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Cardiac Oxidative Stress and Electrophysiological Changes in Rats Exposed to Concentrated Ambient Particles are Mediated by TRP-Dependent Pulmonary Reflexes

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Previous studies suggest that, through the stimulation of pulmonary nervous endings, ambient particles modulate the autonomic tone on the heart leading to cardiac oxidant stress and dysfunction. In this paper we investigated the effect of blockade of vanilloid receptor 1 (Transient Receptor Potential Vanilloid Receptor 1 [TRPV1]) on concentrated ambient particles (CAPs)–induced cardiac oxidative stress and dysfunction in a rat model of inhalation exposure. Capsazepine (CPZ), a selective antagonist of TRPV1, was given ip or as an aerosol immediately before exposure to CAPs. Control and CPZ-treated rats were exposed to filtered air or CAPs aerosols for 5 h using the Harvard Ambient Particle Concentrator (mean $PM_{2.5}$ mass concentration: 218 ± 23 $\mu g/m^3$). At the end of the exposure we measured cardiac oxidative stress (*in situ* chemiluminescence [CL]), lipid peroxidation (thiobarbituric acid reactive substances [TBARS]), and tissue edema. Cardiac function was monitored throughout the exposure. CPZ (ip or aerosol) decreased CAPs-induced CL, lipid TBARS, and edema in the heart, indicating that blocking TRP receptors, systemically or locally, decreases heart CL. CAPs exposure led to significant decreases in heart rate (CAPs 350 ± 32 bpm, control: 370 ± 29), and in the length of the QT, RT, Pdur and Tpe intervals. These changes were observable immediately upon exposure and were maintained throughout the 5 h of CAPs inhalation. Changes in cardiac rhythm and electrocardiogram morphology were prevented by CPZ. These data suggest that current abnormalities in CAPs-exposed rats alter the action potentials leading to changes in conduction velocity and ventricular repolarization, and that triggering of TRPV1-mediated autonomic reflexes in the lung is essential for the observed changes in cardiac rhythms.

Key Words: **unmyelinated C-fibers; TRP receptors; cardiac oxidative stress; ambient particles; PM.**

Epidemiologic studies show an association between short-term increases in the levels of particulate air pollution (PM) and increased cardiovascular hospital admissions and mortality (Pope, 2000; Schwartz, 1994). Although the relative effects of PM are greater for respiratory than cardiovascular mortality, the absolute number of deaths attributable to PM is much higher for cardiovascular than respiratory deaths (Dockery, 2001; Frampton, 2001).

The mechanism(s) leading to PM cardiac effects are not yet completely understood. Ambient particles elicit cardiovascular effects in part through the autonomic nervous system. Short-term exposure to PM is associated with changes in autonomic function as assessed by changes in the cardiac rhythm. Henneberger *et al*. (2005) reported changes in electrocardiogram (ECG) parameters for QT duration in response to exposure to organic carbon and changes in T-wave amplitude and complexity in patients with preexisting coronary heart disease in response to daily variations of PM levels. Rhoden *et al*., showed that PM-induced changes in heart rate variability occurred simultaneously with increases in cardiac oxidants and were prevented by the antioxidant N-acetyl cysteine. Furthermore, both sympathetic and parasympathetic antagonists prevented PM-induced cardiac oxidant stress (Rhoden *et al*., 2005). Consistently, a recent study shows that exposure of aged spontaneously hypertensive rats to highway aerosols leads to short-term alterations in autonomic control of heart rate, that is, elevations in normalized high frequency power and decreased vagosympathetic balance (Elder *et al*., 2007).

Pulmonary chemoreflexes including apnea, bradycardia, and hypotension were first reported by Brodie (1900; Dawes and Comroe, 1954). Cardiopulmonary reflexes are defense mechanism against inhaled toxicants aimed to reduce the amount of inspired pollutants transported into the blood stream (Aviado and Guevara Aviado, 2001). Extensive evidence indicates that bronchopulmonary C-fibers afferents are responsible for these responses (Lee and Pisarri, 2001) and that these afferents are extremely sensitive to chemical irritants (Coleridge and Coleridge, 1984).

Approximately 75% of the afferent fibers in the vagal branches innervating the respiratory tract are nonmyelinated C-fibers (Agostoni *et al*., 1957; Lin *et al*., 2003). C-fibers are characterized by their distinct sensitivity to chemical stimuli and a relatively weak and irregular response to lung inflation

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(Lee and Pisarri, 2001). A variety of inhaled toxicants such as ozone (Lee and Widdicombe, 2001), sulfur dioxide, ammonia, (Wang *et al*., 1996) tobacco, wood smoke (Bonham *et al*., 2001; Lai and Kou, 1998), diesel exhaust (Wong *et al*., 2003), PM (Reilly *et al*., 2003; Veronesi *et al*., 2003) acrolein, volatile anesthetics, and capsaicin (Coleridge and Coleridge, 1984, 1994; Paintal, 1973) have been shown to stimulate bronchopulmonary C-fibers afferents.

Reflexes generated by nocireceptors such as the Transient Receptor Potential Vanilloid Receptor 1 (TRPV1) affect cardiovascular function by increasing and decreasing sympathetic and parasympathetic activity (Klabunde, 2005b). The activation of TRPV1 receptors on sensory fibers and some nonneuronal cells (e.g., respiratory epithelia) produces calcium and sodium influx and the corresponding release of tachykinin neuropeptide such as substance P and inflammatory cytokines *in vitro* (Veronesi *et al*., 2002), and *in vivo* (Richardson and Vasko, 2002). Studies on human respiratory epithelial cells (i.e., BEAS-2B) show that PM-induced cytokine release (e.g., interleukin-6, tumor necrosis factor-alpha) is initiated by activation of TRPV1 receptors (Veronesi *et al*., 1999), because these responses can be reduced by using Capsazepine (CPZ), a selective pharmacological antagonist (Benham *et al*., 2002; Tominaga *et al*., 1998).

In this study we tested the hypothesis that PM deposition in the lung triggers neural reflexes mediated by vagus nervous unmyelinated C-fibers through the activation of vanilloid (TRVP1) receptors. We hypothesized that in this way PM modulates the sympathetic/parasympathetic tone in the heart increasing oxidative stress and leading to functional alterations.

TABLE 1
Analysis of CAPs Concentration and Composition

Parameter	Mean ± SEM
CAPs mass concentration	218 ± 23
Black carbon concentration	5.0 ± 0.5
Particle number concentration	31,000 ± 2000
Na	8 ± 1
Mg	0.7 ± 0.1
Al	5 ± 1
Si	13 ± 2
S	19 ± 3
Cl	7 ± 2
K	2.5 ± 0.3
Ca	6 ± 1
Ti	0.44 ± 0.06
V	0.022 ± 0.008
Cr	0.035 ± 0.005
Mn	0.15 ± 0.02
Fe	9 ± 1
Ni	0.08 ± 0.03
Cu	0.17 ± 0.03
Zn	0.61 ± 0.07
As	0.01 ± 0.05
Se	0.004 ± 0.002
Br	0.056 ± 0.005
Sr	0.04 ± 0.01
Zr	0.04 ± 0.05
Cd	0.007 ± 0.002
Sn	0.13 ± 0.02
Ba	0.37 ± 0.07
Pb	0.08 ± 0.02

Note. All measures are expressed in $\mu g/m^3$ except for particle number concentration, which is expressed in particles/cm^3.

MATERIALS AND METHODS

Adult Sprague–Dawley rats were maintained and studied in accordance with the National Institutes of Health guidelines for the care and use of animals in research and all protocols were approved by the Harvard Medical Area Standing Committee on Animals. In a first experiment, 48 unrestrained, conscious animals were exposed once for 5 h to either to CAPs or filtered air. Immediately after exposure, we assessed cardiac oxidative stress with *in situ* chemiluminescence (CL), lipid peroxidation with thiobarbituric acid reactive substances (TBARS), and tissue edema in each animal, as described below. In a second experiment, 16 rats were exposed for 5 h to either to CAPs or filtered air repeatedly over a 4-month period. During each exposure we used radio telemetry to record the ECG and assessed cardiac function. In both experiments, half of the rats were randomly assigned to pretreatment with CPZ in order to test the hypothesis that observed responses to CAPs are mediated at least in part by TRVP1 receptors.

Experiment 1: effects of CPZ on CAPs-induced cardiac oxidative stress, lipid peroxidation, and tissue edema. Each exposure day, rats were treated with either saline (control and CAPs groups) or CPZ (CPZ and CPZ/CAPs groups) immediately prior to exposure. In a first protocol, rats were pretreated with either ip saline or 10 mg/kg CPZ. Subsequently, we tested the specific effect of inhibiting pulmonary efferent C-fibers by pretreating animals with aerosolized saline or CPZ (500μM × 20 min) immediately prior to exposure.

The stock solution of CPZ (Axxora LLC, San Diego, CA) was prepared by dissolving the drug in dimethylsulfoxide 1% (Sigma) and diluting the concentrated stock in saline added with 10% Tween 80, and 10% ethanol. CPZ for ip injection was prepared daily by diluting the stock solution with PBS without Ca^{++} and Mg^{++} to a final concentration of 10 mg/kg. CPZ for aerosol delivery (500μM in PBS without Ca^{++} and Mg^{++}) was also prepared daily. Animals were exposed to CPZ aerosols generated using a DeVilbiss Reusable Jet Nebulizer model 800 for 20 min at a constant flow of 2 l/min. Compressed air was obtained using a DeVilbiss Compact Compressor 3655 D. The animals were placed awake and unrestrained in single cages for aerosol delivery. The time elapsed between the end of CPZ exposure and the start of the CAPs exposure was ~10 min.

Rats were exposed once for 5 h to either CAPs or filtered air (sham) using the Harvard Ambient Particle Concentrator (HAPC) as previously described (Gurgueira *et al*., 2002). Briefly, the HAPC concentrates ambient particles with aerodynamic diameter < 2.5 μm (size cut 0.1–2.5 μm) for subsequent aerosol exposure of animals without altering particle composition or size distribution (Sioutas *et al*., 1995, 1997). During each exposure, we measured integrated CAPs mass concentration gravimetrically, trace metal concentrations using X-ray fluorescence (Chester LabNet, Tigrad, OR), black carbon, a surrogate for elemental carbon (Aethalometer Model AE-9, Magee Scientific, Berkeley, CA), and particle number concentration continuously (CPC Model 3022A, TSI Incorporated, Shoreview, MN). The average values for mass and composition of the CAPs aerosols used in this study are presented in Table 1.

Immediately following exposure, rats were anesthetized with sodium pentobarbital (50 mg/kg ip). The trachea was cannulated and connected to an animal ventilator (2.5 ml/breath, 80 breaths/min [Harvard Apparatus Model

"687" Mouse Ventilator, Cambridge, MA]). A sternotomy was performed, animals were placed in the measurement compartment and spontaneous CL of the surface of heart was measured as previously described (Gurgueira *et al*., 2002). Briefly, CL was measured using a Thorn EMI CT1 single-photon counting apparatus with an EMI 9816B photomultiplier cooled at −20°C. Body temperature was kept at 37°C using isothermal pads (Braintree Scientific, Braintree, MA). Emission data are expressed as counts per second per unit of tissue surface (cps/cm^2). A high-pass optical filter (Wratten number 25; Eastman Kodak, Rochester, NY) with a cut-off of 600 nm was placed in the optical path to avoid hemoglobin interference.

At the completion of the CL assay, rats were euthanized and their hearts were excised, washed in saline, and flash frozen in a liquid nitrogen bath for the determination of TBARS. Heart tissue samples were homogenized in seven volumes of 120mM KCl, 30mM phosphate buffer (pH = 7.4) added with proteinase inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin and 0.5mM phenylmethylsulphonyl fluoride) at 0–4°C. The suspensions were centrifuged at 700 × g for 10 min at 0–4°C to remove nuclei and cell debris. The pellets were discarded and the supernatants were used as homogenates.

Homogenates were then precipitated with 10% Trichloroacetic acid, centrifuged, and incubated with thiobarbituric acid (Sigma, Chem. Co.) for 1 h at 100°C. TBARS were extracted using butanol (1:1) to eliminate most interferences. After centrifugation, the fluorescence of the butanol layer was measured at 515 nm excitation and 555 nm emission using a spectrofluorometer (Photon Technology International, Lawrenceville, NJ). The amount of TBARS formed is expressed in nmol/mg protein. Malondialdehyde standards were prepared from 1,1,3,3-tetramethoxypropane (Esterbauer and Cheeseman, 1990). Protein concentration in homogenates was measured by the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard. Measurements were carried out using a Perkin–Elmer Lambda 40 spectrophotometer.

For the determination of water content heart samples (100 mg) were weighted and then dried in a convention oven (80°C). Tissues were reweighted 24 h after to obtain the wet/dry ratios.

Experiment 2: effects of CPZ on CAPs-induced changes in cardiac function. An additional 16 rats were exposed for 5 h to either CAPs or filtered air (sham) repeatedly over a 4-month period. Rats were implanted with a radio telemetry transmitter (DSI PhysioTel Transmitter ETA-F20) for the measurement of the ECG. Electrodes were implanted subcutaneously in a Lead II configuration.

After recovery, two groups of eight animals were exposed in the HAPC for a total 21 experiments. Rats were housed at the Harvard School of Public Health animal facility during the 10–15 days between exposures. Each exposure day, rats were treated with either ip saline (control and CAPs groups) or 10 mg/kg CPZ (CPZ and CPZ/CAPs groups) immediately prior to exposure. The 4 groups were exposed and tested simultaneously by using two animals per group on each exposure day. Real-time ECG waveforms were continuously displayed and recorded using a PC-based system (Dataquest ART, Data Sciences, Inc. St Paul, MN). A total of 105 h of ECG recording were available for each animal. At the end of the protocol, rats were euthanized and the hearts were dissected and flash frozen in liquid nitrogen for further analysis.

Standard ECG intervals and waveform amplitudes were measured from the recorded ECGs using a commercial software package (Physiostat ECG Analysis version 4.0, Data Sciences, Inc.). This package segments the ECG into 10 s intervals and identifies and marks normal waveform complexes. The normal complexes are combined to form a composite reference complex. Parameters are reported for waveform amplitudes and intervals based on this composite reference complex. Abnormal beats are excluded from the reference complex. Because missing or inappropriate labeling of even one beat can significantly distort the reference complex, we limited our analyses to one segment 15 min after the start of the exposure and one segment 4 h 45 min after the start of the exposure where all beats were appropriately labeled. Appropriate labeling was visually verified by a study coinvestigator blinded to the exposure status of each animal. Thus, for each parameter of interest, two data points were contributed per animal per exposure. The intervals considered in this study were: PR (time interval between the beginning of the P-wave to the peak of the R-wave), PQ (time interval between the beginning of the P-wave and the beginning of the Q-wave), QT (time interval between the beginning of the Q-wave and the end of the T-wave), RT (time interval between the peak of the R-wave and the end of the T-wave), QT dispersion (QTD, time difference between the longest QT interval and the shortest QT interval within a given waveform segment), QRS (time interval between the beginning of the Q-wave and the peak of the S-wave), RTp (time interval between the peak of the R-wave and the peak of the T-wave), Tpe (time interval between the peak of the T-wave and the end of the T-wave), and P duration (Pdur, time interval between the beginning and the end of the P-wave).

In humans and large animals, QT interval varies strongly and inversely with heart rate. Consequently, in clinical practice and human pharmacologic studies it is typical to correct the measured QT interval for heart rate in order to obtain measures of QT interval which are heart rate independent (QTc). In contrast, it has been shown that in adult Sprague–Dawley rats there is no consistent relationship between heart rate and QT interval (Hayes *et al*., 1994). Accordingly, we did not correct the measured QT interval for heart rate in this study. Likewise, the other intervals measured were not corrected for heart rate because there is no evidence of a consistent relationship between rate and interval length.

Statistical analyses. Values are expressed as means ± SEM. CL, TBARS, and tissue edema data were analyzed statistically by factorial analysis of variance followed by Student–Newman–Keuls' test for comparison of the means. Heart rate and ECG interval data were analyzed using linear mixed models with treatment group (four categories: control, CAPs, CPZ, CPZ/CAPs), time (two categories: 15 min, 4 h 45 min), and time-by-treatment interaction as fixed effects and random rat-specific intercepts. This modeling approach provides a framework for evaluating effects of treatments and time while accounting for the correlation between repeated measures on the same animal. We evaluated the linear association between measures of exposure (CAPs mass, number, and black carbon concentrations) using a similar model with fixed effects for time, CPZ, the chosen exposure metric and all two- and three-way interactions. Analyses were carried out using SAS v9 (Cary, NC) and Statview for MacIntosh. Statistical significance was accepted at $p < 0.05$.

RESULTS

Inhibition of CAPs-Induced Cardiac Oxidant Stress by CPZ (Experiment 1)

For the first protocol, rats were injected with either 10 mg/kg CPZ or saline ip immediately prior to 5 h exposure to either CAPs or filtered air. Heart CL, TBARS accumulation and wet/dry ratio were determined in control, CAPs, CPZ, and CPZ/CAPs rats immediately after each exposure. In saline-treated rats, CAPs significantly increased cardiac oxidative stress as measured by CL (Fig. 1A), accumulation of lipid peroxides (TBARS, Fig. 1B), and tissue edema (Fig. 1C). Pretreatment of rats with CPZ effectively prevented CAPs-dependent oxidative stress, accumulation of oxidized lipids, and heart edema. CPZ treatment did not alter oxidative stress or edema in the hearts of control animals (Fig. 1).

Next we tested the specific effect of inhibiting pulmonary TRP receptors by pretreating rats with aerosolized CPZ (500μM × 20 min) or saline immediately prior to 5 h exposure to CAPs or filtered air. Pretreatment of rats with CPZ aerosol effectively prevented CAPs-dependent increase of oxidative stress (Fig. 2A) and TBARS accumulation in heart tissue (Fig. 2B). CPZ aerosol did not affect the oxidative stress levels in hearts of control rats exposed to filtered air (Fig. 2). Tissue edema was not evaluated in this protocol.

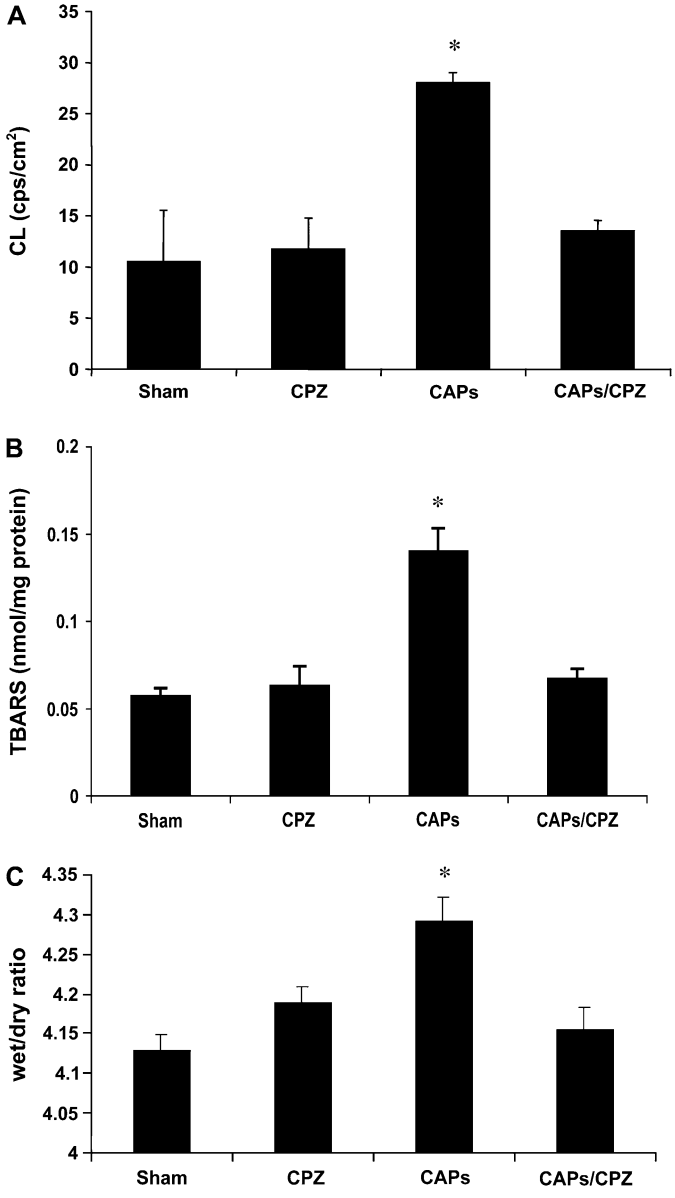

FIG. 1. Intraperitoneal injection of CPZ prevents oxidative stress in the heart of rats exposed to CAPs. Adult Sprague–Dawley rats were injected with 10 mg/kg CPZ ip or saline solution immediately prior to exposure to CAPs or filtered air. (A) *In situ* CL, (B) TBARS accumulation, and (C) Cardiac edema. Values represent the mean of four-six independent determinations ± SEM. $*p < 0.05$.

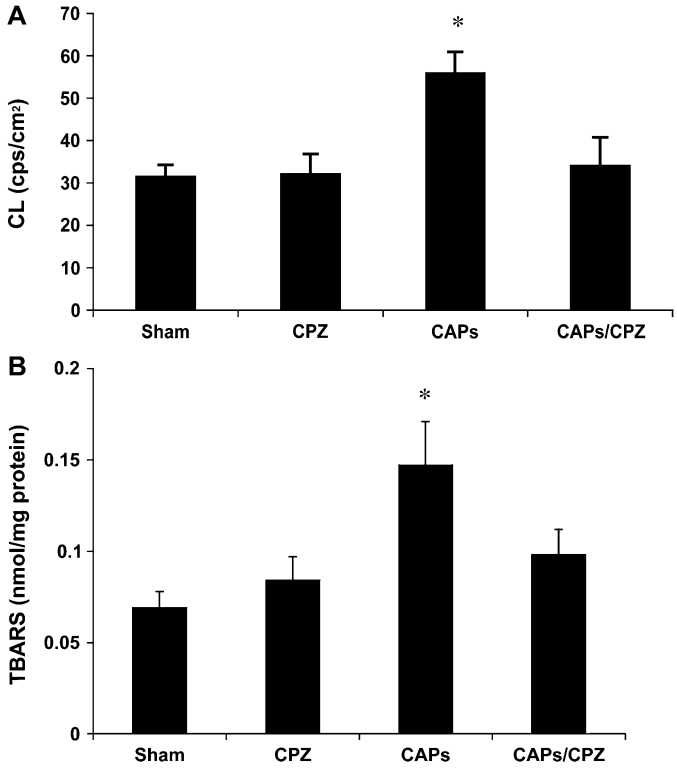

FIG. 2. CPZ aerosolization prevents oxidative stress and damage in the heart of rats exposed to CAPs. Adult Sprague–Dawley rats received aerosols containing either 500μM CPZ or saline for 20 min immediately prior to exposure to CAPs. (A) *In situ* CL. (B) TBARS accumulation. Values represent the mean of eight independent determinations ± SEM. $*p < 0.05$.

Effect of CPZ Treatment on CAPs-Induced Changes in Heart Function (Experiment 2)

We evaluated change in cardiac function by measuring heart rate and ECG waveform morphology in 10-s segments taken from the first and last 15 min of each recording (15 min, and 4 h 45 min, respectively). Because ip injection of CPZ proved to be as effective at preventing CAPs effects as aerosol administration and due to the additional complexity of the experimental procedure needed to aerosolize rats, we chose to use ip injections of CPZ for this protocol.

In rats pretreated with saline, CAPs exposure led to an immediate decrease in heart rate and these changes were prevented by pretreatment with CPZ (Table 2). Heart rate decreased throughout the exposure in all treatment groups ($p < 0.0001$). The change in heart rate was similar in the CAPs and control groups and statistically significantly greater in the CPZ and CAPs/CPZ groups (Table 2).

We evaluated the timing of ECG waveforms to better understand the effects of CAPs on myocardial depolarization and repolarization (Table 3). In saline pretreated animals, CAPs exposure significantly and immediately increased Pdur, and significantly and immediately shortened QRS, QT, and Tpe intervals. In contrast, pretreatment with CPZ lead to immediate shortening of the PR interval as compared with control animals, and an immediate lengthening of the QRS and Tpe intervals. In rats pretreated with CPZ, no CAPs-related changes were observed in any ECG interval.

In the control group no ECG interval changed significantly over the course of the exposure. In CPZ and CAPs/CPZ animals, QRS duration increased significantly throughout the exposure as compared with the change observed in the control group. The temporal pattern of other intervals did not change significantly as compared with the changes in the control group (Table 3).

TABLE 2
Change in Heart Rate during Acute Exposure to either Filtered Air or CAPs

	15 min		4 h 45 min		Δ	
	Mean ± SEM	*p* Value*	Mean ± SEM	*p* Value*	Mean ± SEM	*p* Value†
Control	370.2 ± 6.2	—	317.3 ± 6.3	—	−52.9 ± 7.6	—
CAPs	349.3 ± 5.7	**0.014**	291.5 ± 5.8	**0.003**	−57.8 ± 7.2	0.64
CPZ	396.4 ± 5.5	**0.002**	317.7 ± 5.6	0.96	−78.7 ± 6.8	**0.013**
CAPs/CPZ	380.2 ± 5.6	0.23	299.1 ± 6.3	**0.043**	−81.1 ± 7.4	**0.009**

Note. Δ: Change in heart rate over time. **p* value comparing heart rate in each group versus control group at the same time point. †*p* value comparing change in heart rate over time in each group versus change in heart rate observed in control group. Values in bold are statistically significant at the alpha = 0.05 level.

TABLE 3
Change in ECG Parameters during Acute Exposure to either Filtered Air or CAPs

	15 min		4 h 45 min		Δ	
	Mean ± SEM	*p* Value*	Mean ± SEM	*p* Value*	Mean ± SEM	*p* Value†
QT (ms)						
Control	59.6 ± 1.5	—	57.9 ± 1.6	—	−1.6 ± 1.3	—
CAPs	50.4 ± 1.4	**< 0.001**	51.8 ± 1.5	**0.007**	1.4 ± 1.4	0.10
CPZ	55.7 ± 1.4	0.064	56.8 ± 1.4	0.59	1.1 ± 1.1	0.12
CAPs/CPZ	58.2 ± 1.5	0.52	57.2 ± 1.6	0.76	−1.0 ± 1.3	0.72
Tpe (ms)						
Control	25.9 ± 1.3	—	25.3 ± 1.3	—	−0.6 ± 1.2	—
CAPs	19.9 ± 1.2	**0.001**	21.9 ± 1.2	0.064	2.0 ± 1.2	0.13
CPZ	21.8 ± 1.1	**0.018**	23.9 ± 1.1	0.42	2.0 ± 1.0	0.10
CAPs/CPZ	25.7 ± 1.2	0.90	23.6 ± 1.3	0.36	−2.1 ± 1.2	0.37
Pdur (ms)						
Control	14.8 ± 0.6	—	16.3 ± 0.6	—	1.4 ± 0.7	—
CAPs	17.1 ± 0.6	**0.007**	17.2 ± 0.6	0.31	0.0 ± 0.8	0.21
CPZ	15.7 ± 0.6	0.27	16.8 ± 0.6	0.54	1.0 ± 0.7	0.71
CAPs/CPZ	16.3 ± 0.5	0.070	16.2 ± 0.7	0.99	0.0 ± 0.8	0.20
QRS (ms)						
Control	19.6 ± 0.2	—	19.1 ± 0.2	—	−0.4 ± 0.2	—
CAPs	18.7 ± 0.2	**0.003**	18.8 ± 0.2	0.24	0.1 ± 0.2	0.09
CPZ	18.5 ± 0.2	**0.001**	18.9 ± 0.2	0.34	0.3 ± 0.2	**0.016**
CAPs/CPZ	19.3 ± 0.2	0.32	19.7 ± 0.2	0.069	0.5 ± 0.2	**0.007**
PR (ms)						
Control	55.6 ± 1.0	—	56.6 ± 1.0	—	1.0 ± 1.3	—
CAPs	55.6 ± 1.0	0.99	58.9 ± 1.1	0.11	3.3 ± 1.3	0.20
CPZ	51.7 ± 0.9	**0.005**	52.9 ± 1.0	**0.013**	1.3 ± 1.2	0.86
CAPs/CPZ	53.6 ± 0.8	0.13	56.9 ± 1.2	0.83	3.3 ± 1.4	0.21

Note. Δ: Change in parameter value over time. **p* value comparing value of parameter in each group versus control group at the same time point. †*p* value comparing change in parameter value over time in each group versus change in parameter value observed in control group. Values in bold are statistically significant at the alpha = 0.05 level.

Composition as a Determinant of CAPs Cardiac Effects

One of the advantages of working with CAPs aerosols is that they reflect the day-to-day changes in composition typical of urban environments, and in that way they provide a set of samples with a wide range of mass concentrations suitable for statistical analyses (Table 1). Using univariate regression analyses we identified statistically significant ($p < 0.05$) associations between QT and Tpe duration and CAPs mass concentration, average black carbon concentration and particle number concentration (Table 4). Heart rate (HR) and QRS were linearly associated with CAPs mass and black carbon levels ($p < 0.05$). Pdur showed a trend of association but the results show no statistical significance. Consistent with their lack of change as a result of exposure to CAPs as a binary variable, QTD and PR showed no association with the continuous exposure measures.

DISCUSSION

Exposure to ambient air particles leads to changes in autonomic nervous system activity in the heart and increased production of reactive oxygen species (Rhoden *et al.*, 2005). In the current study we show that particle-induced oxidative stress and changes in cardiac electrophysiology are prevented by CPZ. Specifically, we found that in addition to the already reported increases in cardiac oxidative stress, lipid peroxidation, tissue edema, and heart rate, inhalation exposure to CAPs alters electrocardiographic parameters related to ventricular repolarization (QT and Tpe intervals), depolarization (QRS), and velocity of propagation (Pdur). Notably, all of these changes are abrogated by ip injection of CPZ. CPZ is a selective antagonist of TRPV1 receptors and shows broad inhibitory action for other TRP receptors present in the lung. Therefore our data suggest a central role for TRP receptors, probably TRPV1, in eliciting the observed responses.

Our finding that inhalation exposure to CAPs increases cardiac oxidative stress is consistent with the results of previous work (Gurgueira *et al.*, 2002; Okayama *et al.*, 2006; Rhoden *et al.*, 2005). However, the mechanism linking inhaled particles to cardiac oxidative stress is unknown. Our observation that the CAPs-induced increase in cardiac oxidative stress is abrogated by pretreatment with CPZ provides evidence in support of the primary role of pulmonary irritant receptors in mediating this response.

It has been hypothesized that the cardiotoxicity of PM could be mediated by (1) increased autonomic influence on the heart, (2) proinflammatory cytokines or chemokines, and/or (3) direct effect of ultrafine particles or soluble particle components. We have previously reported that in this inhalation model inflammatory responses are detectable 24 h after the end of the exposure (Rhoden *et al.*, 2004). The responses we report here are detectable within the first few minutes of exposure and therefore are unlikely to be attributable to inflammatory mediators.

TABLE 4
ECG Parameter Changes per Interquartile Range Increase in Continuous Measures of CAPs Concentrations

	CAPs mass		Black carbon		Particle number	
	Slope (SEM)[a]	*p* Value	Slope (SEM)[a]	*p* Value	Slope (SEM)[a]	*p* Value
Heart rate (bpm)	−6.7 (3.4)	0.051	−11.0 (4.4)	**0.014**	−5.9 (3.2)	0.072
PR (ms)	0.0 (0.5)	0.93	−0.3 (0.8)	0.74	−0.2 (0.5)	0.68
QRS (ms)	−0.3 (0.1)	**0.031**	−0.4 (0.2)	**0.017**	−0.2 (0.1)	0.061
QT (ms)	−2.7 (0.9)	**0.002**	−4.0 (1.2)	**0.001**	−2.6 (0.8)	**0.002**
Pdur (ms)	0.6 (0.3)	0.074	0.8 (0.5)	0.081	0.6 (0.3)	0.065
Tpe (ms)	−2.2 (0.7)	**0.002**	−3.3 (1.0)	**0.001**	−2.2 (0.7)	**0.002**

Note. Interquartile ranges were 112.8 μg/m^3 for CAPs mass, 3.3 μg/m^3 for black carbon, and 12,100 particles/cm^3 for particle number. Values in bold are statistically significant at the alpha = 0.05 level.

[a]Slope represents change in ECG parameter per interquartile increase in exposure parameter.

The direct effects of ultrafine particles or soluble components that may leach out of the particle surface and reach the heart through the blood stream cannot be ruled out, but they seem unlikely to be responsible for the observed effects due to the low concentrations that are expected to reach the heart in this model. The amount of ultrafines particles estimated to be present in the $PM_{2.5}$ aerosols on this study is approximately 0.4% of the CAPs mass on average; corresponding to about 5 μg/m^3, a concentration ~30-fold lower than the amounts used to mimic episodes of high increases in urban air (Elder *et al*., 2000, 2004). Nonetheless, the possible role of ultrafine particles in mediating these effects must be considered given that we did find statistically significant associations between particle number concentrations and QT interval length.

Similarly, the amount of metals carried in the particles is estimated to be much lower than the trace amount present in biological systems. For example, a calculation of the total amount of iron potentially delivered by the average CAPs aerosols during a 5-h exposure at a flow rate of 1.5 l/min yields an estimate of 4.05 μg or 70 μmol. Assuming an intrapulmonary deposition fraction in rats similar to that of cigarette smoke particles (12%; Chen *et al*., 1989) the estimated maximal levels of Fe would be 8.4 nmol. Deposition of Boston CAPs in healthy humans is estimated to be slightly higher, with an ~20% for fine particles which still yields a maximum of 14 nmol of Fe (Montoya *et al*., 2004). Because iron was present at a higher concentration in CAPs (Table 1), we would expect to see even lower amounts of potentially bioavailable metals for all the other components. Copper complexes and strong oxidizing agents (diamide and chloramines) activate TRPV1 receptors at mM concentrations via irreversible oxidation of Cys621 at the extracellular side of the receptors (Susankova *et al*., 2006; Tousova *et al*., 2004). Although it is hard to imagine that concentrations on the higher micromolar or millimolar range could be delivered by CAPs aerosols, it seems possible to envision that a combination of redox-active components could approach those levels at the hot spots for PM deposition in the lung, where the concentrations are expected to be thousands of times higher than the average PM dose (Phalen *et al*., 2006). This could explain the activation TRPV1 receptors in the lung. Activation of TRVP1 by this mechanism in tissues other than the lung seems unlikely.

Challenge with exogenous H_2O_2 also evokes airway reflexes involving lung vagal afferents (Ruan *et al*., 2003). Aerosolized H_2O_2 (60 and 120mM, calculated from (Ruan *et al*., 2003) produced an antioxidant-sensitive, TRPV1/TRPM2-dependent stimulation of vagal lung afferents in rats (Ruan *et al*., 2003). Furthermore, Lee and colleagues demonstrated that inhalation of cigarette smoke elicits immediate respiratory and cardiovascular changes that are abolished when the cervical vagus nerves are cut or when vagal conduction is blocked reversibly by cooling the nerves below 0°C (Lee and Pisarri, 2001; Lee *et al*., 1989). These effects are similar to those associated with pulmonary chemoreflexes evoked when pulmonary C-fibers are activated by capsaicin, a pungent ingredient of hot pepper (Lee and Pisarri, 2001; Lee *et al*., 1989). In our model, the decreases in HR and myocardial repolarization produced by CAPs inhalation are completely abrogated by CPZ, suggesting that activation of TRVP1-initiated reflexes plays a central role in the mediation of these responses. However, the possible involvement of other CPZ-sensitive TRP channels expressed in C-fibers and other neurons in the respiratory tract cannot be ruled out and warrants further investigated. TRVP1 receptors are also present in lung epithelial and endothelial cells and vascular endothelial cells, where they trigger biochemical, mostly pro-inflammatory responses. However, as discussed above, these seem to be unlikely to contribute to the cardiac effects observed in this model given the short time of the responses.

Decreased heart rate in response to particulate exposure has been demonstrated in animals and humans by several groups (Cheng *et al*., 2003; Elder *et al*., 2007; Gold *et al*., 2000; Graff *et al*., 2004; Ibald-Mulli *et al*., 2004; Rhoden *et al*., 2005), although not all studies have been consistent (e.g., Pope *et al*., 1999). For example, in a previous study by our laboratory rats instilled with PM showed a decrease in heart rate and alterations in sympatho-vagal balance with a shift toward predominance of parasympathetic stimulation (Rhoden *et al*., 2005). Similarly, Elder *et al*., (2007) reported elevation of high frequency power of heart rate variability and decreased heart rate in rats exposed to highway aerosol, when compared with clean air exposed animals.

Additionally, we found electrocardiographic evidence suggesting that ventricular depolarization, repolarization, and conduction are acutely altered by CAPs exposure and that this response is probably mediated by TRVP1-dependent activation of pulmonary reflexes.

We found that CAPs exposure decreased the QT interval and that this response was probably mediated through activation of

pulmonary TRVP1 receptors. An experimental study in humans found a decrease in QT interval following exposure to laboratory-generated ultrafine carbon particles in healthy exercising subjects but not in those with mild asthma (Frampton *et al*., 2004). In contrast, two observational epidemiologic studies in patients with coronary artery disease found that exposure to traffic-related particles and organic carbon was associated with a significant lengthening of the QT interval (Henneberger *et al*., 2005; Yue *et al*., 2007).

The QT interval provides a measure of ventricular repolarization and is determined by the balance of the repolarizing inward sodium and calcium currents, and the outward potassium and chloride currents. Inhibition of K currents by mixofloxacin, quinidine, or verapamil leads to prolongation of the QT interval (i.e., slower repolarization) and arrhythmias, whereas inhibition of Na or Ca channels by propranolol or lidocaine leads to shortening of the QT interval and increases the risk of ventricular fibrillation. The QT interval correlates with measurements of cardiac autonomic function, with cardiac vagal dysfunction resulting in prolongation of the QT interval (Takahashi *et al*., 2004), and vagal stimulation in shortening of QT (Zamotrinsky *et al*., 2001). Shortened QT intervals are encountered in patients with hypercalcemia (Gussak *et al*., 2002), in the short-QT syndrome (Extramiana and Antzelevitch, 2004), and in the deceleration-dependent shortening of the QT Interval syndrome (Gussak *et al*., 1999, 2002) and may be associated with an increased risk for arrhythmia. The shortening in these conditions is attributed to alteration of cardiac calcium or potassium currents due to gain-of-function mutations (Tanabe *et al*., 2006) or high vagal tone (Gussak *et al*., 1999, 2002). Given these results, it is tempting to speculate that the mechanism by which CAPs exposure leads to shortening of the QT interval involves increased vagal tone to the heart and inhibition of Na, Ca, or K channels. This hypothesis is supported by the published changes in correlates of vagal tone, and by the prevention of these changes by CPZ reported here.

In humans, the Tpe interval is the clinical counterpart of the transmural dispersion of repolarization (TDR). In experimental models of the long-QT syndrome TDR/Tpe has been linked to the genesis of *torsade de pointes*, a life-threatening form of ventricular tachycardia associated with increased risk of sudden death (Viitasalo *et al*., 2002). The CAPs-related decrease in Tpe found in the current study is suggestive of changes in Na currents and is consistent with the observed decreases in QT duration.

Pdur reflects intra-atrial conduction times and is influenced by changes in autonomic tone (Cheema *et al*., 1995). The statistically significant increase in P-wave duration reported here for CAPs-exposed rats once again suggests a shift in sympatho-vagal balance toward parasympathetic dominance. The combination of simultaneous increases in sympathetic and parasympathetic stimulation on the heart is consistent with our previous observation in rats exposed to urban air particles (Rhoden *et al*., 2005), as well as dogs exposed to CAPs (Godleski *et al*., 2000). However, the lack of significant changes in the PR interval suggests that changes in conduction velocity, at least through the bundle of His, are not affected by activation of these reflexes.

CAPs exposure led to decreases in QRS duration, a measure of ventricular depolarization. Changes in the width of the QRS complex indicate changes in conduction velocity within the His-Purkinje system. Shortening of QRS is found in cardiac pathologies such as supraventricular tachycardia (Oreto *et al*., 2001), and in healthy patients subjected to a regime of exercise strong enough to increase sympathetic tone and enhance ventricular conduction (Goldberger and Bhargava, 1983). In rat models, QRS duration shortens in response to moderate hypercalcemia, which accelerates conduction by reducing the differences between resting membrane potential and threshold potential (Kuwahara *et al*., 1992).

Variations in PM composition are an important source of variability in the biological effects among different studies. Boston ambient $PM_{2.5}$ is typical of northeastern urban fine particle pollution, impacted by both local and regional pollution sources including among others: oil combustion; traffic-derived PM; secondary (photochemical) production of sulfates, nitrates and organics; soil/crustal material and marine aerosol (Godleski *et al*., 2002). The average CAPs composition in the present study was similar to the average composition in previous exposures carried out between 2001 and 2003 (Gurgueira *et al*., 2002; Rhoden *et al*., 2004, 2005) albeit with twofold higher concentrations of Cl and half the values for Br, Mn, V, and S. The CAPs mass concentrations in this study ranged from 100–550 $\mu g/m^3$, equivalent to those reached in human "real world" exposures in some transport microenvironments (Adams *et al*., 2001) and highly populated cities (Qian *et al*., 2001). The observed changes in ECG morphology showed strong associations with particle mass, number and black carbon content. Further associations with elemental components are currently under study using a larger dataset that includes the data reported here (in preparation).

CPZ treatment also showed consistent effects on ECG intervals. Specifically, we observed a shortening of the QRS and PR intervals. Shortening of these intervals may results from a decrease in vagal activity and/or an increase in sympathetic activity. Thus, the observed decreases are supportive of the hypothesized inhibition of the activation of vagus unmyelinated C-fibers afferents via blockade of TRVP1 receptors by CPZ. Consistent with previous reports (Schultz and Ustinova, 1998) CPZ had no scavenger effects on ROS in control animals (Figs. 1 and 2)

Taken together these results suggest that inhaled CAPs stimulate TRVP1, and maybe other pulmonary irritant receptors, and activate autonomic nervous system reflexes. The end result of this reflex activation is increased cardiac oxidative stress, and functional cardiac electrophysiologic changes including increased P-wave duration and QT interval

and decreased QRS and Tpe durations. Thus, we suggest that CAPs exposure results in cardiac current abnormalities leading to changes in conduction velocity and ventricular repolarization, and that triggering of TRPV1-mediated autonomic reflexes in the lung is essential for the observed changes in conduction, repolarization and cardiac rhythms. The particle components or sources responsible for these effects remain to be identified.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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