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TRPA1 and Sympathetic Activation Contribute to Increased Risk of Triggered Cardiac Arrhythmias in Hypertensive Rats Exposed to Diesel Exhaust

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Running title: Role for TRPA1 in Diesel-Induced Arrhythmia Risk

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Keywords – Air pollution, diesel exhaust, cardiac, arrhythmia, TRPA1, sympathetic

Abbreviations –

CA – cardiac arrest

DE – diesel exhaust

ECG - electrocardiogram

fDE – filtered diesel exhaust

HF – high frequency

HR – heart rate

HRV – heart rate variability

LF – low frequency

PM – particulate matter

RR – ruthenium red

SH – Spontaneously hypertensive

TRPA1 – transient receptor potential cation channel, member A1

TRPV1 – transient receptor potential cation channel, member V1

VF – ventricular fibrillation

VPB – ventricular premature beat

wDE – whole diesel exhaust

Abstract

Background - Diesel exhaust, which is emitted from on- and off-road sources, is a complex mixture of toxic gaseous and particulate components that results in triggered adverse cardiovascular effects like arrhythmias.

Objective – We hypothesized that increased risk of triggered arrhythmias one day after diesel exhaust exposure is mediated by airway sensory nerves bearing transient receptor potential (TRP) channels (e.g., TRPA1) that, when activated by noxious chemicals, can cause a centrally-mediated autonomic imbalance and heightened risk of arrhythmia.

Methods - Spontaneously hypertensive (SH) rats implanted with radiotelemeters were exposed whole-body to either 500 $\mu g/m^3$ (high) of whole (wDE) or filtered (fDE) diesel exhaust , or 150 $\mu g/m^3$ (low) of wDE or fDE (4 hours). Arrhythmogenesis was assessed 24hrs later by continuous intravenous infusion of aconitine, an arrhythmogenic drug, while heart rate (HR) and electrocardiogram (ECG) were monitored.

Results - Rats exposed to wDE or fDE had slightly higher HR and increased LF/HF (sympathetic modulation), when compared to controls; ECG showed prolonged ventricular depolarization and shortened repolarization periods. Rats exposed to wDE developed arrhythmia at lower doses of aconitine than controls; the dose was even lower in rats exposed to fDE. Pretreatment of low wDE-exposed rats with a TRPA1 antagonist or sympathetic blockade prevented the heightened sensitivity to arrhythmia.

Conclusions - These findings suggest that a single exposure to diesel exhaust increases the sensitivity of the heart to triggered arrhythmias. It appears the gaseous components play an important role in the pro-arrhythmic response, which may be mediated by activation of TRPA1, and subsequent sympathetic modulation. As such, toxic inhalants may partly exhibit their toxicity by lowering the threshold for secondary triggers complicating assessment of their risk.

Introduction

Whole diesel exhaust (wDE) has been identified as a significant hazard to human health in a 2003 IRIS assessment by the U.S. Environmental Protection Agency (USEPA 2003). Acute exposures to wDE elicit a spectrum of effects on the respiratory tract which include inflammation and congestion, as well as physiological symptoms such as coughing and shortness of breath (Ris 2007). The concern over these multiple respiratory effects coincides with a growing awareness that wDE exposure causes adverse cardiac events as well, particularly in people who have underlying diseases such hypertension and heart disease. Recently, a study by Mills et al. indicated that wDE could impact cardiac function in humans with pre-existing heart disease (Mills et al. 2007). However only a few epidemiological studies have reported that a single exposure to diesel dominated traffic can compromise the electrical function and compensatory mechanisms of the heart, particularly in the 24 hours immediately following exposure. Among them are reports of increased incidence of cardiac arrhythmias immediately after exposure to air pollution (Link and Dockery 2010; Peters et al. 2000), including diesel exhaust (Anselme et al. 2007). These findings have stirred great interest in these potentially serious outcomes, but much is still unknown about the mechanisms underlying them.

We previously demonstrated that in normal rats a single exposure to particulate or gaseous air pollutants has the potential to “sensitize” the heart to subsequent arrhythmogenic stimuli, which is further worsened by the presence of underlying cardiovascular disease (Hazari et al. 2009). Furthermore, it has become clear that underlying cardiovascular disease plays an important role in the body’s ability, or inability, to maintain cardiac homeostasis in the presence of stressful stimuli hours to days after exposure. The American Heart Association statement on particulate matter (PM) air pollution and cardiovascular disease recently proposed several biological pathways by which air

pollution, PM in particular, can produce such a compromised state and impact cardiovascular disease (Brook et al. 2010). Of the known mechanisms, acute exposure-related increase in "arrhythmia potential" is often linked to autonomic nervous system reflex arcs, which are putatively initiated when sensory irritant nerves are activated in the nose and lungs, and which result in brainstem-derived changes in sympathetic and parasympathetic balance thereafter. The airways are innervated by sensory nerves bearing transient receptor potential (TRP) channels, namely TRPA1 and TRPV1, which detect different types of noxious chemicals, including many of those found in the complex mixtures of common air pollutants such as diesel exhaust. Activation of these nerves by airborne irritants like ozone or acrolein, causes centrally-mediated autonomic "imbalance", which produces ventilatory, pulmonary and cardiovascular function changes (Bautista et al. 2006; Bessac and Jordt 2008; Ghelfi et al. 2008).

In this study, aconitine, which targets voltage-dependent sodium channels in the myocardium, suppresses their inactivation, and thereby interferes with repolarization of the cardiomycyte membrane in preparation for the next beat, was employed as a challenge test (Hazari et al. 2009) to measure the arrhythmia sensitivity of spontaneously hypertensive (SH) rats following a single exposure to wDE. The aim of these experiments was to examine the role of TRP channels in the heightened arrhythmia sensitivity after wDE exposure, and determine if autonomic changes mediate the response. We hypothesized that a single exposure to wDE would sensitize the heart to aconitine-induced arrhythmia, a response which could be prevented by blocking TRPA1 (and possibly TRPV1) or by sympathetic modulation. It is believed that blockade of this receptor would prevent activation of airway sensory nerves and the autonomic reflex arc; and would thus prevent the heightened response to aconitine by severing the outgoing signal that connects the autonomic nervous system to the heart.

Materials and Methods

Animals. Eighteen to twenty-week old, male Spontaneously Hypertensive (SH) rats (Charles River, Wilmington, MA) weighing 300-400g were studied. Upon arrival, animals were housed two per cage with food and water available ad libitum in an AAALC-approved facility; all animals were treated humanely, particularly with regard to alleviation of suffering. All experimental protocols were approved by and in accordance with the guidelines of the Institutional Animal Care and Use Committee of the United States Environmental Protection Agency, Research Triangle Park, North Carolina.

Implantation of radiotelemeters and electrocardiogram acquisition/analysis. Methods are described in detail in Supplemental Material, Section 1A. In brief, Radiotelemeters were implanted in all animals as previously described (Hazari et al. 2009); this methodology was used to track changes in cardiovascular function by monitoring electrocardiogram (ECG) and heart rate (HR). Each animal was aseptically implanted with a radiotelemetry transmitter (Model TA11CTA-F40, Data Sciences International, St. Paul, MN) in the abdominal cavity and electrode leads were guided and secured in positions that approximated those of the lead II of a standard ECG. Using a remote receiver (DataART2.1: Data Sciences International, Inc., St. Paul, MN), HR and ECG waveforms were continuously acquired and saved during the 5-min baseline period and aconitine challenge.

ECGAuto software (EMKA technologies USA, Falls Church, VA) was used to visualize individual ECG signals, analyze and quantify ECG segment durations, calculate time-domain and frequency-domain measures of heart rate variability (HRV), and identify cardiac arrhythmias. The Lambeth

conventions (Walker et al. 1988) were used as guidelines for the identification of cardiac arrhythmic events in rats. Arrhythmias were identified as occurring sequentially during aconitine challenge as ventricular premature beats (VPBs), ventricular tachycardia (VT) and ventricular fibrillation (VF) (see Supplemental Material, Figure 1).

Groups. Table 1 shows the experimental groups. SH rats (n = 6/group) were assigned to a group within one of the following three experiments:

I. *Whole vs. filtered diesel exhaust* – These experiments were conducted to determine if lowering the concentrations of wDE would decrease arrhythmia risk, and assess the impact of only the gaseous components of wDE.

II. *Diesel exhaust and TRP* – Animals were pretreated [before exposure to either filtered air (FA) or wDE] with a TRPA1 antagonist (HCO30031), a general TRP antagonist (ruthenium red), or a TRPV1 antagonist (SB366791). These experiments were conducted to assess the role of TRP channels, which are located on the irritant sensory nerves of the upper and lower airways, on arrhythmia sensitivity after exposure to wDE (150μg/m^3 for 4hrs).

III. *Diesel exhaust and Autonomics* – Animals received bilateral vagotomy, the sympathetic adrenergic blocker guanethidine, or the muscarinic (parasympathetic) antagonist atropine, after exposure to either FA or wDE (30mins before aconitine challenge). These experiments were conducted to examine the role of the parasympathetic and sympathetic branches, which regulate heart function, in the arrhythmia response after exposure to wDE (150μg/m^3 for 4hrs).

Diesel exhaust generation and exposure. The method for generation of wDE has been previously described (Sharkhuu et al. 2010 and Supplemental Material Section 1B). Briefly, wDE for exposure experiments was generated using a Yanmar diesel generator using low sulfur diesel fuel (32 ppm). From the engine, the exhaust was mixed with particulate (HEPA) filtered room air. wDE concentrations were based on the fine particulate matter ($PM_{2.5}$; Mass Median Aerodynamic Diameter < 2.5 microns) fractions of the diluted exhaust. Target concentrations were 500 μg/m^3 (high) and 150 μg of PM/m^3 (low) which were routed to a filtered and unfiltered exposure chamber. The filtered chamber had almost no PM present but contained all the diluted combustion gases present in the unfiltered chamber. Control animals were placed in a third chamber supplied with HEPA filtered room air (FA). Continuous emission monitors (CEMs) were used to measure chamber concentrations of PM, oxygen, carbon monoxide, nitrogen oxides, and sulfur dioxide (SO_2) (see Supplemental Material, Table 1). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges.

Aconitine challenge. Twenty-four hours after exposure, all animals were anesthetized with urethane (1.5g/kg, *ip*) and underwent the aconitine challenge; supplemental doses of the anesthetic were administered intravenously when necessary to abolish pain reflex. Animal body temperature was maintained at ~36ºC with a heating pad. The left jugular vein was cannulated with P.E. 50 polyethylene tubing for the administration of aconitine. Aconitine (10 μg/ml) was continuously infused at a speed of 0.2ml/min while ECG was continuously monitored and timed. Sensitivity to arrhythmia was measured as the threshold dose of aconitine required to produce VPBs, VT, and VF was calculated using the following formula:

> Threshold dose (μg/kg) for arrhythmia = 10 μg/ml x 0.2ml/min x time required for inducing arrhythmia (min)/body weight (kg)

Drugs. The specific TRPA1 antagonist HC030031 (Chembridge, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO). The general TRP antagonist ruthenium red (RR) (Sigma-Aldrich, St. Louis, MO) and the specific TRPV1 antagonist SB366791 (Tocris, Ellisville, MO) were dissolved in 50% DMSO and 50% saline. Guanethidine monosulphate (U.S. Pharmacopeia, Rockville, MD) and atropine (Sigma-Aldrich, St. Louis, MO) were dissolved in saline. Stock solutions of aconitine (Sigma-Aldrich, St. Louis, MO) were dissolved in ethanol and then diluted to the desired concentration with saline.

Statistics. The statistical analyses for all data in this study were performed using SAS version 9.1.3 software, (SAS Institute Inc, Cary, NC). PROC MIXED and PROC GLIMMIX procedures were used to analyze all ECG and HRV-generated data. Tests of normality were performed for all continuous variables and parametric methods of analysis were used. A linear mixed model with restricted maximum-likelihood estimation analysis (SAS) and least squares means post hoc test were used to determine statistical differences for all data. All aconitine dose-response data were analyzed using an analysis of variance (ANOVA) for repeated measures. $P < 0.05$ was considered as statistically significant. Reported values represent means ± standard error (SE).

Results

Heart rate. Twenty-four hours after exposure, urethane anesthetized SH rats exposed to high or low wDE or fDE had slightly higher heart rates (HR) than FA controls (see Supplemental Material, Figure 2). HR did not increase in response to low wDE in rats pretreated with the TRPA1 antagonist, but rats pretreated with either the TRP antagonist or TRPV1 antagonist had a larger (non-significant) increase in HR following wDE than untreated rats. HR increased significantly

following low wDE exposure ($p < 0.05$ relative to FA controls) in vagotomized rats and rats treated with atropine, but the increase in HR was less pronounced in rats treated with guanethidine.

Electrocardiogram. Following exposure to wDE or fDE we observed a prolongation of ventricular depolarization and a shortening of ventricular repolarization, indicated by an increase in QRS duration and decrease in ST segment duration, respectively (see Supplemental Material, Figure 3). There was no indication of heterogeneity of repolarization, as indicated by QTc. Pretreatment of rats with either the TRPA1 antagonist, TRP antagonist or the TRPV1 antagonist prevented the increase in QRS duration, and in fact, pretreatment with the TRPA1 or TRP antagonist actually caused a significant increase in the ST segment duration. Vagotomy and atropine appeared to prevent the increase in QRS duration caused by low wDE, but not the decrease in ST segment duration. Post-exposure guanethidine treatment seemed to partially reverse effects of wDE on ST segment duration, but did not appear to alter QRS duration or QTc in response to exposure. There were no significant effects of the drugs on FA rats (not shown).

Heart rate variability. Rats exposed to wDE or fDE had non-significant decreases in R-R intervals compared with FA controls (see Supplemental Material, Table 2). When adjusted for HR, only rats exposed to low wDE and low fDE had increases in the time-domain HRV measures [standard deviation of the time between normal-to-normal beats (SDNN), and root mean squared successive differences (RMSSD)] compared to controls. The low frequency/high frequency (LF/HF) ratio, an estimate of the relative balance between sympathetic (LF) and vagal (parasympathetic, HF) activity, was increased in all DE-exposed rats compared with controls, but the difference was significant for high fDE only. Treatment of rats with the TRPA1 antagonist appeared to prevent effects of low wDE on R-R, SDNN, RMSSD and the LF/HF ratio. The decline in R-R was more pronounced

following low wDE exposure in rats pretreated with TRP and TRPV1 antagonists than in rats that were not pretreated. SDNN, RMSSD and LF/HF increased following low wDE exposure in rats without pretreatment, but decreased following exposure in rats pretreated with TRP or TRPV1 antagonists. As expected, vagotomy decreased RR, SDNN, and RMSSD, and increased LF/HF in FA rats. In low wDE-exposed rats, vagotomy caused similar but even more pronounced changes in these parameters, which were significantly different from untreated low wDE rats. Atropine and guanethidine had little effect in FA rats. However, atropine and guanethidine decreased all parameters, some significantly, with respect to untreated low wDE-exposed rats.

Aconitine challenge. During aconitine infusion, the first arrhythmia to be manifested was the VPB, continued infusion of aconitine then elicited 3 or more successive VPBs or VT, and then VF. Figure 1A shows the dose of aconitine that elicited arrhythmia in SH rats exposed to high wDE. The cumulative dose of aconitine necessary to trigger VPB, VT, and VF was lower in rats exposed to high wDE compared to controls. High fDE appeared to be as potent as high wDE, or more so in the case of VF. The cumulative dose of aconitine needed to trigger the same arrhythmias was lower, but significantly only for VT, in rats exposed to low wDE than in FA rats; and like the high fDE, low fDE was either as potent as the low wDE in sensitizing animals to arrhythmogenesis or more (Fig. 1B).

Figure 2 shows the responses to aconitine in the low wDE and TRP experiments. Rats pretreated with the TRPA1 antagonist did not show increased responsiveness to aconitine following low wDE exposure (Fig. 2A), whereas pretreatment with the general TRP antagonist appeared to make the animals hyporesponsive to aconitine following low wDE (Fig. 2B). The TRPV1 antagonist appeared to prevent increased sensitivity to aconitine caused by low wDE based on VPB and VT,

but aconitine doses required to elicit VF and CA following wDE were comparable to those in rats without pretreatment (Fig. 2C). None of the drugs influenced responsiveness to aconitine in the FA rats. Treatment with the sympathetic blocker guanethidine prior to aconitine challenge prevented the potentiated arrhythmic response to aconitine and significantly increased the dose necessary to cause CA in low wDE-exposed rats; it had no effect on controls (Fig. 3A). On the other hand, the muscarinic antagonist atropine did not affect sensitivity to aconitine following low wDE, and vagotomy only prevented it for VBP. More significantly, FA rats treated with atropine or those that were vagotomized developed arrhythmia at lower cumulative doses of aconitine than vehicle-treated controls; these responses resembled those of low wDE-exposed rats (Fig. 3B and C).

Discussion

The data presented in this study suggest that a single exposure to a complex air pollutant increases susceptibility to triggered cardiac arrhythmias one day after exposure. Although the degree to which the high wDE increased arrhythmia sensitivity was expected, the comparable potency of the low wDE was surprising. These experiments also suggest that the chemosensor TRPA1, which is stimulated by irritant gases (e.g. acrolein) typically present in wDE (Krivoshto et al. 2008), likely contributes to the pro-arrhythmic response by causing autonomic imbalance and a shift towards sympathetic activation. These results, considered with the strong effect of the fDE (gases alone), point to the complexity of studying the short-term cardiac effects of multi-pollutant mixtures and the interaction of their components at certain concentrations.

Few studies have demonstrated that a single air pollution episode directly alters cardiac electrophysiology in a manner that would be potentially detrimental. However, as we report here,

the effect of one exposure on heart rhythm may be latent and indirect, altering the degree to which the cardiovascular system can withstand stress and/or lowering the threshold for initiation of adverse ventricular arrhythmias in response to a stimulus, particularly in compromised individuals. As far as wDE is concerned, Anselme et al. previously showed a 200 to 500% increase in VPB arrhythmias in chronic heart failure (CHF) Wistar rats exposed to 500 $\mu g/m^3$ wDE, which persisted for more than 5 hours, but they saw no change in wDE-exposed healthy rats (Anselme et al. 2007). Therefore, the challenge approach employed in our study reveals a unique way to identify subtle impacts of air pollution exposure that may be effective not only in animals with underlying cardiovascular disease, such as the CHF Wistar rats or SH rats, but also as we have previously shown (Hazari et al. 2009), in healthy strains such as the WKY.

The finding that low wDE, which had PM concentrations comparable to human studies (Kipen et al. 2010; Mills et al. 2007; Mills et al. 2010), caused comparable arrhythmia sensitivity to high wDE was unexpected. A search of the relevant literature indicates that this lack of dose-dependency has been reported by others. Hansen et al. (2007) found that low wDE exposure caused significant impairment of endothelium-dependent vasorelaxation in mildly atherosclerotic mice, whereas the high wDE had no effect. Studies have shown that lower particle masses of DE contain a greater fraction of ultrafine and nano-sized particles (Bagley et al. 1996; Johnson and Baumgard 1996), which could penetrate more distally and increase the degree of sensory activation; thus accounting for the effects observed with the low wDE. Additionally, these smaller particles have a larger surface area onto which known toxic organic compounds from diesel exhaust, such as polycyclic aromatic hydrocarbons (PAH), can adsorb and ultimately be transported deeper into the lung (Ris 2007). Thus it is likely that chemical composition, as demonstrated with concentrated ambient particles (CAPs) with higher metals and organic carbon concentrations (Kodavanti et al. 2005), may be a more important determinant of biological effects than shear particle mass. To be clear

however, our results should not be taken to suggest that the higher wDE concentrations are less toxic, rather it may be that the toxicity profile is different for the high and low concentrations. Similar inversed responses were observed with wDE exposures in mice, where 100 $\mu g/m^3$ wDE caused an increase in interleukin-4 (IL4) in mouse lungs, which the authors suggested contributed to development of allergic airways, whereas 3 mg/m^3 wDE resulted in suppression (Saito et al. 2002).

Along with PAHs, wDE also consists of highly toxic gases, which include nitrogen oxides (NOx), sulfur oxides (SOx), ozone, carbon monoxide (CO) and various aldehydes (e.g. acrolein) (Krivoshto et al. 2007; Ris 2007). Although many studies and assessments stress the adverse effects of particulates (Metzger et al. 2007; Wellenius et al. 2002), our findings suggest an important role for the gases, the concentrations of which were not affected by particle removal, in promoting cardiac arrhythmogenesis in the short term (Hesterberg et al. 2009). It may be that the presence of particles changes the composition, and therefore toxicity, of the gaseous components of wDE due to adsorption or chemical transformation. Regardless, several epidemiological studies have shown a significant association between increases in ventricular arrhythmias and increased levels of NO_2 and CO (Peters et al. 2000), and SO_2 (Ljungman et al. 2008; Rich et al. 2006). Most of these gases are considered to be serious respiratory irritants, yet information on the short-term consequences of exposure is limited, particularly with respect to reflex control of the cardiovascular system. The levels of CO and NOx were significantly higher in our wDE exposures when compared to the FA. However, even though the level of these gases was higher in the high wDE than the low wDE, the latter caused greater sensitivity to arrhythmia, suggesting the response may be driven more by other components like the aldehydes, which were not measured during exposure but should not be ruled out given their irritant characteristics (Hazari et al. 2008).

Exposure to inhaled irritants (Nishino et al. 1996), including air pollution, has repeatedly been shown to cause immediate cardiac changes such as decreased heart rate, alterations in heart rate variability and ECG, and dysrhythmia in humans (Gold et al. 1999; Pope et al. 1999). These reflexive effects are also seen in animals. However, it has also been demonstrated in animals that significant activation of these sensory receptors causes a sensitization or priming of the reflex (Widdicombe and Lee 2001). For instance, ozone not only causes acute irritant responses through the airway sensory receptors, it sensitizes and enhances their excitability to subsequent stimuli (Ho et al. 1998; Joad et al. 1998). Similarly, exposure to sidestream tobacco smoke was found to not only sensitize bronchopulmonary C-fibers, but also transmitted this enhanced excitability to the nucleus tractus solitarius (NTS), which regulates outgoing autonomic signals, as well as other efferent information, to the heart and lungs (Mutoh et al. 2000). Thus based on these data, we assumed that exposure to wDE would not only cause immediate exposure-related physiological changes (not measured), but also sensitize the airway autonomic reflex arc (see Figure 4), resulting in autonomic imbalance and increased sensitivity to developing arrhythmia.

In the current study, we indirectly examined the role of airway C-fibers in increasing the sensitivity of animals to aconitine-induced arrhythmia after exposure to wDE by testing the involvement of TRPA1 and TRPV1 ion channels, which are the chemical-sensing structures colocalized to these fibers. TRPA1 is activated by irritants commonly found in wDE such as acrolein (Bautista et al. 2006), ozone (Taylor-Clark and Undem 2010), oxidizing agents, and other aldehydes, whereas TRPV1 is activated by capsaicin, the pungent chemical found in red chilies. It was not entirely a surprise that TRPA1 antagonism prevented the wDE-induced heightened sensitivity to arrhythmia. Multiple studies have shown that an initial challenge may activate TRPA1 channels, and in the presence of inflammation result in hypersensitivity to a host of other chemical stimuli thereafter

(see review by Bessac and Jordt 2008). So, we assumed that blockade of TRPA1 not only inhibited sensitization of the afferent pathways, but also prevented any acute or short-term changes in centrally-mediated autonomic imbalance. The partial effects of TRPV1 may be explained by the fact that it can be activated secondary to TRPA1-mediated Ca^{++} mobilization or through the release of inflammatory mediators (Bessac and Jordt 2008). As such, a role for TRPV1-mediated lung reflexes in triggering cardiac rhythm disturbances and oxidative stress after CAPs has already been shown (Ghelfi et al. 2008). Finally, our future experiments will seek to determine if TRPA1 mediates heightened sensitivity to arrhythmia in animals exposed to filtered DE; this would clarify whether the gases in wDE drive this response or not.

Air pollution has been shown to dysregulate the autonomic nervous system (see review Simkhovich et al. 2008), linking exposure to both decreases (Gold et al. 2000) and increases (Pope et al. 1999) in HR. Most, but not all studies have demonstrated a decrease in HRV, which has also been observed in animals exposed to ROFA (Wellenius et al. 2002) or CAPs (Godleski et al. 1997); these types of responses are consistent with increased sympathetic activity. Blockade of sympathetic activity using guanethidine, which targets peripheral adrenergic neurons, prevented heightened aconitine-induced arrhythmia, which seems to confirm the predominant finding of exposure-related enhancement of sympathetic tone in both humans and animals. It is yet unclear whether this protective effect is due to reduced cardiac rate/contractility, or vasodilatation, both of which are the result of guanethidine treatment. Regardless, autonomic shift towards increased sympathetic drive following exposure to wDE predisposes the animal to triggered arrhythmia; and as such, represents a disruption of homeostatic balance one day after exposure. This is further illustrated by the fact that both atropine or vagotomy (removal of parasympathetic influence), which had minimal to no effect on aconitine sensitivity in response to wDE, increased arrhythmia sensitivity in rats exposed to air,

suggesting the balance of activity from these autonomic nerves is important in maintaining normal function both in a healthy and "challenged" state.

Paradoxically, time-domain HRV (SDNN and RMSSD) was decreased in rats exposed to high wDE or high fDE but increased in rats exposed to low wDE or low fDE. However, all DE-exposed rats had small increases in LF/HF, except the significant increase observed with high fDE. Peretz et al. observed similar opposite HRV effects of high and low diesel exhaust concentrations in healthy individuals and concluded that they did not observe a consistent effect on the autonomic control of the heart (Peretz et al. 2008). Similarly, Anselme et al. found that HRV changes due to wDE exposure were only transient when compared with persistent ventricular pro-arrhythmic effects, indicating HRV may only be a marker of exposure and not so much directly involved in the mechanism of arrhythmogenesis (Anselme et al. 2007). Instead, the authors suggested that the immediate pro-arrhythmic response was due to direct cardiac effects of certain wDE constituents that enter the circulation, while the persistent effect was due to inflammation and oxidative stress. We cannot address the role of inflammation or oxidative stress in mediating these heightened arrhythmia responses since they were not measured. However, it is likely that these conditions alter the local tissue environment which controls the excitability of cardiac tissue and the neurons innervating it. Therefore it remains to be determined whether the response of the autonomic nervous system is directly due to airway-mediated reflexes or secondary to inflammation and oxidative stress, or a combination of the two.

In this study, blockade of TRPA1 prevented the increase in HRV induced by low wDE. These effects on HRV were also observed in vagotomized animals and those treated with atropine or guanethidine. However, in these data, the LF/HF, which represents the balance between sympathetic and parasympathetic tone, may be the main indicator of protection against DE-induced

arrhythmogenesis. Treatment with the TRPA1 antagonist or sympathetic blockade lowered the LF/HF and prevented wDE-induced arrhythmia sensitivity. Additionally, the air-exposed rats that were vagotomized developed increased arrhythmia sensitivity to aconitine had higher LF/HF, suggesting that autonomic imbalance marked by increased sympathetic drive may be mediating the pro-arrhythmic response.

ECG changes were observed one day following exposure, but it is unclear whether these changes would predispose to greater risk of developing arrhythmia. Exposure to wDE caused prolongation of the QRS duration, which represents lengthening of ventricular depolarization and is believed to predispose individuals with underlying heart disease to an increased risk of ventricular arrhythmias (Kashani and Barold 2005), and decreased ST segment length. Decreased ST segment length in response to wDE was prevented by both TRPA1 and sympathetic blockade, suggesting the shorter duration of repolarization after wDE may also contribute to greater arrhythmia sensitivity. Similar ST segment decreases have been observed in human beings exposed to wDE (Mills et al. 2007). However, there was no evidence of repolarization abnormalities or ST depression.

To conclude, traditional environmental health evaluations focus on the monotonic exposure-response paradigm, and although valuable, in the case of some physiological outcomes as might be observed in the cardiovascular system, such an approach does not take into account exposure-induced “sensitization” or “priming” of secondary responses from other triggers. The findings of this study demonstrate that a single exposure to wDE increases the sensitivity of the cardiac electrical conduction system (perhaps lowering a threshold) such that it increases the risk of triggered arrhythmias. How reversible these effects are remains to be determined. Additionally, evidence that fDE (i.e., gases alone) elicits more cardiotoxic effects at these exposure levels than

wDE suggests that source apportionment for multi-pollutant mixtures is more complex than simply attributing the effects to the sum total of individual components. It also appears that heightened arrhythmia sensitivity may be mediated by activation of TRPA1 (on airway sensory nerves), which are particularly sensitive to inhaled irritants, and autonomic imbalance with a shift towards sympathetic activation. Taken together, this work highlights the importance of air pollution-induced irritation in the nose and lungs on the persistent short-term effects of a single exposure, particularly for people with underlying cardiovascular disease, and warrants further investigation.

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Table 1. Experimental Groups

	EXPERIMENT	EXPOSURE	TREATMENT
I.	Whole vs. filtered DE	Filtered air (FA)	
		500 μg/m^3 whole diesel exhaust (high wDE)	
		500 μg/m^3 filtered diesel exhaust (high fDE)	
		150 μg/m^3 whole diesel exhaust (low wDE)	
		150 μg/m^3 filtered diesel exhaust (low fDE)	
II.	wDE and TRP	150 μg/m^3 whole diesel exhaust (low wDE)	Vehicle
			TRPA1 antagonist (5mg/kg i.p.)
			TRP antagonist (2.5mg/kg i.p.)
			TRPV1 antagonist (5mg/kg i.p.)
		Filtered Air (FA)	TRPA1 antagonist (5mg/kg i.p.)
			TRP antagonist (2.5mg/kg i.p.)
			TRPV1 antagonist (5mg/kg i.p.)
III.	wDE and Autonomics	150 μg/m^3 whole diesel exhaust (low wDE)	Vehicle
			Vagotomy
			Atropine (0.5mg/kg i.p.)
			Guanethidine (5mg/kg i.p.)
		Filtered Air (FA)	Vagotomy
			Atropine (0.5mg/kg i.p.)
			Guanethidine (5mg/kg i.p.)

TRPA1 antagonist – HC030031
TRP antagonist – Ruthenium red
TRPV1 antagonist – SB366791

Figure legends

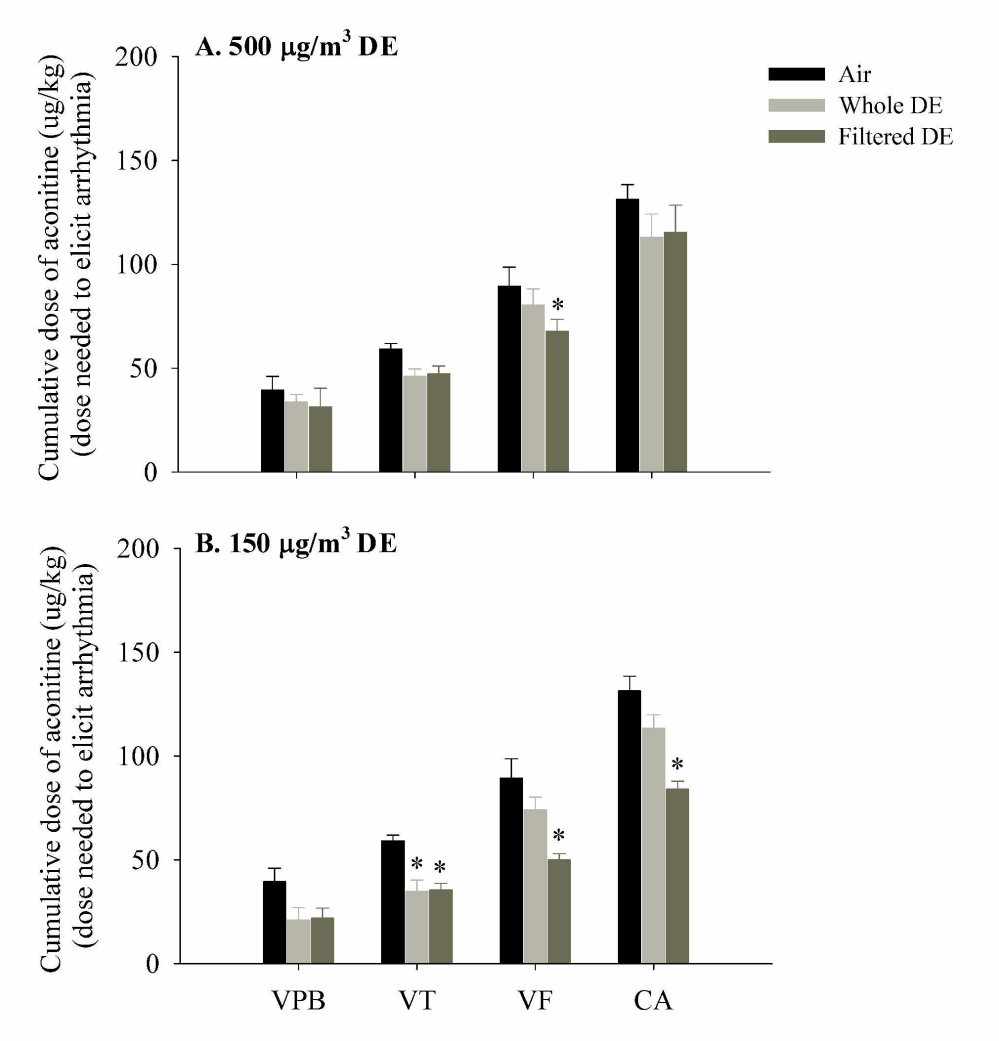
Figure 1. A single exposure to diesel exhaust increases aconitine-triggered arrhythmia in hypertensive rats. Constant infusion of aconitine (2μg/min) triggers VPB, followed by VT, VF and progression to cardiac arrest (CA) in FA-exposed SH rats. The cumulative dose of aconitine necessary to trigger arrhythmia and CA in SH rats exposed to high wDE or fDE (1A), or low wDE or fDE (1B) was lower than FA-exposed rats. Values are mean ± SEM; * significantly different from FA controls; $p < 0.05$, n = 5-6.

Figure 2. Heightened arrhythmia sensitivity after diesel exhaust exposure is mediated by TRPA1. Constant infusion of aconitine (2μg/min) triggers VPB, followed by VT, VF and progression to cardiac arrest (CA) in FA-exposed SH rats. The cumulative dose of aconitine necessary to trigger arrhythmia and CA in SH rats exposed to low wDE was lower than FA; this response was blocked by the TRPA1 antagonist (**A.**). The TRP antagonist caused a significant decrease in sensitivity relative to FA (**B.**), however the TRPV1 antagonist only reduced sensitivity to VPB and VT (**C.**). There was no effect of the drugs on controls. Values are mean ± SEM; * significantly different from control; $p < 0.05$.

Figure 3. Heightened arrhythmia sensitivity after diesel exhaust exposure is mediated by sympathetic activation. Constant infusion of aconitine (2μg/min) triggers VPB, followed by VT, VF and progression to cardiac arrest (CA) in FA-exposed SH rats. The increased arrhythmia response to aconitine after low wDE was prevented by acute sympathetic blockade with guanethidine (**A.**), but not by muscarinic blockade with atropine (**B.**). Vagotomy only partially reduced the low wDE-induced pro-arrhythmic response (**C.**). There was no effect of sympathetic blockade in FA-exposed animals (**A.**); however, muscarinic blockade (**B.**) and vagotomy (**C.**) increased aconitine-induced

arrhythmia sensitivity in FA rats. Values are mean ± SEM; * significantly different from control; $p < 0.05$.

Figure 4. Simplified depiction of how exposure to air pollutants is proposed to sensitize the sensory-to-autonomic reflex arc, and alter subsequent responses. Activation of airway sensory nerves (A) causes local effects and stimulates neurons in the midbrain (B). Neural circuits within the midbrain process the signals and then activate the preganglionic autonomic neuron (C); postganglionic autonomic neurons (D) carry the signal to the heart. Air pollution may increase the excitability of neuron A (sensitization), which would potentiate transmission in neuron B, and in turn increase/decrease CNS outflow by neuron C thereafter (autonomic imbalance). Adapted from Undem et al 2000.



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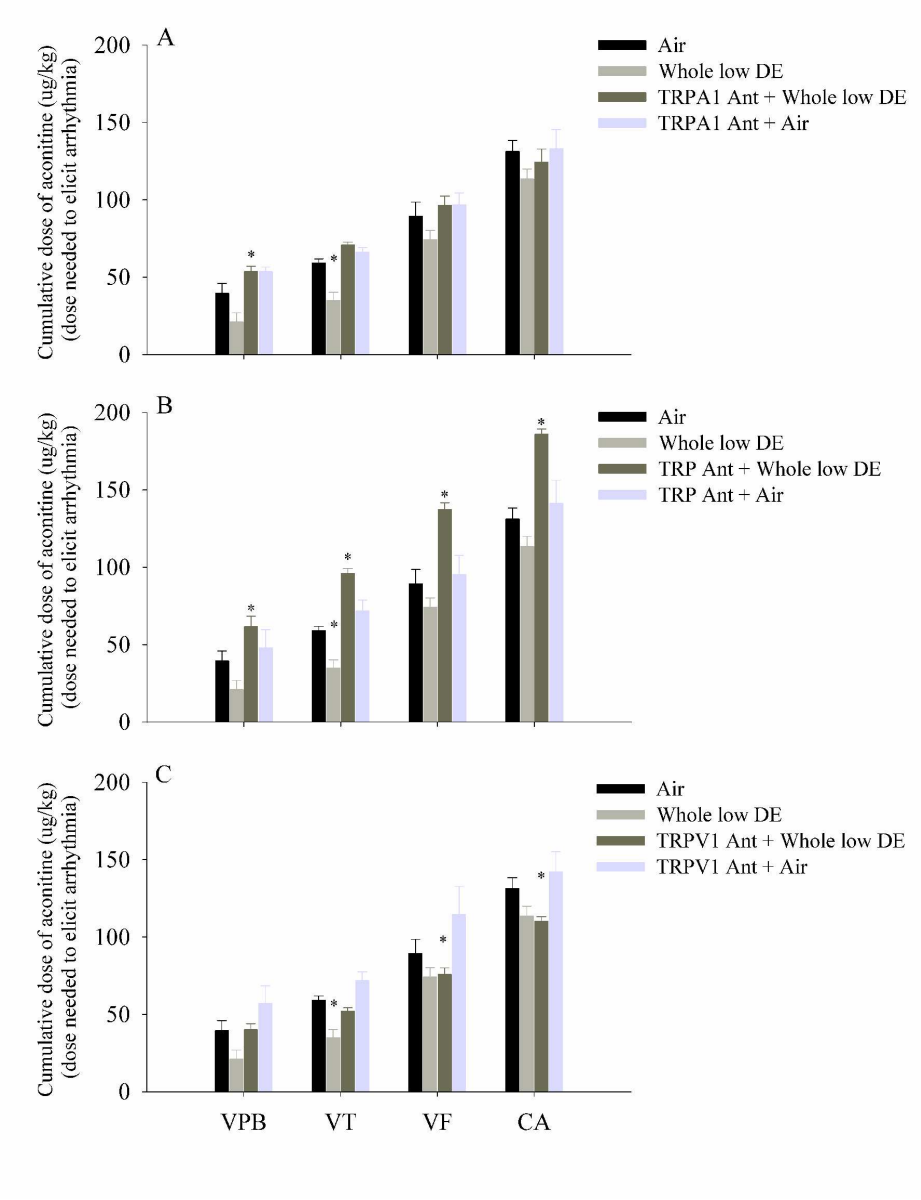

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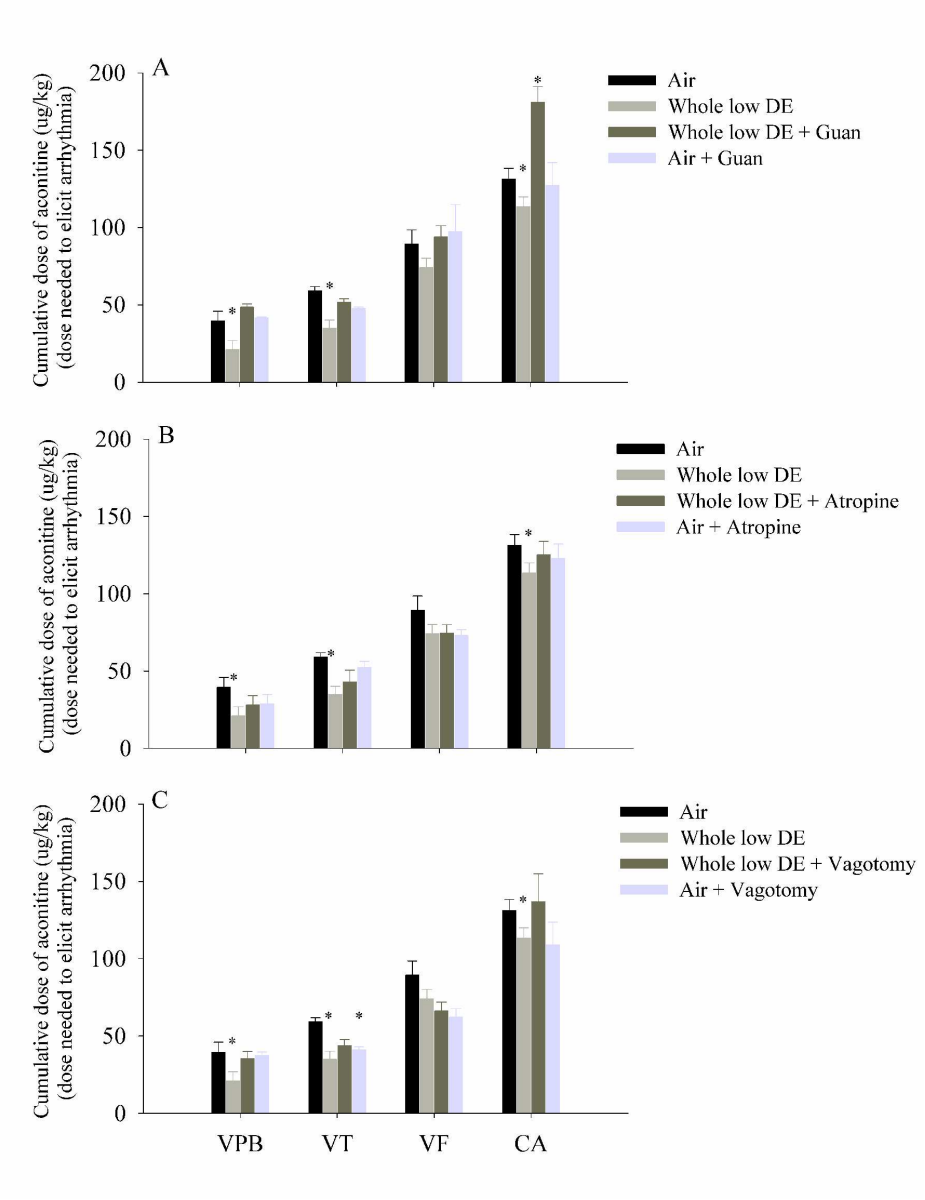

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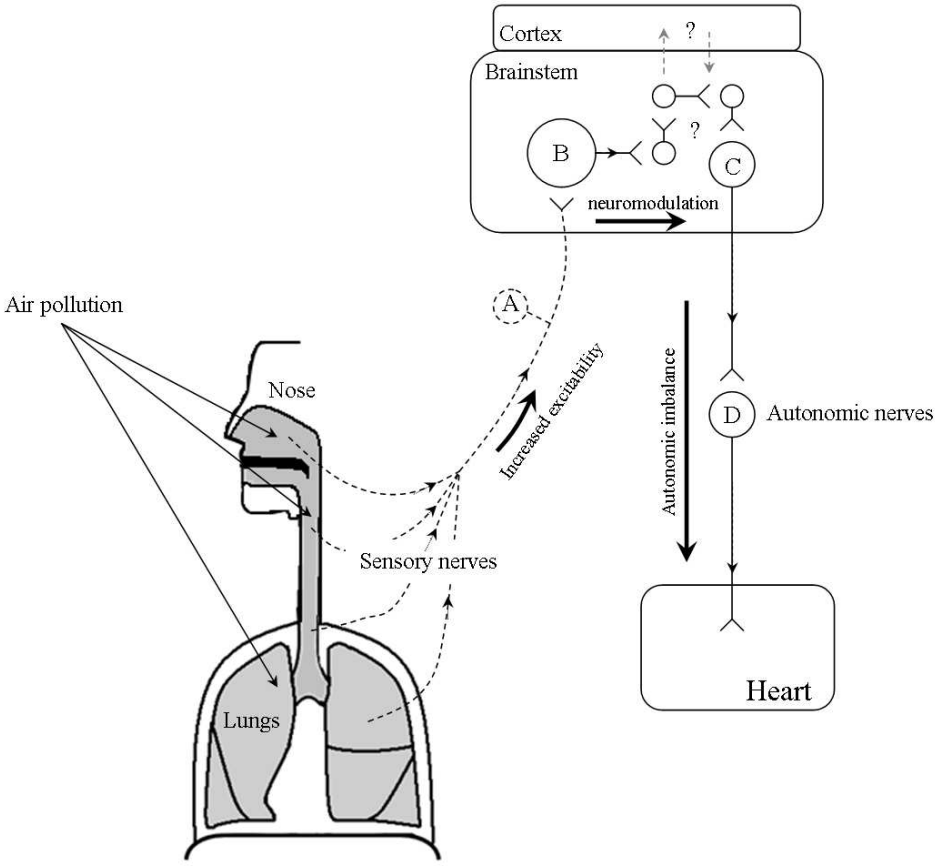

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186x177mm (150 x 150 DPI)