receiving pretreatment. Furthermore, atenolol prevented
Figure 6.8: Atropine prevented the AITC-induced bradycardia in the WKY rats, but did not prevent the AITC-induced tachycardia in the SH rat. (A) Populational consecutive bin averages of RR-I during ambient, vehicle and AITC (30mM) exposures in the WKY rats. (B) Mean ± SEM RR-I in the WKY rats. (C) Populational consecutive averages of RR-I during ambient, vehicle and AITC (30 mM) exposures in the SH rats. (D) Mean ± SEM RR-I in the SH rats. Black data: Control animals; aqua data: Animals given atropine (1 mg/kg, i.p.; WKY: n=6, SH: n=9). * denotes significant difference to vehicle response (p<0.05). # denotes significant difference between control and atropine responses (p<0.05).
Discussion

We found that exposure to AITC caused differential cardiovascular responses between healthy WKY rats and cardiovascular diseased SH rats, such that AITC-induced responses switched from purely bradycardic in the WKY rats, to also evoking positive chronotropic effects (and PVCs) in the SH rats. The cardiac effects evoked by AITC mimic those found in previous PM basic and clinical studies: i.e. bradycardia in normal, healthy animals (Nakamura & Hayashida, 1992; Lamb et al., 2012) and brady-tachy with PVCs in cardiovascular disease (Wellenius et al., 2002; Simkhovich et al., 2008; Brook et al., 2010; Zanobetti et al., 2014). Differences in responses to the TRPV1
agonist capsaicin (600 µM) were also seen as capsaicin caused a significant RR-I increase in the WKY rats (185 ± 8 ms compared to 175 ± 9, p<0.05, n=5), while having no effect on RR-I in the SH rats (157 ± 15 ms compared to 157 ± 10, n.s., n=3). The apparent lack of response in the SH rat to capsaicin could be a consequence of increased sympathetic activity functionally antagonizing increases in parasympathetic activity, however, an increase in SH sample size and capsaicin concentration would be required to confirm this possibility. Nevertheless, the difference in SH and WKY responses to AITC inhalation suggests that pre-existing cardiovascular disease alters pulmonary-cardiac reflexes so that TRP agonists induce tachyarrhythmias following a reflex increase in sympathetic drive. This could be occurring through cardiovascular disease-induced plasticity of the afferent arm of the reflex: e.g. *de novo* expression of

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**Figure 6.10:** Atropine exacerbated the AITC evoked tachycardia in the SH rat. Mean ± SEM % tachycardic beats in the WKY (left) and SH (right) rats. Black data: Control animals; aqua data: Animals given atropine (1 mg/kg, i.p.). * denotes significant difference to vehicle response (p<0.05). # denotes significant difference between control and atropine responses (p<0.05).
TRPA1 in SAR fibers, which are the only pulmonary afferents that stimulate sympathetic drive (Coleridge et al., 1989; Schelegle, 2003). Evidence for this possibility was seen in a study that found de novo TRPV1 expression on SAR fibers in animals with increased levels of inflammation (Zhang et al., 2008). However, there is also the possibility that normal C-fiber reflexes (parasympathetic) cause tachyarrhythmia in SH hearts due to their intrinsic electrical dysregulation (Tsuboi et al., 1987; Kaseda & Zipes, 1988; Ferrari et al., 1992). We favor the former hypothesis, as vagal electrical stimulation does not evoke tachyarrhythmia in SH rats (Lee et al., 1980; Head, 1994). To determine whether the differential responses seen in SH rats were due to plasticity of the reflex (stimulating sympathetic drive), or the heart’s inability to deal with exaggerated parasympathetic drive, we performed AITC exposures after muscarinic (atropine) and adrenergic (atenolol) inhibition. We found that atenolol inhibited the tachycardia and PVC responses, without affecting the bradycardic responses evoked by AITC. These results
indicate that AITC evokes positive chronotropic effects via activation of sympathetic reflexes and negative chronotropic effects via parasympathetic activation. The results from our atropine studies argue against exaggerated parasympathetic output being responsible for the AITC-induced tachyarrhythmia in SH rats, as these responses were found to be unaffected by atropine. We were also able to provide evidence for increased sympathetic dominance in the SH rat, as baseline HR was found to be significantly higher in SH rats compared to WKY rats. Furthermore, the resting HR of SH rats was unaffected by the administration of atropine, while atenolol caused a significant decrease in resting HR.

Figure 6.12: Atenolol inhibited AITC-induced PVCs and tachycardia in the SH rat. Left: Mean ± SEM % tachycardic beats. Right: Mean ± SEM PVCs. Black data: Control animals; red data: Animals given atenolol (0.5 mg/kg, i.p.). * denotes significant difference to vehicle response (p<0.05). # denotes significant difference between control and atenolol responses (p<0.05).
Results from these studies provide evidence for the differential responses seen in SH rats to be due to plasticity of the afferent arm of the reflex (stimulating sympathetic drive). Given that PM activates TRPA1, it is possible that PM evokes tachyarrhythmia in cardiovascular diseased models through an increase in sympathetic tone via de novo expression of TRPA1 on SAR-fibers. Studies have found increased inflammatory mediators in the lungs as a consequence of systemic inflammation known to be associated with cardiovascular disease. These studies found an increase in endothelin in the lungs of rats after MI (Zhang et al., 2008), activated macrophages and neutrophils in the lungs of SH rats (Kodavanti et al., 2000), and increases in inflammatory cytokines such as MCP-1 and IL-6 (Yndestad et al., 2006). Inflammatory mediators have been shown to lead to de novo synthesis and expression of TRP channels on myelinated pulmonary afferents (SAR) (Zhang et al., 2008). Altered excitability of pulmonary afferents due to inflammation is supported by a study which found air pollution effects on reduced HRV to be stronger in elderly individuals with elevated systemic inflammation (Luttmann-Gibson et al., 2010). Therefore, individuals with pre-existing cardiovascular disease could be predisposed to cardiac arrhythmia upon PM inhalation due to exaggerated sympathetic reflexes evoked by pulmonary afferent activation.
CHAPTER 7
Blood Pressure Responses to Inhaled AITC in Spontaneously Hypertensive Rats

Introduction

As mentioned previously, individuals with pre-existing cardiovascular disease are more likely to have negative cardiovascular outcomes after inhaling PM than healthy individuals. Apart from the tachyarrhythmic effects evoked by PM exposure, studies have also shown PM-evoked effects on BP. Chronic exposure to concentrated ambient PM2.5 (CAP) has been shown to significantly increase BP in SH rats in a reversible manner, such that cessation of CAP exposure results in restoration of BP (Chang et al., 2007; Ying et al., 2015). In the same studies, CAP exposure was also found to markedly increase expression of pro-inflammatory cytokines in the lungs and heart, and the inflammation was shown to resolve in the heart, but not the lungs when the CAP exposures were stopped. Acute PM exposures have also been shown to evoke increases in blood pressure of individuals with cardiovascular disease (Dvonch et al., 2009; Kannan et al., 2010). The increase in BP seen in chronic PM exposure studies is believed to be a factor of the associated increase in systemic inflammation, while very little is known as to how acute PM exposures lead to an increase in BP. The current theory is that PM activates nociceptive C-fibers in the airways, which instigate reflex autonomic responses and alter the cardiovascular sympathetic/parasympathetic
balance (Widdicombe & Lee, 2001). Sympathetic hyperactivity and parasympathetic insufficiency characterize BP control in hypertensive animals and humans. Evidence of this is found in studies showing that the baroreceptor reflex has diminished sensitivity due mainly to reduced maximum capacity of the cardiac vagal component rather than a change to the sympathetic (Head, 1994). To further investigate the differential cardiovascular responses evoked by AITC in the SH rat, we recorded BP from SH and WKY rats during exposures to AITC. Based on a review of relevant literature and results from the previous ECG studies, we believe that exposure to AITC causes increases in sympathetic and parasympathetic activity in SH rats. Therefore, we performed additional AITC exposures on SH and WKY rats pretreated with either the muscarinic antagonist atropine, or the α1-antagonist terazosin.

Results

Based on results from our SD BP studies, we hypothesized that AITC would evoke a biphasic response in the WKY rat similar to that seen in the SD rat. However, unlike BP responses seen in the SD rats, no consistent BP responses to AITC were observed in the WKY rats (Fig. 7.1, see page 90). Nevertheless, PI responses were similar as AITC was found to evoke a significant increase in PI (275 ± 26.1 ms compared to vehicle, 167.7 ± 13.1, p<0.05). We further investigated BP responses to AITC in the WKY rats by pretreating them with the muscarinic antagonist atropine (1 mg/kg, i.p.). Similar to BP responses seen in SD rats pretreated with atropine, AITC evoked substantial hypertension in the WKY rats given atropine (159.2 ± 8 mmHg compared to vehicle, 134.5 ± 4, p<0.05) (Fig. 7.2, see page 91). Additionally, we found that responses to AITC in WKY rats pretreated with the peripheral α1 antagonist
terazosin (0.3 mg/kg, i.p.) were consistent with our previous SD data (see Chapter 4). Specifically, terazosin prevented AITC-induced BP changes (75.4 ± 13 mmHg compared to vehicle, 78.4 ± 6.9, n.s.), but not AITCs ability to evoke bradycardia in the WKY rats (252.7 ± 43.6 ms compared to vehicle, 148.1 ± 2.5, p<0.05) (Fig. 7.3, see page 92). Thus, indicating that the bradycardia was not secondary to AITC-induced hypertension.

We next investigated BP responses to AITC in the SH rat. Based on results from our ECG studies that suggested AITC was causing increases in sympathetic activity and parasympathetic activity in the SH rats, we predicted that AITC would evoke a hypertensive response in the SH rat. As expected, the baseline BP was significantly higher in the SH rats compared to the WKY rats (185.9 ± 6.7 mmHg vs. 106.9 ± 2.9, p<0.05) and their PI was significantly lower (145.2 ± 5.6 ms vs. 175.5 ± 15.9, p<0.05). In agreement with our hypothesis, exposure to AITC (30 mM) evoked a hypertensive reflex.
in the SH rats (208.5 ± 5.4 mmHg compared to vehicle, 186.9 ± 6.8, p<0.05) (Fig. 7.4, see page 93).

Similar to the ECG responses shown in our previous SH studies, analysis of the AITC-induced PI responses showed a significant increase in PI in the SH rats (199.1 ± 5.6 ms compared to vehicle, 155.1 ± 7.2, p<0.05), but the increase in PI was not as great as that seen in the WKY rats (Fig. 7.5, see page 94). These results are in agreement with an AITC-induced increase of sympathetic activity in the SH rats. Therefore, we hypothesized that the hypertension evoked by AITC in the SH rats would be accentuated after pretreating the rats with the muscarinic antagonist atropine. Indeed, we found that exposing the SH rats to AITC (30 mM) after pretreatment with atropine (1 mg/kg, i.p.) led to a substantial hypertensive reflex (206 ± 13.7 mmHg

Figure 7.2: Atropine potentiated the hypertensive response to AITC in the WKY rat. Left: Populational consecutive bin averages of MABP during ambient, vehicle and AITC (30mM) exposures. Right: Mean ± SEM MABP. Black data: Control animals; aqua data: Animals given atropine (1 mg/kg, i.p., n=5). * denotes significant difference to vehicle response (p<0.05). # denotes significant difference between control and atropine responses (p<0.05).
Figure 7.3: Terazosin prevented AITC-induced BP responses without preventing the AITC-induced bradycardia in WKY rats. (A) Populational consecutive bin averages of MABP during ambient, vehicle and AITC (30mM) exposures. (B) Mean ± SEM MABP. (C) Populational consecutive averages of PI during ambient, vehicle and AITC (30 mM) exposures. (D) Mean ± SEM PI. Black data: Control animals; red data: Animals given terazosin (0.3 mg/kg, i.p., n=4). * denotes significant difference to vehicle response (p<0.05). # denotes significant difference between control and terazosin responses (p<0.05).
compared to vehicle, 162 ± 7.5, p<0.05) (Fig. 7.6, see page 95). This AITC-induced hypertensive reflex was similar to that seen in WKY rats, however, differential PI responses to AITC were seen in the SH and WKY rats pretreated with atropine. PI decreased in the SH rats (138.9 ± 0.7 ms compared to vehicle, 151.5 ± 2.1, p<0.05), but increased in the WKY rats (219.5 ± 15.5 ms compared to vehicle, 171.7, p<0.05). This differential response provides further evidence for an AITC-evoked increase in sympathetic activity in the SH rats, but not the WKY rats.

To confirm that the bradycardia seen in SH rats upon AITC inhalation was not secondary to the AITC-induced hypertension, we exposed the rats to AITC (30 mM) after pretreatment with the peripheral α1 antagonist terazosin (0.3 mg/kg, i.p.). We hypothesized that terazosin would have the same affects on the SH rats as the WKY

Figure 7.4: AITC evoked a hypertensive response in the SH rat. Left: Populational consecutive bin averages of MABP during ambient, vehicle and AITC (30mM) exposures. Right: Mean ± SEM MABP. * denotes significant difference to vehicle response (p<0.05), n=5.
rats and prevent BP responses evoked by AITC without inhibiting the AITC-induced bradycardia. As expected, AITC did not affect BP in the SH rats pretreated with terazosin (105.6 ± 4.7 mmHg compared to vehicle, 106.9 ± 3.8, n.s.) (Fig. 7.7, see page 96), but did evoke bradycardia (177 ± 22.6 ms compared to vehicle, 147.9 ± 2.7, p<0.05) (Fig. 7.8, see page 97). These findings suggest that the bradycardia seen in SH and WKY rats upon inhalation of AITC is a result of reflexes initiated in the airways and not the baroreceptors.

**Discussion**

We have shown that exposure to AITC caused BP changes in both healthy WKY rats and cardiovascular diseased SH rats. Although we were unable to establish
significant differences in the populational BP responses induced by AITC in the WKY rats compared to vehicle, we did see a biphasic BP response to AITC exposure in each individual that was similar to that seen in the SD rats. However, the onset of the biphasic BP response was inconsistent between animals and therefore, affected the ability of the mean data to accurately show BP responses to AITC in the WKY rats. AITC was also shown to evoke an initial hypertension in the SH rats, however, unlike the WKY or SD rats, BP gradually returned to baseline without the occurrence of a hypotensive phase. Furthermore, the AITC evoked BP increase in the SH rats was found to be two times greater than that seen in the WKY or SD rats. Although the AITC-induced hypertension was greater in the SH rats, in agreement with findings from our
ECG studies, PI analysis showed that AITC evoked bradycardia in SH and WKY rats with the bradycardic effects being greater in the WKY rats. These findings of differential BP and PI responses evoked by AITC in WKY and SH rats are indicative of sympathetic hyperactivity and parasympathetic insufficiency in the SH rats, which has been shown to characterize BP control in hypertensive animals and humans (Head, 1994). As mentioned previously, the initial hypertension evoked by AITC could be a consequence of increased sympathetic outflow caused by upper airway C-fiber activation (Yavari et al., 1996; Mutoh et al., 2000; Liu et al., 2013), while the consequent drop in BP seen in the WKY rats is likely a consequence of increased parasympathetic outflow resulting from lower airway C-fiber activation (Coleridge et al., 1964; Rinkema et al., 1982; Lee et al., 1996).
al., 1987). This would explain the greater hypertensive reflex evoked by AITC in the SH rats (sympathetic hyperactivity) and the absence of a consequent hypotension (parasympathetic insufficiency). However, this does not explain why AITC evoked less bradycardia in the SH rats as both upper and lower airway C-fiber activation evoke bradycardia (Coleridge et al., 1964; Lee et al., 1987; Yavari et al., 1996; Mutoh et al., 2000; Liu et al., 2013). Based on findings from our ECG studies, it is likely that the apparent decreased bradycardic response seen in the SH rats is due to a concomitant increase in tachycardic beats evoked by AITC. As discussed previously, it is possible that AITC-induced tachycardic responses were initiated by SAR fibers exhibiting de novo expression of TRPA1 (Zhang et al., 2008).

Figure 7.8: Terazosin did not prevent AITC-induced PI responses in the SH rat. Left: Populational consecutive averages of PI during ambient, vehicle and AITC (30 mM) exposures. Right: Mean ± SEM PI. Black data: Control animals; red data: Animals given terazosin (0.3 mg/kg, i.p.). * denotes significant difference to vehicle response (p<0.05).
To further investigate the differential responses evoked by AITC in the SH and WKY rats, we performed AITC exposures after pretreating the rats with the muscarinic antagonist atropine. The administration of atropine caused a significant increase in baseline BP, but only a mild increase in HR in the WKY rats. A slight increase in HR was also seen in the SH rats after atropine administration, however, baseline BP was found to be decreased. Given that atropine evoked mild tachycardia throughout these studies, the decrease in BP was unexpected. Previous studies have noted a variety of effects (hypotension, no effect and hypertension) in SH rats given atropine (Caputi et al., 1980; Abraham et al., 1981; Lazartigues et al., 1999; Raji et al., 2013). As SH rats have altered cardiac function with decreased ejection fraction (Kokubo et al., 2005), it is possible that the atropine-induced HR increase led to a reduction in cardiac output, thus decreasing BP. Nevertheless, as we have not studied the effect of atropine in paired experiments, we cannot rule out the involvement of other factors such as variations in surgical procedure. In regards to responses evoked by AITC in WKY and SH rats pretreated with atropine, we found that the AITC-induced pressor response was potentiated in both strains. An AITC evoked increase of sympathetic outflow caused by upper airway C-fiber activation could explain the increased pressor response in rats pretreated with atropine, in that these responses were not subject to concomitant increases in parasympathetic activity opposing sympathetic activation. Additionally, similar to the results found in our SD studies, atropine prevented the hypotensive phase evoked by AITC in the WKY rats further suggesting that this phase of the response is due to parasympathetic activation initiated by lower airway C-fiber activation. Analysis of AITC-induced PI responses showed similar results to those seen in the ECG studies,
wherein AITC had no effect on PI in the WKY rats, but evoked tachycardia in the SH rats. These findings suggest that the AITC-induced bradycardia is not dependent upon baroreflexes responding to the hypertension evoked by AITC. However, as atropine has been shown to affect baroreflexes (Knuepfer et al., 1989), it is difficult to make this assumption from these results alone.

To determine if the AITC-induced cardiac effects occur independent of baroreflex activation, we performed AITC exposures on rats pretreated with the peripheral α1 antagonist terazosin. As expected, terazosin caused a significant drop in baseline BP, but did not have an effect on baseline HR in either strain. The decrease in BP seen in the SH rats was more than twice that seen in the WKY rats, suggesting that peripheral vascular resistance is greater in the SH rats at rest. This is in agreement with findings from previous studies that showed SH rats to have increased vascular resistance due to increased basal sympathetic activity (Yamori, 1977). We found that the administration of terazosin prevented the AITC-induced BP effects in both strains, without affecting the bradycardic responses to AITC. As no significant differences were found between the AITC-induced PI increases seen in control animals and those pretreated with terazosin, it is unlikely that these bradycardic responses occur secondary to an increase in vascular tone resulting from increased baroreceptor firing. Furthermore, studies have shown that baroreceptor sensitivity with respect to HR control is attenuated in the SH rat (Frohlich & Pfeffer, 1975; Modolo Rda et al., 1995; Du et al., 1998; Bibevski & Dunlap, 2011). Current evidence suggests that this is a result of increased activity of the hypothalamic defense area selectively blocking the efferent parasympathetic component of the reflex bradycardia caused by baroreceptor activation (Ricksten &
Thoren, 1981). Therefore, the ability of AITC to evoke significant bradycardic responses in SH controls provides further evidence for the pathways involving the AITC-induced bradycardia to be separate from those initiated by baroreceptor activation. In agreement with this argument, studies have shown that NTS cells excited by bronchopulmonary C-fibers are rarely responsive to inputs from arterial baroreceptors or cardiac mechanoreceptors (Paton, 1998; Paton et al., 2000). Thus, such variation in afferent signal input could allow for selective modulation of transmission in pathways from distinct peripheral receptors.
CHAPTER 8

Conclusion

There is an increasing amount of evidence that short-term elevations in daily particulate matter (PM) levels pose a greater absolute risk for cardiovascular disease-related mortality than for all other causes (Pope, 2000). Furthermore, the deleterious effects of PM have been shown to be greater in individuals with pre-existing cardiovascular conditions such as hypertension, heart failure and myocardial ischemia (Pope & Dockery, 2006; Simkhovich et al., 2008; Brook et al., 2010). The mechanisms through which inhalation of PM leads to increases in cardiovascular morbidity and mortality are separated into two main pathways: chronic systemic inflammation (humeral response to PM uptake) and aberrant autonomic nervous system (ANS) balance resulting from activation of airway sensory nerve reflexes. The majority of current research into PM effects is focused mainly on the inflammatory pathways, but we would argue that more effort should be directed towards its effects on the ANS as cardiovascular mortality is linearly associated with day-to-day changes in PM levels. Acute PM exposures have been shown to alter ANS balance through its activation of pulmonary afferent fibers as evidenced by alterations in control of mean heart rate (HR (Elder et al., 2007)), heart rate variability (HRV (Pope et al., 1999)), and overall heart rhythm (Brook et al., 2010). However, our current understanding of how these effects
occur is lacking, as no approach to date has been effective in elucidating the mechanism of action by which PM inhalation leads to ANS imbalance resulting in arrhythmias and exacerbation of existing cardiovascular disease. Numerous previous studies (Brook et al., 2010) have used PM and a plethora of other pollutants to evoke the desired responses in various models, however, due to the many actions (inflammatory and direct binding) of these particles on various systems, the specific role of the pulmonary cardiac reflex has not been identified.

Recently, studies have provided evidence that could explain how PM initiates pulmonary-cardiac reflexes. These studies found that TRP channels expressed on nociceptive bronchopulmonary afferent C-fibers are activated by various forms of PM including coal fly ash (CFA), diesel exhaust (DE), wood smoke and residual oil fly ash (ROFA) (Bautista et al., 2006; Deering-Rice et al., 2011; Fariss et al., 2013). Additionally, PM-induced ECG changes were found to be prevented by TRPA1 inhibition in rats (Ghelfi et al., 2008), suggesting that arrhythmia evoked by PM inhalation occurs through TRP channel-mediated autonomic reflexes in the lung. To provide further evidence for this possibility, we recorded ECG and BP responses in healthy (SD and WKY) and cardiovascular diseased (SH) rats during controlled exposures to the selective TRPA1 agonist AITC. In healthy animals, the only nerve fibers innervating the airways found to express TRPA1 are slowly-conducting C-fibers (Hooper et al., 2015). Thus, the use of AITC as opposed to a PM variant allows for the specific activation of PM neural pathways/reflexes without the confounding effects of PMs other biological actions (Deering-Rice et al., 2011). By using this approach, we hoped to determine the mechanisms through which selective activation of the PM/TRPA1 neural pathways
evokes differential arrhythmic profiles in cardiovascular disease models (compared to healthy animals).

Given that the activation of airway C-fibers initiates parasympathetic reflexes (Coleridge & Coleridge, 1984; Lee & Pisarri, 2001), we hypothesized that AITC would evoke bradycardia in the healthy animals. As expected, exposing conscious SD and WKY rats to nebulized AITC evoked immediate and substantial bradycardia accompanied by PR-I prolongation and AV block. We found similar bradycardic responses in SD rats exposed to the selective TRPV1 agonist capsaicin, suggesting that these responses result from activation of nociceptive C-fibers innervating the airways, given that these are the only airway nerves to express both TRPA1 and TRPV1 (Nassenstein et al., 2008; Nassenstein et al., 2010).

To further investigate the level of involvement of TRP channels in the bradycardic responses, we exposed SD rats to AITC after pretreatment with the TRP channel inhibitor ruthenium red. Ruthenium red greatly reduced ECG responses to AITC, providing evidence for the direct involvement of TRP channels in these responses. The activation of TRPA1 and TRPV1 likely results in increased parasympathetic drive to the heart, given that similar responses were seen after direct stimulation of cardiac parasympathetic efferents (Levy & Zieske, 1969; Rinkema et al., 1982). Indeed, AITC-induced RR-I responses were almost entirely abolished after pretreating the SD and WKY rats with the muscarinic antagonist atropine. That atropine was not seen to completely prevent an AITC-induced increase in RR-I, could simply be explained by the dose not being sufficient for total muscarinic blockade. It is also possible that some
parasympathetic activity remained due to contributions from non-adrenergic, non-cholinergic (NANC) nerves (Rand, 1992).

In agreement with studies demonstrating that bradycardia evoked by smoke inhalation was completely abolished (Nakamura & Hayashida, 1992), or attenuated (White & McRitchie, 1973) by pentobarbital and chloralose anesthesia, we found that the administration of anesthesia prevented the bradycardia evoked by inhalation of AITC in the SD rats. This is likely due to the effects of anesthesia on the ANS, as anesthesia is known to cause variable block of homeostatic control of cardiovascular and respiratory rhythms (Elsner et al., 1966; Cullen et al., 1987; Watkins & Maixner, 1991; Sellgren et al., 1994). Furthermore, no ECG responses were seen after performing ANS reflex function tests in SD rats under ketamine anesthesia. Although anesthesia had substantial effects on ANS activity, we did see some persistence of defensive reflexes, as anesthesia was found not to inhibit the AITC-induced bradypnea. Additionally, tonic parasympathetic control of the heart was maintained after urethane administration, as a significant reduction in RR-I was seen after bilateral vagotomy. The contrasting effects of anesthesia on AITC-induced cardio-respiratory reflexes suggests significant differences between the central neural circuits involved with reflexes initiated by the airways.

Isoflurane anesthesia was also found to inhibit AITC-induced ECG responses with inhibition persisting even after apparent recovery from the anesthetic. This suggests that residual anesthesia has a profound effect on pulmonary-cardiac reflexes evoked by AITC. Nevertheless, we found that isoflurane inhibits responses to AITC in a reversible manner as responses to AITC returned following a recovery period of 45
minutes. The results from the isoflurane studies gave us confidence that we could effectively record responses to AITC in a decerebrate rat model as long as exposures were performed at least 45 minutes following decerebration and cessation of isoflurane administration. We found that exposing nebulized AITC into the lower airways of decerebrate rats evoked significant bradycardia associated with AV block. These responses to AITC seen after removal of the somatosensory brain regions suggest that sensation is not required for the AITC-induced bradycardia (Xing et al., 2015). Additionally, by isolating AITC exposures to the lower airways, we were able to show that AITC-induced responses occur through activation of TRPA1 channels expressed on bronchopulmonary C-fibers and do not require activation of TRPA1 expressed in the upper airways. It is possible that nebulized AITC may have entered the pulmonary circulation leading to activation of TRPA1 channels expressed on the heart, however, removal of the brainstem abolished responses to AITC suggesting that its effects were not due to a direct effect on the heart. It is also possible that the effects of AITC on respiration rate contribute to the effects seen on HR. While it is likely AITC-induced bradypnea contributes to some degree in the AITC-induced bradycardia, results from our decerebrate studies suggest that its contributions are minor as respiration rate was controlled through mechanical ventilation throughout the duration of these studies.

In addition to TRPV1 and TRPA1, the expression of the purinergic P2X\(_{2/3}\) receptor is also found on C-fibers innervating the airways (Kwong et al., 2008). However, the expression of P2X\(_{2/3}\) differs in that it is found only in nodose C-fibers with no expression seen in jugular C-fibers. Therefore, by selectively activating P2X\(_{2/3}\) with ATP (Burnstock, 2000; Undem & Carr, 2001), we hoped to determine if variations in
contributions from nodose and jugular C-fibers were occurring in the responses evoked by TRP channel activation. ATP evoked only minimal responses in the SD rats, suggesting that activation of nodose C-fibers has minimal effects, and activation of jugular C-fibers are responsible for the bradycardic reflex. However, we are unable to confirm this possibility given that no studies to date have investigated cardiac responses to selective activation of nodose C-fibers in conscious animals. It is also possible that the nature of the performed exposures allowed for the reduction of ATP to ADP prior to reaching the airways. If this was occurring, it would explain the lack of response to ATP as ADP does not activate P2X receptors (Spelta et al., 2003).

In regards to the effects of AITC inhalation on BP, we found that AITC in conscious SD and WKY rats yielded a biphasic blood pressure response, with an early hypertensive phase followed by a late hypotensive phase. Although populational BP responses of the WKY rats were affected by inconsistencies in the onset of the biphasic BP response between animals, we are confident that an increase in sample size would result in populational means similar to those seen in the SD rats. The initial hypertension is most likely due to activation of upper airway nasotrigeminal afferent C-fibers whose activation initiates the diving reflex resulting in bradycardia (parasympathetic) and hypertension (sympathetic) through increased peripheral vasoconstriction (Yavari et al., 1996; Mutoh et al., 2000; Liu et al., 2013). In agreement with this, we saw that pretreatment with atropine potentiated the AITC-induced hypertensive reflex while preventing the AITC-induced bradycardia. Similarly, previous studies have shown that atropine prevented bradycardic responses to intravenous AITC administration, but did not affect the pressor response (Pozsgai et al., 2010).
Hypertensive responses to activation of TRPA1 channels expressed in skeletal muscle by systemic administration of AITC have also been shown (Koba et al., 2011), however, the instantaneous nature of the hypertension argues against this possibility. The late hypotensive phase is best explained by AITC-induced activation of lower airway bronchopulmonary C-fibers evoking a parasympathetic depressor effect (Coleridge et al., 1964; Rinkema et al., 1982; Lee et al., 1987). The inhibition of AITC-induced hypotensive responses seen after pretreatment with atropine gives credence to this possibility. Whether the hypotension evoked by AITC is exclusively due to a drop in HR, or a decrease in peripheral resistance is difficult to say.

Acute increases in BP can cause reflex bradycardia secondary to baroreflex activation. We have provided evidence arguing against baroreflex involvement, as pretreatment with the α1 antagonist terazosin blocked the AITC-evoked changes in BP, but did not prevent the AITC-induced bradycardia. This is in agreement with previous studies that found prazosin prevented hypertensive responses of the diving reflex without affecting the bradycardic responses (Yavari et al., 1996), suggesting that the bradycardic responses occur independent of baroreceptor firing. Furthermore, the baroreflex has been shown to account for a 15 ms PI increase for every 10 mmHg in BP (Head & McCarty, 1987; Ferrari et al., 1991; Oliveira et al., 1996; el-Mas & Abdel-Rahman, 1998). The responses we found were 10 times greater than that, thus baroreflex activation cannot solely be responsible for the AITC-induced bradycardia. Taken together, our results suggest that much of the bradycardia is a direct effect of AITC on reflex parasympathetic signaling to the heart and not secondary to baroreceptor activity.
Interestingly, we found that AITC-induced cardiovascular responses differed in the cardiovascular diseased SH rat, such that AITC-induced HR effects switched from purely bradycardic (parasympathetic) in the healthy rats, to also evoking positive chronotropic (sympathetic) effects (and PVCs) in the SH rats. These differential responses evoked by AITC are similar to those seen in previous PM basic and clinical studies that showed PM evoked bradycardia in healthy animals (Nakamura & Hayashida, 1992; Lamb et al., 2012) and brady-tachy with PVCs in animals with cardiovascular disease (Wellenius et al., 2002; Simkhovich et al., 2008; Brook et al., 2010; Zanobetti et al., 2014). Differences as such, suggest that pre-existing cardiovascular disease alters pulmonary-cardiac reflexes so that PM and AITC induce tachyarrhythmias following a reflex increase in sympathetic drive. Responses to capsaicin were also found to be different in the SH rats, as capsaicin failed to evoke any ECG changes in the SH rat. This could be a consequence of a capsaicin-induced increase in sympathetic activity functionally antagonizing concomitant increases in parasympathetic activity evoked by capsaicin, however, an increase in sample size would be needed to confirm this possibility. Alterations in pulmonary-cardiac reflexes of SH rats allowing for TRP agonist evoked increases in sympathetic activity could be occurring through cardiovascular disease-induced plasticity of the afferent arm of the reflex. In agreement with a TRP agonist-evoked increase in sympathetic activity, we found that administration of the β1 antagonist atenolol prevented tachycardia and PVC responses, but did not affect the bradycardic responses evoked by AITC in the SH rats. Studies have shown de novo expression of TRP channels on pulmonary afferent SAR fibers (Zhang et al., 2008), which stimulate sympathetic drive (Coleridge et al., 1989;
Neuronal plasticity has been shown in stroke (Segal et al., 2016) and in the NTS and RVLM brain regions of hypertensive animals (Mischel et al., 2015; Gouraud et al., 2016), such that increases in sympathetic outflow alter regulation of cardiovascular autonomic activity. Indeed, baseline HR was unaffected by atropine, while atenolol caused a significant decrease in resting HR, agreeing with the notion that sympathetic activity controls HR in SH rats. It is well established that SH rats exhibit decreased parasympathetic activity and there is some evidence that stimulation of post-ganglionic parasympathetic cardiac efferents of SH rats results in exaggerated parasympathetic responses (Ferrari et al., 1992). Thus, normal C-fiber reflexes evoked by the TRP agonists could be causing tachyarrhythmia in SH hearts due to their intrinsic electrical dysregulation (Tsuboi et al., 1987; Kaseda & Zipes, 1988). We believe that this is unlikely, as vagal electrical stimulation does not evoke tachyarrhythmia in SH rats (Lee et al., 1980; Head, 1994). Furthermore, the AITC-evoked tachycardia increased in SH rats pretreated with atropine arguing against these responses being caused by exaggerated parasympathetic output.

Apart from tachyarrhythmic effects evoked by PM exposure, acute PM exposures have also been shown to evoke BP increases in cardiovascular diseased individuals (Dvonch et al., 2009; Kannan et al., 2010). It is believed that PM activates nociceptive C-fibers innervating the airways, which instigate reflex autonomic responses that alter ANS balance (Widdicombe & Lee, 2001). In agreement with these findings, we found that AITC evoked substantial hypertension in the SH rats without the occurrence of a hypotensive phase like that seen in the healthy animals. Furthermore, the AITC evoked BP increase in the SH rats was found to be two times greater than that seen in the WKY
or SD rats. These findings are indicative of sympathetic hyperactivity and parasympathetic insufficiency in the SH rats, which has been shown to characterize BP control in hypertensive animals and humans (Head, 1994). This would explain the greater hypertensive reflex evoked by AITC in the SH rats (sympathetic hyperactivity) and the absence of a consequent hypotension (parasympathetic insufficiency).

As seen in the healthy animals, pretreatment with atropine potentiated the AITC-induced pressor response in the SH rats. An AITC evoked increase of sympathetic outflow caused by upper airway C-fiber activation could explain the increased pressor response in rats pretreated with atropine, in that these responses were not subject to concomitant increases in parasympathetic activity opposing sympathetic activation. As in the ECG studies, AITC induced tachycardia in the presence of atropine in the SH rats. Therefore, some contributions to the AITC-induced hypertension likely came from the increase in HR.

Administration of the peripheral α1 antagonist terazosin caused a significant drop in baseline BP, but did not have an effect on baseline HR in SH rats. The decrease in BP seen in the SH rats was more than twice that seen in the healthy rats, suggesting that peripheral vascular resistance is greater in the SH rats at rest. This is in agreement with findings from previous studies that showed SH rats to have increased vascular resistance due to increased basal sympathetic activity (Yamori, 1977). Similar to the effects seen in healthy animals, terazosin prevented the AITC-induced BP effects, but did not prevent the bradycardic responses to AITC in the SH rats. Thus, it is unlikely that these bradycardic responses occur secondary to an increase in vascular tone resulting from increased baroreceptor firing. Furthermore, studies have shown that baroreceptor
sensitivity with respect to HR control is attenuated in the SH rat (Frohlich & Pfeffer, 1975; Modolo Rda et al., 1995; Du et al., 1998; Bibevski & Dunlap, 2011). Current evidence suggests that this is a result of increased activity of the hypothalamic defense area selectively blocking the efferent parasympathetic component of the reflex bradycardia caused by baroreceptor activation (Ricksten & Thoren, 1981). Therefore, the ability of AITC to evoke significant bradycardic responses in SH controls provides further evidence for the pathways involving the AITC-induced bradycardia to be separate from those initiated by baroreceptor activation. In agreement with this argument, studies have shown that NTS cells excited by bronchopulmonary C-fibers are rarely responsive to inputs from arterial baroreceptors or cardiac mechanoreceptors (Paton, 1998; Paton et al., 2000). Thus, such variation in afferent input could allow for selective modulation of transmission in pathways from distinct peripheral receptors.

We have provided significant evidence for the inhalation of the TRPA1 agonist AITC to be capable of evoking substantial cardiovascular effects in normal and cardiovascular diseased rats. Through pharmacological and surgical intervention studies, we were also able to show that the AITC-induced effects likely occur through direct activation of TRPA1 channels expressed on nerves innervating the airways with major contributions coming from the bronchopulmonary C-fibers. Although our results are in agreement with this conclusion, there are some future studies that could be done in order to strengthen this argument. Firstly, we are not confident that our ATP studies were sufficient for determining the relative contributions of nodose and jugular airway C-fibers in the AITC-induced responses, as ATP may have been metabolized or caused activation of A-fibers. To address this concern, we would expose the rats to adenosine.
and 2-methyl-5-hydroxytryptamine (5-HT), which will selectively activate nodose C-fibers (Chuaychoo et al., 2005; Chou et al., 2008). Secondly, we would like to perform AITC studies in a working heart-brainstem preparation (WHBP), which is an *in situ* preparation that retains cardiovascular response functionality and a eupneic respiratory motor pattern that can be implemented in adult rats (Paton, 1996; Wilson et al., 2001). By directly applying AITC onto the rat heart in a WHBP, we would hope to provide additional evidence that the AITC-induced responses are not a consequence of AITC entering the pulmonary circulation leading to activation of TRPA1 channels expressed on the heart. No studies to date have applied AITC to an adult rat WHBP, however, our hypothesis is supported by previous WHBP studies that found the application of capsaicin (TRPV1) onto the epicardial surface of the heart produced reflex effects that were prevented by vagotomy and, therefore, not direct (S. Cerritelli, 2012). Lastly, we would like to further investigate our hypothesis that the differential cardiovascular responses evoked by AITC in the SH rats is due to altered TRPA1 expression profiles. In agreement with this hypothesis, previous studies have shown that SH rats have enhanced expression of neurotrophic factors such as BDNF and GDNF when compared to normotensive controls (Atanasova & Lazarov, 2014). Furthermore, BDNF and GDNF have been shown to cause increased expression of TRPV1 and TRPA1, respectively, in cultured DRG neurons (Ciobanu et al., 2009). In order to further investigate this intriguing possibility, we would perform immunohistochemical analysis of TRPA1 expression in afferents innervating the airways and, in a separate study, expose SH rats receiving long-term treatment with an angiotensin-converting enzyme (ACE) inhibitor (captopril) to AITC. For the immunohistochemical studies, the cell bodies of afferents
innervating the airways would be identified by retrograde labeling using fluorescent Dil. The labeled neurons would then be incubated in primary antibody against TRPA1 and neurofilament (for identification of myelinated neurons). These triple staining experiments would determine the number of Dil-labeled, neurofilament-positive neurons that express TRPA1 and enable us to see if differences exist between the expression profiles of TRPA1 in WKY and SH rats. In regards to the captopril studies, we would give the SH rats captopril for a period of 10 weeks prior to exposing them to AITC, as this has been shown to be sufficient for lowering MABP to normotensive levels and reverse cardiac remodeling (Gupta et al., 2005; Mao & Li, 2015). Results from these studies would provide further evidence as to whether altered airway afferent TRPA1 expression and cardiac remodeling play a role in the differential responses induced by AITC in the SH rat.

In addition to the future SH rat studies, we would also like to investigate the effects of AITC inhalation in the chronic ischemia heart disease (CIHD) rat model. Post-MI patients are at-risk for PM-induced arrhythmia (Folino et al., 2009). Ligation of the left anterior descending (LAD) coronary artery is a well-characterized model of CIHD (Bader, 2010), which results in a decrease in blood supply to the left ventricle giving localized transmural MI with the typical morphological and functional cardiovascular changes seen in humans post-MI (e.g. ST elevation, PVCs, AV block, necrosis of myocytes and neutrophil infiltration) (Ren et al., 1998; Miyauchi et al., 2003; Li et al., 2010). The LAD ligation CIHD rat model has become an ideal model for identifying the mechanisms of PM-induced arrhythmias due to their increases in PVCs and decreased HRV seen upon exposure to PM (Wellenius et al., 2002; Anselme et al., 2007; Carll et
The surgery is relatively simple and has a low incidence of post-surgical mortality (Samsamshariat et al., 2005; Li et al., 2010). Given that PM activates TRPA1 and evokes similar responses in the SH and CIHD rats, we expect that exposing CIHD rats to AITC will result in similar cardiovascular responses to those seen here in the SH rat. In addition to performing the various methods of analysis shown here, we would like to also look into investigating a parameter known as heart rate turbulence (HRT). Although we were unable to perform HRV analysis for reasons discussed earlier, measurement of HRT provides another method for determining the status of ANS balance in healthy and cardiovascular diseased animals by measuring the hearts ability to recover after a single PVC. Immediately after the occurrence of a PVC in a normal heart, heart rate speeds for a few beats and then slows to baseline over the next 10 beats. The healthier the heart, the quicker this process is completed. The ability of HRT analysis to assess ANS balance is due to the opposing roles the sympathetic and parasympathetic nervous systems play in the hearts response to a PVC. The early ventricular contraction causes a decrease in cardiac output and consequent drop in BP due to the ventricles not having enough time to fill prior to the contraction. Baroreflexes respond to the drop in BP by increasing sympathetic outflow, which in turn causes an increase in heart rate and BP. Immediately, the increases in HR and BP lead to withdrawal of sympathetic activity and an increase in parasympathetic activity, which returns the HR to baseline (Wichterle et al., 2002). A potential issue with performing this analysis is that it requires the animals to have PVCs. PVCs occur commonly in healthy and diseased individuals, however, we have only recorded a PVC in 3 of our healthy
rats. This is likely due to the short duration of our recordings and as such, we will increase the time of our recordings if necessary.

The results of our studies presented show that the activation of TRP channels expressed on airway nerves evoke substantial cardiovascular responses in healthy and cardiovascular diseased rats. Additionally, TRP agonist-induced arrhythmia associated with negative cardiovascular outcomes (e.g. PVCs and tachycardia) was only seen in the cardiovascular diseased rats. This is similar to the findings of PM studies which found that individuals with pre-existing cardiovascular disease were more susceptible to negative cardiovascular outcomes upon inhalation of PM than healthy individuals (Brook et al., 2010). Currently, individuals with cardiovascular disease (e.g. hypertension and MI) are treated with one or varying combinations of ACE inhibitors, beta-blockers and calcium channel blockers depending on the individuals’ disease presentation. The prevention of PVCs and tachycardia evoked by AITC by the beta-blocker atenolol, suggest that these treatments can potentially attenuate deleterious cardiovascular effects evoked by PM in individuals with cardiovascular disease and should be given if they are not already receiving such treatment. Furthermore, our current lack in understanding as to how acute PM exposures leads to negative cardiovascular outcomes leaves us only able to simply advise patients to avoid going outside on days when PM levels are high. Here, we have provided evidence for the potential involvement of TRP channels in the acute effects evoked by PM inhalation, thus giving a potential therapeutic target more substantive than recommendations. Given that the mechanisms of activation of TRPV1 and TRPA1 have only recently begun to be elucidated, it is not surprising that there are currently no TRP channel inhibitors that
would be feasible for prescribing on days when PM levels are high. This is due to issues involving selectivity, bioavailability, toxicity and side effects of current TRP inhibitors that have hindered clinical development. However, we are confident that such inhibitors will be available in the near future, as our mechanistic understanding of these channels has improved greatly in recent years. Additionally, should the mechanism underlying the plasticity turn out to be due to neurotrophins, they could be targeted for therapy in the future. It is our hope that the results from our studies discussed here, in combination with results from future studies mentioned previously, will provide additional therapeutic targets for preventing the deleterious effects evoked by acute PM inhalation by aiding in the elucidation of its mechanisms of action.

In conclusion, TRPA1 (and to a lesser extent TRPV1) is the target of multiple inhaled pollutants including particulate matter, cigarette smoke, ozone, diisocyanates, formaldehyde, crotonaldehyde and acrolein. Inhibition or knockout of TRPA1 substantially limits neuronal responses to these pollutants. Inhalation of these pollutants is associated with acute exacerbation of cardiovascular and respiratory disease. Here, we have shown that AITC causes robust bradycardia and biphasic blood pressure responses in healthy animals via reflex modulation of the autonomic nervous system. Furthermore, we have provided evidence for differential cardiovascular responses evoked by AITC in cardiovascular diseased animals, wherein AITC evoked tachycardia and hypertension. These findings are in agreement with previous studies that found inhalation of PM caused an increase in heart rate variability (suggestive of increased parasympathetic drive) and second degree AV block in healthy animals, while a decrease in heart rate variability (suggestive of increased sympathetic drive) and
tachyarrhythmia was seen in cardiovascular diseased animals (Watkinson et al., 1998; Pope et al., 1999; Nalivaiko et al., 2003; Ghelfi et al., 2008; Luttmann-Gibson et al., 2010). Therefore, it is likely that similar acute reflexes occur with TRPA1-activating pollutants via pulmonary afferent activation.
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