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Whole and Particle-Free Diesel Exhausts Differentially Affect Cardiac Electrophysiology, Blood Pressure, and Autonomic Balance in Heart Failure–Prone Rats

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Epidemiological studies strongly link short-term exposures to vehicular traffic and particulate matter (PM) air pollution with adverse cardiovascular (CV) events, especially in those with preexisting CV disease. Diesel engine exhaust is a key contributor to urban ambient PM and gaseous pollutants. To determine the role of gaseous and particulate components in diesel exhaust (DE) cardiotoxicity, we examined the effects of a 4-h inhalation of whole DE (wDE) (target PM concentration: 500 µg/m^3) or particle-free filtered DE (fDE) on CV physiology and a range of markers of cardiopulmonary injury in hypertensive heart failure–prone rats. Arterial blood pressure (BP), electrocardiography, and heart rate variability (HRV), an index of autonomic balance, were monitored. Both fDE and wDE decreased BP and prolonged PR interval during exposure, with more effects from fDE, which additionally increased HRV triangular index and decreased T-wave amplitude. fDE increased QTc interval immediately after exposure, increased atrioventricular (AV) block Mobitz II arrhythmias shortly thereafter, and increased serum high-density lipoprotein 1 day later. wDE increased BP and decreased HRV root mean square of successive differences immediately postexposure. fDE and wDE decreased heart rate during the 4th hour of postexposure. Thus, DE gases slowed AV conduction and ventricular repolarization, decreased BP, increased HRV, and subsequently provoked arrhythmias, collectively suggesting parasympathetic activation; conversely, brief BP and HRV changes after exposure to particle-containing DE indicated a transient sympathetic excitation. Our findings suggest that whole- and particle-free DE differentially alter CV and autonomic physiology and may potentially increase risk through divergent pathways.

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Key Words: **air pollution; cardiovascular; particulate matter; arrhythmia; heart rate variability; electrocardiogram; diesel exhaust.**

Exposure to vehicular traffic air pollution poses a significant threat to public health, especially in individuals with preexisting cardiovascular (CV) disease (Brook, 2008). Diesel engine exhaust is a major source of urban fine and ultrafine particulate matter (PM), as well as volatile organics, carbonyls, and gases such as sulfur dioxide (SO_2), nitrogen oxides (NO and NO_2), and carbon monoxide (Krivoshto *et al.*, 2008; Peretz *et al.*, 2008). Moreover, diesel exhaust (DE) is an important contributor to vehicular emissions attendant to biochemical and physiological responses and adverse clinical outcomes near roadways. For instance, in a study spanning 15 cities within the United Kingdom, Bhaskaran *et al.* (2011) demonstrated an association between exposure to NO_2 and onset of myocardial infarction 1–6 h later. Further, ischemic heart disease hospitalizations in eight European cities have been attributed to DE exposure (Le Tertre *et al.*, 2002). In addition, Mills *et al.* (2007) found that DE exposure exacerbated exercise-induced electrocardiographic ST depression in human subjects with known coronary artery disease. Several mechanisms underlying the acute CV toxicity of DE exposure have been implicated, including electrophysiological dysfunction, autonomic imbalance, vascular dysfunction, coagulation, and low-level systemic inflammation (Anselme *et al.*, 2007; Brook, 2008; Campen *et al.*, 2005; Lucking *et al.*, 2011; Mills *et al.*, 2007; Peretz *et al.*, 2008).

Although many components of DE are suspected to play a role in DE-induced CV dysfunction, recent investigations using relatively healthy individuals have implicated particles

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

Because the correlations between air pollution and adverse cardiac events are strongest among populations with preexisting CV disease, it is important to model this in animal toxicity studies. We have previously demonstrated that exposures to residual oil PM (Carll *et al.*, 2010; Farraj *et al.*, 2009; Farraj *et al.*, 2011), the gaseous irritant acrolein (Hazari *et al.*, 2009), or DE (Lamb *et al.*, 2012) cause a number of alterations in cardiac physiology including increased parasympathetic tone, ST depression, and cardiac arrhythmia (e.g., atrioventricular [AV] block) in rat models of hypertension or heart failure. Because of the continued uncertainty regarding the precise role of specific DE constituents in the elicitation of CV effects, we investigated previously undescribed electrocardiographic and BP effects of acute exposure to particle-free filtered diesel exhaust (gases alone; fDE) and wDE (particles plus gases) in heart failure–prone rats. We hypothesized that wDE exposure would provoke greater changes in CV physiology than fDE exposure in Spontaneously Hypertensive Heart Failure (SHHF) rats. Electrocardiogram and BP radiotelemetry were used to monitor autonomic balance (measured by heart rate variability [HRV]), cardiac arrhythmia, and indicators of altered myocardial conduction, before, during, and after a single whole-body inhalation exposure to either wDE or fDE. Cardiopulmonary injury, inflammation, and oxidative stress were also assessed.

MATERIALS AND METHODS

Animals and radiotelemetry implantation. Lean male SHHF rats (*McCcrl-Lepr^{cp}*; $n = 20$, 9 weeks old; Charles River Laboratories, Kingston, NY) were implanted with radiotelemeters (model TL11M2-C50-PXT; Data Sciences International, St Paul, MN) capable of transmitting electrocardiography (ECG), heart rate (HR), aortic BP, and core body temperature wirelessly to a computer receiver. Telemeter implantation was performed by surgeons at Charles River Laboratory in adherence with preoperative, anesthetic, and surgical procedures described previously (Carll *et al.*, 2010). Lean male SHHFs acquire cardiac hypertrophy by 3 months of age and transition into dilated cardiomyopathy and heart failure (HF) at 18 months of age as a consequence of hypertension and hyperleptinemia (Carll *et al.*, 2011b). Rats were shipped after a 10-day recovery period to our Association for Assessment and Accreditation of Laboratory Animal Care International-approved animal facility, housed individually in 42- × 21- × 20-cm Plexiglas cages with pine-shave bedding in a room (22°C ± 1°C, 50% ± 5% relative humidity, 12-h light:dark cycle 0600:1800 h), and provided standard Purina rat chow (5001; Brentwood, MO) and water *ad libitum*. All studies conformed to the guidelines of the U.S. Environmental Protection Agency (EPA) Institutional Animal Care and Use Committee. Three days later, rats were transferred to the EPA High Bay facility's satellite animal-holding room and maintained under the same conditions as previously stated but in smaller, 33- × 18- × 19-cm Plexiglas, cages. Rats were weighed and assigned blindly to one of three exposure groups (clean filtered air, "Air"; "wDE"; and "fDE") while maintaining equivalent mean body weights per group.

DE exposure and generation. Rats were acclimated to exposure conditions in 20- × 12.5- × 17-cm metal wire cages within the clean filtered air exposure chamber for 1 h at 2 days preceding exposure. On the exposure day, rats were allowed to acclimate to the chambers for 20 min, and then baseline data was recorded for the next 40 min. Rats were then exposed to wDE (target of 500 µg $PM_{2.5}/m^3$), fDE (target of 0 µg $PM_{2.5}$ $/m^3$), or clean filtered air (Air) for 4 h in whole-body exposure chambers. Thereafter, DE exposures were stopped for a 1-h recovery period in which clean filtered air was circulated through exposure chambers. Rats were returned to home cages immediately after recovery period. DE exposures were at ultrafine PM concentrations comparable to those found in traffic tunnels and (at brief moments) on roadways (Anselme *et al.*, 2007; Zhu *et al.*, 2007) and NO_2 and SO_2 concentrations comparable to those observed in traffic tunnels or in cities within the United States and Europe (Danzon, 2000; Svartengren *et al.*, 2000).

DE was generated using a 4.8 kW (6.4 hp) direct injection single-cylinder 0.320 L displacement Yanmar L70V diesel generator operated at a constant 3600 rpm on low sulfur diesel fuel (32 ppm) as previously described (Lamb *et al.*, 2012). Resistance heating elements provided a constant 3 kW load. Engine lubrication oil (Shell Rotella, 15W-40) was changed before each set of exposures. From the engine, the exhaust was mixed with clean air previously passed through high-efficiency particulate air (HEPA) filters. Air dilution of wDE was adjusted periodically to maintain target $PM_{2.5}$ mass concentration. The diluted DE was delivered to an isolated animal exposure room and was either delivered unfiltered to a Hazelton 1000 (984 L) exposure chamber (wDE) or diverted through a HEPA canister filter and delivered to a similar exposure chamber (fDE). The HEPA canister filter featured a 99.97% removal efficiency standard to 0.3 µm. Although the fDE chamber was relatively absent of PM, its concentrations of diluted combustion gases remained comparable to the unfiltered chamber (Table 1). Control animals were placed in a third chamber supplied with the same HEPA-filtered room air as that used to dilute DE. The chambers were operated at the same flow rate (424 l/min, resulting in approximately 25 air exchanges per hour), temperature, and pressure. Integrated 4-h filter samples (14.1 l/min) were collected daily from each chamber and analyzed gravimetrically to determine particle concentrations. Chamber concentrations of PM, oxygen (O_2), carbon monoxide (CO), nitrogen oxides (NO and NO_2), and sulfur dioxide (SO_2) were measured as previously described (Lamb *et al.*, 2012). Chamber temperatures, relative humidity, and noise were also monitored and maintained within acceptable ranges.

Radiotelemetry data acquisition and analysis. Radiotelemetry was used to track changes in CV and thermoregulatory function by continuously monitoring core body temperature, BP, ECG, and activity in awake, unrestrained rats beginning at 1 day before inhalation exposure and continuing through exposure until euthanasia 24 h after exposure. Data were monitored by remote receivers (Model RPC-1; Data Sciences International, Inc.) positioned under the home cages within the animal facility and beside cages within exposure chambers. Arterial BPs (mean, systolic, diastolic, and pulse), HR, and QA interval were derived from pressure and ECG waveforms collected at a sample rate of 1000 Hz for 2 min of every 10 min and automatically analyzed by computer software (DataART 3.01; Data Sciences International) as previously described (Carll

TABLE 1
Inhalation Exposure Characterization

	Air	fDE	wDE
$PM_{2.5}$ (µg/m^3)	—	3 ± 2	472 ± 2
$PM_{2.5}$ number (n/cm^3)	—	$1.4 \times 10^3 \pm 9$	$2.1 \times 10^6 \pm 3 \times 10^3$
Number median diameter of PM (nm)	34 ± 3	21 ± 3	61 ± 0
Volume median diameter of PM (nm)	124 ± 0	125 ± 0	91 ± 0
O_2 (%)	21.0 ± 0.2	20.6 ± 0.0	20.6 ± 0.2
CO (ppm)	BDL	9.7 ± 0.7	9.5 ± 4.2
NO (ppm)	0.1 ± 0.0	10.0 ± 0.8	10.3 ± 1.9
NO_2 (ppm)	0.0 ± 0.0	0.4 ± 0.1	0.3 ± 0.2
SO_2 (ppm)	BDL	0.6 ± 0.1	0.4 ± 0.2

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

et al., 2010). QA interval provides an index of contractility determined by a measure of the aortic preejection period. Specifically, QA interval is the delay between onset of left ventricular depolarization and ejection, which are respectively indicated by the initializations of the R-wave and the following increase in aortic pressure (Cambridge and Whiting, 1986). Averages were calculated for BPs on an hourly basis over the 4 h of exposure (mid-inhalation, within exposure chambers), the roughly 40-min baseline and 1-h recovery periods (within exposure chambers), the 4-h periods in home cages immediately pre- and postexposure, and a home cage period from the day preceding exposure that was time-matched with exposure.

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

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

Arrhythmias were verified from time-matched BP and identified (while blinded to treatment group) as ventricular, supraventricular, junctional, and atrial premature beats, sinoatrial blocks, or AV blocks using the Lambeth Conventions (Walker *et al.*, 1988) as a guideline and according to additional, more specific criteria (Carll *et al.*, 2010). Each AV block Mobitz II arrhythmia was marked by a nonconducted P-wave that lacked the following four features: (1) an RR interval less than twice the average of the preceding 3 RR intervals, (2) a progressive PR interval prolongation in the preceding three PQRST complexes, (iii) a PR shortening in the first subsequent PQRST complex, and (iv) a PP interval shortening immediately prior to the dropped R wave. To facilitate statistical analysis of each arrhythmia type and allow the data to converge under the Poisson distribution, zero values for each arrhythmia type within a sample interval were converted to 0.1. Arrhythmia frequencies were calculated over specific periods in home cages (preexposure and postexposure, 6 h each) and in exposure chambers (baseline, mid-exposure, recovery), normalized to adjust for time differences between periods and gaps in data, and presented as number of events per hour of theoretically continuous ECG waveforms.

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

Tissue collection and analysis. At approximately 24 h after onset of the 4-h inhalation exposure, rats were deeply anesthetized with an intraperitoneal injection of euthasol (200 mg/kg Na pentobarbital and 25 mg/kg phenytoin; Virbac Animal Health, Fort Worth, TX). Whole blood was collected from the descending abdominal aorta in serum separator tubes and microcentrifuge tubes containing either buffered sodium citrate or K2EDTA (Becton Dickinson and Company, Franklin Lakes, NJ) as previously described (Carll *et al.*, 2011a). Hearts were excised, trimmed free of arterial tissue and fat, and weighed. Right tibia length was measured by caliper for heart-weight normalization. The trachea was cannulated, and the lungs were lavaged with a total volume of 20 ml/kg of Ca^+, Mg^+, and phenol red-free Dulbecco's phosphate-buffered saline (SAFC Biosciences, Lenexa, KS) that was divided into two equal aliquots and processed for cell differentials (Carll *et al.*, 2010). Lavage, serum, and plasma samples were collected, centrifuged, stored, and subsequently analyzed according to previously published procedures for the following biomarkers: lavage supernatants for albumin, lactate dehydrogenase, N-acetyl-b-d-glucosaminidase, total antioxidant status, and total protein; serum for creatine kinase, C-reactive protein, total protein, and glutathione peroxidase, reductase, and –S-transferase; and supernatants from plasma for angiotensin converting enzyme, albumin, blood urea nitrogen, creatinine, and total protein (Carll *et al.*, 2011a). Serum was also analyzed for α-hydroxybutyrate dehydrogenase, glucose, total cholesterol, and triglycerides (Sigma–Aldrich, St Louis, MO), as well as for alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase-1, myoglobin, high- and low-density lipoprotein cholesterol, and sorbitol dehydrogenase according to previous procedures (Carll *et al.*, 2010), whereas commercially available kits were used in analysis of serum for D-dimer, ferritin, and insulin (Kamiya Biomedical Company, Seattle, WA), lipoprotein (a), superoxide dismutase (SOD), manganese SOD, and copper-zinc SOD (Randox Life Sciences, Antrim, U.K.), and lipase (Genzyme Diagnostics, Framingham, MA). Lavage was also analyzed for gamma-glutamyl transferase (Fisher Diagnostics, Middletown, VA).

Statistics. The statistical analyses for all data in this study were performed using Prism version 4.03 (GraphPad Software, Inc., San Diego, CA). One-way ANOVA with Tukey's *post hoc* test was used to detect significant differences between groups in biochemical endpoints and tissue weight. Repeated measures two-way ANOVA with Bonferroni's *post hoc* test was performed on (1) arrhythmia frequency data, which were collected at pre-, mid-, and postexposure periods (spanning approximately 6, 4, and 6 h, respectively) and normalized by sampling duration; (2) HRV, ECG morphology, BP, and BP parameters during the exposure period, including exposure hours 1–4, baseline (40 min), and recovery periods (30 min); and (3) HRV, ECG morphology, and BP to analyze for intragroup differences between time-matched periods (separated by exactly 24 h) collected on the exposure day and on the previous day. Two-way ANOVA with Bonferroni's *post hoc* test was also used to analyze for intergroup differences in percent change in HRV and ECG parameters at postexposure relative to the day before exposure. BP data from the day of exposure were analyzed both by treatment period and by individual hour for significant time-matched intergroup differences by two-way ANOVA with Bonferroni's *post hoc* test. $p < 0.05$ was considered statistically significant. Linear regressions were performed to test for correlations between various physiological endpoints.

TABLE 2
CV Physiology During Baseline Period Within Exposure Chamber

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

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

RESULTS

Physiological Responses During Inhalation Exposure

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

HR and HRV. There were no group differences in HR or HRV at baseline (Table 2). All groups were upright and active at the beginning of the exposure and became recumbent and inactive during exposure to Air or DE. As would be expected with decreased activity, HR decreased from baseline at multiple hours of exposure for the Air, fDE, and wDE groups ($p < 0.05$; Figure 1). LF/HF paralleled changes in HR, significantly declining from baseline for the Air (2–3 h), fDE (2 h), and wDE (2–4 h) groups ($p < 0.05$). In contrast to Air or wDE, fDE significantly increased triangular index during hour 4 of exposure relative to baseline ($p < 0.05$). During the recovery period, while all rats remained in exposure chambers and were breathing clean filtered air, only the wDE-exposed rats had a significant change in RMSSD, which decreased by 24% relative to baseline ($p < 0.05$). Simultaneously, LF/HF for only the DE-exposed groups significantly rebounded from their mid-exposure values (exceeding 1–4 h for fDE and 2–4 h for wDE; $p < 0.05$).

Hemodynamics and thermoregulation. There were no group differences in BP at baseline (Table 2). At hour 2 of exposure, fDE decreased systolic BP relative to baseline (Figure 2; −8.5 mmHg, $p < 0.05$). In contrast, the Air group had increased pressure at hour 4 relative to baseline (+ 9.0 mmHg in mean arterial pressure [MAP], + 9.5 mmHg in diastolic BP; $p < 0.05$) and the fDE-exposed group (+ 12.2 mmHg in MAP, + 13.2 mmHg in systolic BP, + 11.7 mmHg in diastolic BP; $p < 0.05$). During the recovery period when all groups were provided clean filtered air within exposure chambers, the Air group still had significantly increased BP relative to baseline (+ 8.8 mmHg in MAP, $p < 0.05$). Meanwhile, the wDE group also exceeded its own baseline diastolic pressure during recovery (+7.0 mmHg, $p < 0.05$). There was no such increase in the fDE-exposed group. There were no significant changes in pulse pressure, QA interval (an index of contractility), or core body temperature during exposure ($p > 0.05$).

ECG Morphology. There were no group differences in ECG morphology at baseline (Table 2). During hour 3 of exposure, fDE increased PR interval by 2.7 ms and decreased T-wave amplitude by 25% relative to baseline, whereas wDE also prolonged PR by 2.0 ms from baseline (Figure 3; $p < 0.05$). At hour 4, fDE exposure prolonged PR interval relative to baseline (+ 3.8 ms; $p < 0.05$) and relative to Air at the same time (+ 3.3 ms; $p < 0.05$). During recovery, the fDE-exposed group had a significant increase in corrected QT interval (QTc) relative to its own baseline (+ 5.0 ms) and relative to Air (+ 6.9 ms), and it also continued to have significantly prolonged PR relative to baseline (+ 2.4 ms; all comparisons $p < 0.05$). Notably, among all rats, changes from baseline in PR during hour 4 and recovery correlated positively with the subsequent rate of postexposure Mobitz II AV block in home cages (vs. hour 4 PR r^2: 0.41, $p = 0.003$; and vs. recovery PR r^2: 0.22, $p = 0.042$). As well, the change from baseline in QTc during recovery significantly correlated with the change from baseline in PR during hour 4 of exposure (r^2: 0.42, $p = 0.006$) and with the change from baseline in T amplitude during recovery (r^2: 0.51, $p = 0.002$) and over the entire exposure period (r^2: 0.35, $p = 0.016$).

Physiological Responses in Home Cages After Exposure

Cardiac arrhythmia. Over the 6 h following exposure while the animals were in home cages, the fDE group had an increased frequency of second degree Mobitz Type II AV block relative to (1) itself during the same time on the previous day while in home cages, (2) itself during the exposure period

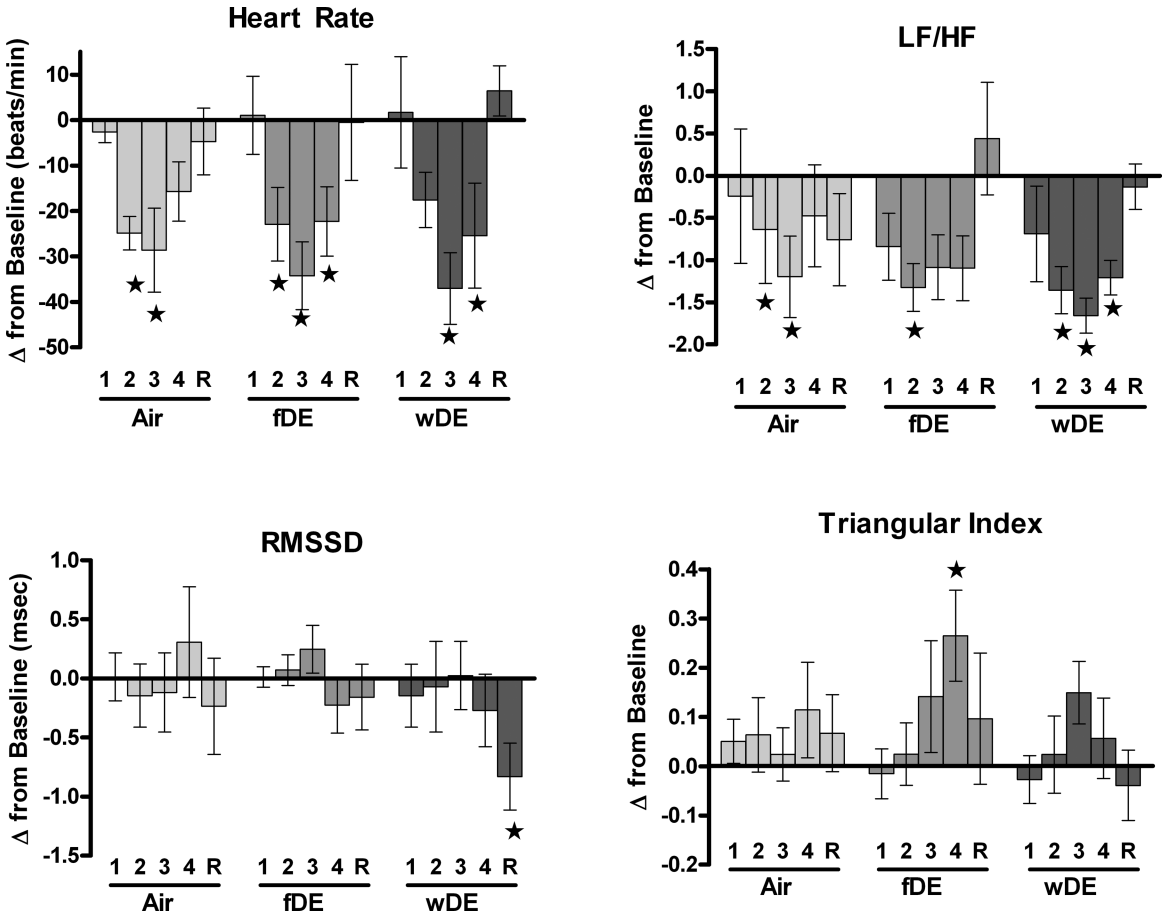

FIG. 1. Change from baseline in HR and HRV endpoints (mean + standard error [SE]) during whole-body exposure. 1, 2, 3, 4, and R represent hours 1–4 of exposure and postexposure Recovery within the chamber, respectively. All measurements were taken from conscious rats temporarily housed within exposure chambers. Significant differences are indicated by stars (relative to baseline) and diamonds (relative to Air group at the same hour; $p < 0.05$). See Table 2 for baseline values.

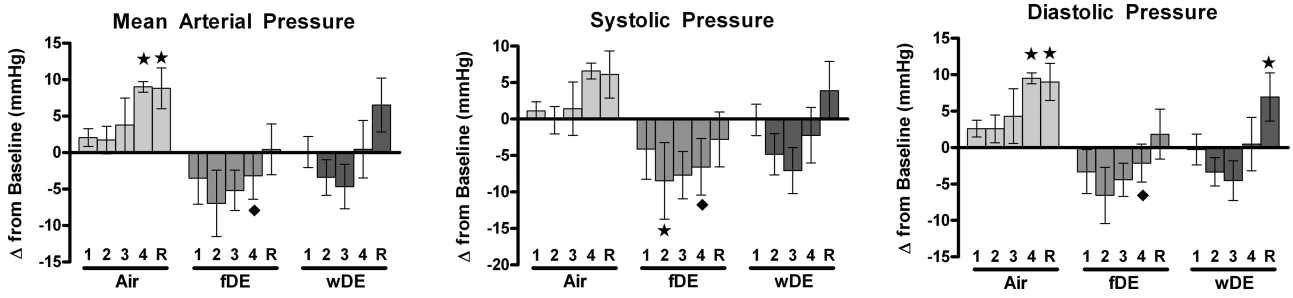

FIG. 2. Change from baseline in BP during whole-body exposure (mean + SE). 1, 2, 3, 4, and R represent hours 1–4 of exposure and postexposure Recovery within the chamber, respectively. All measurements, including baseline, were taken from conscious rats temporarily housed within exposure chambers. Significant differences are indicated by stars (relative to baseline) and diamonds (relative to Air group at the same hour; $p < 0.05$). See Table 2 for baseline values.

within chambers, and (3) the wDE group during the same postexposure period (Figure 4; $p < 0.05$). Notably, 77% of the fDE group's postexposure bradyarrhythmias occurred within the first 2 h of this 6-h period. After the first 6 h of postexposure, AV block arrhythmias were rare for all groups.

HR and HRV. Within the first hour after animals were returned to home cages for postexposure monitoring, LF/HF increased in each group relative to the corresponding hour on the prior day ($p < 0.05$). Simultaneously, all groups also had trends of increased HR ($p > 0.05$; Table 3). At hour 4 of

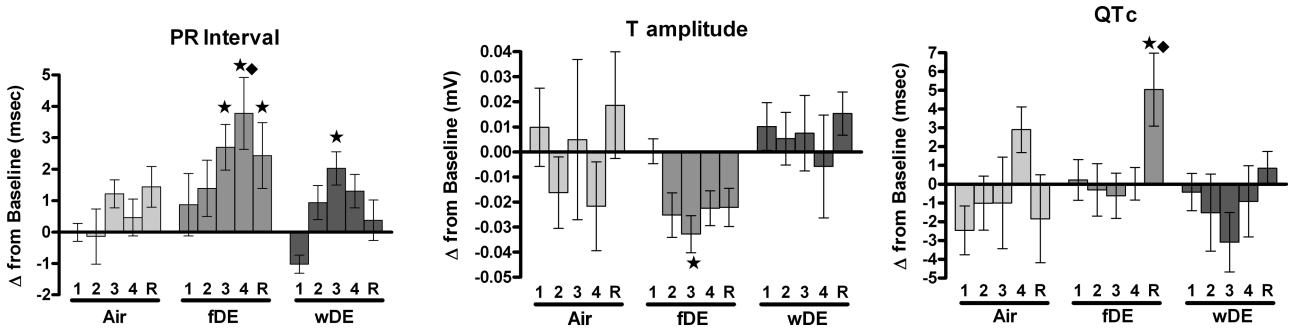

FIG. 3. Change from baseline in ECG endpoints during whole-body exposure (mean + SE). 1, 2, 3, 4, and R represent hours 1–4 of exposure and postexposure Recovery within the chamber, respectively. All measurements were taken from conscious rats temporarily housed within exposure chambers. Significant differences are indicated by stars (relative to baseline) and diamonds (relative to Air group at the same hour; $p < 0.05$). See Table 2 for baseline values. QTc: Fridericia-corrected QT interval.

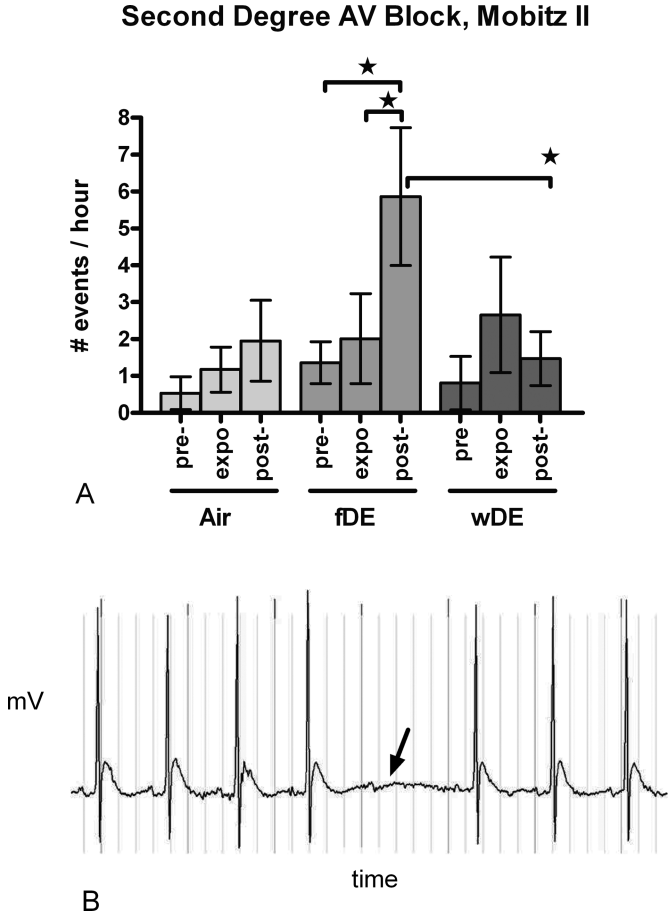

FIG. 4. Filtered DE-induced AV block Mobitz II arrhythmia. Hourly rate of Mobitz II AV block per rat, mean ± SE (A); ECG waveform with representative second degree AV block Mobitz II arrhythmia following fDE exposure (B). "pre-", "expo-", and "post-" represent periods of ECG analysis conducted before (6 h), during (4 h), and after (6 h) inhalation exposure, respectively. Stars and brackets above SE bars indicate significant differences between periods or groups ($p < 0.05$). Arrow indicates individual arrhythmia. Vertical grey lines behind ECG waveform indicate time in 50 ms intervals.

postexposure, HR for both DE-exposed groups decreased from the preceding day, but only the wDE group's change was statistically significant (wDE −38 beats/min, $p < 0.05$; fDE −37 beats/min, $p > 0.05$; Table 3). There was an apparent difference in HR between the DE groups and the Air group at hour 4 of the prior day (Table 3; $p = 0.22$), but the postexposure decreases in HR were roughly 2–3 times larger than these preexisting differences. LF/HF also appeared to decrease for both DE groups at hour 4 postexposure, but these changes were not statistically significant (fDE: −32% and wDE: −31% from prior day; $p > 0.05$).

Hemodynamics, ECG morphology, and thermoregulation. In the first postexposure hour in home cages, the Air group had an increased MAP relative to its own time-matched values from the prior day (+ 17.4 mmHg, $p < 0.05$), whereas the wDE group had a trend of similarly increased MAP (+ 17.3 mmHg; $p > 0.05$), which the fDE group lacked (+ 8.5 mmHg; $p > 0.05$). At 4 h postexposure, no group significantly differed in BP from itself on the prior day. There were no significant postexposure changes in pulse pressure, QA interval (an index of contractility), body temperature, or ECG morphology.

Biochemical markers of cardiopulmonary and circulatory injury, oxidative stress, and inflammation. HDL cholesterol increased in the fDE-exposed group by 25% ($p < 0.05$; Table 4). There were no significant effects of DE inhalation on pulmonary inflammatory cells, pulmonary and circulating antioxidants, or cardiopulmonary markers of injury. There were trends of decreased plasma and serum glutathione S-transferase with wDE exposure (−29% and −21% from Air, respectively; $p > 0.05$), increased lactate dehydrogenase-1 with exposure to fDE or wDE (+ 81% and + 101% from Air, respectively; $p > 0.05$), and increased serum ferritin with fDE or wDE exposure (+ 23% and + 12% from Air, respectively; $p > 0.05$).

DISCUSSION

We present evidence that a single 4-h exposure to DE in a rat model of hypertension and mild (pre-heart-failure) cardiomyopathy differentially alters cardiac rhythmicity, BP, and autonomic modulation of the heart based on the DE constituents present. DE caused a decrease in BP and concomitant PR prolongation during exposure regardless of the presence of particles, and these effects remained with exposure to DE gases alone. Only

TABLE 3
Time-Matched Comparison of HR Before and After Exposure

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

Notes. HR at postexposure was compared to the time-matched period 24 h before exposure (while in home cages). Values represent means (standard error mean).
*Significant difference from Air group ($P < 0.05$).
**A single group's significant decrease from itself on prior day ($p < 0.05$).

TABLE 4
Circulating Endogenous Antioxidants and Markers of CV Risk and Injury

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

Notes. Means (SE). HDL, high-density lipoprotein.
See *Methods* section for other markers of cardiopulmonary toxicity, risk, inflammation, and antioxidants measured in serum, plasma, and bronchoalveolar lavage fluid. All measures were unaffected by exposure. U and IU denote units and international units, respectively.
*Significant difference from Air group ($p < 0.05$).

the filtered DE (fDE) group had increased HRV triangular index and T-wave flattening during exposure, as well as postexposure QT prolongation and increased Mobitz II AV block arrhythmias. Collectively, changes in PR and BP and a unique increase in HRV and bradyarrhythmias within the fDE group suggest that DE gases cause parasympathetic (vagal) dominance. This was further evidenced by a postexposure bradycardia in both DE groups. Meanwhile, the wDE group's less overt responses during exposure and significantly fewer arrhythmias thereafter (relative to fDE group) suggest that these specific effects of DE gases are partly inhibited either by physico-chemical interactions with DE particles or by competing autonomic impacts of the two constituents. In further support of the latter, HRV decreased and diastolic BP increased immediately after wDE exposure, indicating sympathetic excitation.

fDE caused a 3-h PR prolongation upon exposure and a unique increase in second degree Mobitz type II AV block arrhythmias thereafter, indicating markedly impaired AV conduction. In contrast, wDE caused only a 1-h PR prolongation and did not elicit any arrhythmia. There was a correlation between mid-exposure PR prolongation and postexposure AV block arrhythmias, indicating that PR prolongation may by several hours precede (or perhaps even predict) air pollutant-induced AV block arrhythmias and that DE gases may promote spontaneous bradyarrhythmia through impaired conduction along the AV pathway. Although the capacity for DE to elicit this specific type of arrhythmia in humans is unclear, these findings suggest that DE exposure may increase vulnerability to spontaneous cardiac arrhythmia.

Increased vagal tone has been shown to prolong PR, cause nitric oxide-mediated vasodilation (Hotta *et al.*, 2009; Katz, 2006), increase HRV triangular index (Kouidi *et al.*, 2002), provoke AV block Mobitz II arrhythmia (Castellanos *et al.*, 1974; Hotta *et al.*, 2009; Massie *et al.*, 1978), decrease HR, and inhibit sympathetic-mediated norepinephrine release and vasoconstriction (Katz, 2006; Vanhoutte and Levy, 1980). We have previously shown that, relative to wDE, fDE causes PR prolongation and bradycardia, a more pronounced increase in HRV, and greater or equal susceptibility to provocation of arrhythmia, ventricular fibrillation, and cardiac arrest by a pro-arrhythmic drug (Hazari *et al.*, 2011; Lamb *et al.*, 2012). As well, Campen and colleagues found that fDE and wDE cause equally marked bradycardia in mice (2005). Fittingly, others have demonstrated by way of increased HRV that DE may cause parasympathetic dominance (Mills *et al.*, 2011a; Peretz *et al.*, 2008). Meanwhile, one study has found that DE decreases HRV in a rat model of advanced heart failure (Anselme *et al.*, 2007), whereas another provides evidence that ultrafine PM may mediate this effect (Chuang *et al.*, 2005). This study expands the body of research suggesting that gaseous exhaust can mediate several of DE's effects on cardiac rhythmicity and autonomics (Table 5). Additional inhalation studies that examine the effect of DE particles alone (currently beyond the capacity of our inhalation facilities) are needed to disentangle the various effects of DE constituents.

Exposure to fDE altered the ECG (i.e., QTc and T-wave amplitude), indicating changes in the spatiotemporal pattern of ventricular repolarization. These findings are not unprecedented; acute exposure to "particle-free" DE gases has been shown to decrease T-wave amplitude in two separate studies involving atherosclerotic mice and hypertensive rats but not in healthy controls (Table 5) (Campen *et al.*, 2005; Lamb *et al.*, 2012). In humans, air pollutant exposure has also been associated with prolonged QTc and/or decreased T-wave amplitude (Henneberger *et al.*, 2005; Liao *et al.*, 2010). Ventricular repolarization may be impeded by parasympathetic dominance (Conrath and Opthof, 2006; Katz, 2006; Murakawa *et al.*, 1992), inflammation (Zhang *et al.*, 2011), and myocardial ischemia (Channer and Morris, 2002). The inhalation of "particle-free" DE or one of its primary gases (NO_2) has been shown to promote inflammation and pro-ischemic vascular effects (Campen *et al.*, 2010; Channell *et al.*, 2012). Others have found that nonparticulate components may mediate vehicular emission-induced aortic remodeling in entirety (Lund *et al.*, 2007) or pulmonary inflammatory signaling in part (Elder *et al.*, 2004). On the other hand, some have demonstrated that particle filtration inhibits vascular effects of DE (Lucking *et al.*, 2011; Mills *et al.*, 2011b). These discrepancies may point to the divergent vascular and cardiac effects of specific constituents of DE.

TABLE 5
Prior Evidence Linking DE Gases to Alterations in Cardiac Rhythmicity, Repolarization, and Autonomic Balance

Study	Disease model	Exposures (PM conc.)	Findings	Interpretations
Campen *et al*. (2005)	Atherosclerosis: ApoE$^{-/-}$ mice on high-fat diet	fDE and wDE ("low" - 500 µg/m^3 "high" - 3600 µg/m^3)	fDE and wDE equally ↓heart rate & ↓T-wave area during exposure.	Filtration did not alter mid-exposure effects of DE on cardiac rhythm and repolarization.
Hazari *et al*. (2011)	Hypertension: Spontaneously hypertensive rats	fDE and wDE ("low" - 150 µg/m^3 "high" - 500 µg/m^3)	1 day post-exposure: low fDE ↑RMSSD & ↑SDNN; high fDE ↑LF/HF. wDE did not affect HRV. fDE ↑ sensitivity to drug-induced ventricular fibrillation and cardiac arrest, but wDE did not. fDE ↑ sensitivity to drug-induced ventricular tachycardia equal to wDE.	1 day after exposure, filtered DE exclusively caused autonomic imbalance and increased sensitivity to drug-induced fatal arrhythmia.
Lamb *et al*. (2012)	Hypertension: Spontaneously hypertensive rats	fDE and wDE ("low" - 150 µg/m^3 "high" - 500 µg/m^3)	fDE but not wDE ↓heart rate, ↑PR, ↓ST area and ↓T-wave area during exposure.	Filtered DE exclusively caused parasympathetic activation, slowed AV conduction, and altered repolarization.

Both DE groups had decreased BP during exposure, suggesting mediation by DE gases. Although the specific gases mediating this effect remain unclear, there is mounting evidence that inhaled NO is converted to nitric oxide (NOx), which remains in the blood up to 2 h postexposure (Knuckles *et al.*, 2011). NOx decreases BP through vasodilation and facilitates parasympathetic control over cardiac function (Conlon and Kidd, 1999; Yabe *et al.*, 1998). In turn, parasympathetic activation inhibits stress-induced catecholamine release (Katz, 2006; Vanhoutte and Levy, 1980). Conversely, clean air exposure increased BP, likely due to stress-induced catecholamine release, which has been demonstrated under conditions similar to our control exposure (e.g., confinement) (Morimoto *et al.*, 2000). Incidentally, increased catecholamines decrease HDL cholesterol (Kjeldsen *et al.*, 1992), whereas parasympathetic activation inhibits this effect (Benthem *et al.*, 2001), perhaps explaining why air-exposed rats had decreased HDL (and fDE-exposed rats had normal HDL) relative to naive unconfined SHHF rats from a previous study (Carll *et al.*, 2011a). We have previously found "increases" in HDL concomitant with similar parasympathetic-associated effects from PM (Carll *et al.*, 2010). The mechanisms underlying the hemodynamic and biochemical effects of DE gases may bear important implications for the CV risks of DE.

Ultimately, our findings of altered CV function identify potentially disparate responses to the gaseous and particulate components of DE with equally relevant implications toward CV risk and autonomic balance. Increased sympathetic tone is a major putative mechanism of PM-induced CV pathogenesis (Brook, 2008). Consistent with this prevailing hypothesis, inclusion of particles in DE caused a decrease in HRV and diluted the parasympathetic-associated effects of DE gases (AV conduction delay, Mobitz II AV block, HRV increase, BP decrease), suggesting increased sympathetic tone. There is some evidence that oxidative stress mediates particulate-induced sympathetic excitation and decreased HRV (Rhoden *et al.*, 2005), but this mechanism remains underexplored. Regardless, it is important to note that our data neither suggest inherently protective nor prove directly autonomic effects of DE particles. Meanwhile, the stimulation of pulmonary irritant receptors (e.g., C-fibers, transient receptor potential ankyrin-1) is known to cause parasympathetic activation and resulting CV reflexes, including bradycardia and hypotension (Widdicombe and Lee, 2001). Although there is evidence that wDE (Hazari *et al.*, 2011; Wong *et al.*, 2003) and several of its components, including SO_2 (Wang *et al.*, 1996) and particles (Deering-Rice *et al.*, 2011) stimulate pulmonary irritant receptors, it remains unclear to what extent each component factors into physiological reflexes. Our findings imply a predominant role for DE gases in pulmonary irritant reflexes and a potentially divergent mode for PM-induced autonomic effects; however, mechanistic studies are required to discern whether the effects of fDE and wDE result from differing autonomic influences of components or other factors, such as physicochemical interactions between particles and gases. For instance, particles could alter the dynamics of gas deposition, thereby modifying the stimulation of irritant receptor subgroups, which can mediate opposing physiological reflexes (Widdicombe and Lee, 2001).

The SHHF strain is derived from the spontaneously hypertensive (SH) rat, which we have recently shown has enhanced susceptibility to DE exposure (Lamb *et al.*, 2012). Yet, the effects of fDE that we report here on AV block arrhythmia and HRV were not seen in our companion study involving SH rats (Lamb *et al.*, 2012). Thus, the SHHF appears to be even more sensitive to the cardiophysiological impacts of DE exposure, potentially stemming from its underlying pathology including more severe hypertension than the SH rat and more rapid myocardial remodeling (Carll *et al.*, 2011b). The 10-week-old SHHF rats used in the present study had a significant cardiac hypertrophy (tibia-normalized heart weight 15% greater than 19-week-old normotensive Wistar Kyoto [WKY] rats; $p < 0.01$)

that was equivalent to 23-week-old SH rats (Lamb *et al.*, 2012; Carll, *unpublished data*). Additionally, AV conduction rate (PR) seems to be slower in the SHHF than the SH rat, which itself has a longer PR interval than age-matched WKYs (Hazari *et al.*, 2009). Notably, PR duration correlates with age and arterial stiffness (Gosse *et al.*, 2011). Despite being less than half the age of the SH rats used in our companion study (Lamb *et al.*, 2012), the SHHF rats herein had a baseline PR interval that was 5.2 ms longer. Further investigation is required to determine if aspects of cardiac and vascular remodeling mediate the SHHF's heightened sensitivity to air pollutant exposure. Nevertheless, the parallels between the SHHF and hypertensive, hypertrophic humans combined with our findings of enhanced responses in the SHHF relative to SH and WKY rats, further indicate that CV disease confers sensitivity to the effects of air pollution on cardiac conduction.

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

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