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Hypoxia Stress Test Reveals Exaggerated Cardiovascular Effects in Hypertensive Rats After Exposure to the Air Pollutant Acrolein

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Exposure to air pollution increases the risk of cardiovascular morbidity and mortality, especially in susceptible populations. Despite increased risk, adverse responses are often delayed and require additional stress tests to reveal latent effects of exposure. The goal of this study was to use an episode of "transient hypoxia" as an extrinsic stressor to uncover latent susceptibility to environmental pollutants in a rodent model of hypertension. We hypothesized that exposure to acrolein, an unsaturated aldehyde and mucosal irritant found in cigarette smoke, diesel exhaust, and power plant emissions, would increase cardiopulmonary sensitivity to hypoxia, particularly in hypertensive rats. Spontaneously hypertensive and Wistar Kyoto (normotensive) rats, implanted with radiotelemeters, were exposed once for 3 h to 3 ppm acrolein gas or filtered air in whole-body plethysmograph chambers and challenged with a 10% oxygen atmosphere (10 min) 24 h later. Acrolein exposure increased heart rate, blood pressure, breathing frequency, and minute volume in hypertensive rats and also increased the heart rate variability parameter LF, suggesting a potential role for increased sympathetic tone. Normotensive rats only had increased blood pressure during acrolein exposure. The hypoxia stress test after acrolein exposure revealed increased diastolic blood pressure only in hypertensive rats and increased minute volume and expiratory time only in normotensive rats. These results suggest that hypertension confers exaggerated sensitivity to air pollution and that the hypoxia stress test is a novel tool to reveal the potential latent effects of air pollution exposure.

Key Words: **spontaneously hypertensive rat; hypoxia; acrolein; blood pressure.**

Exposure to air pollution increases cardiovascular morbidity and mortality, especially in individuals with pre-existing cardiovascular diseases (Brook *et al*., 2010). Epidemiological studies indicate that these effects are not immediate, usually manifesting some time after exposure. For example, Peters *et al*. (2004) showed that humans exposed to traffic were more susceptible to myocardial infarction up to 24 h after exposure. In addition, patients with implanted cardioverter defibrillators had increased incidence of life-threatening arrhythmias up to 2 days after air pollution exposure (Peters *et al*., 2000). These findings suggest that exposure to air pollution alters cardiovascular physiology and increases conditional susceptibility to triggers of thrombosis and arrhythmia. Moreover, the fact that these effects happen at lower exposure concentrations than those shown to elicit effects in animal studies indicates that the responses are more complicated than the standard monotonic dose-response relationship of traditional toxicology. The reduced capacity to compensate to daily stressors may account for the "lagged" responses associated with air pollution exposure. The study of the factors that determine the capacity to compensate fully presents a potentially informative approach when seeking to uncover and explain the latent effects of air pollution exposure.

A stress test is a common way to reveal enhanced sensitivity or the potential for adverse responses because increased cardiovascular effort causes reduced oxygen availability to tissues and may provoke autonomic imbalance (Goldberger *et al*., 2006). Numerous studies have shown that stress tests trigger many adverse cardiovascular effects including increased cardiac arrhythmias and deleterious changes in heart rate (HR), heart rate variability (HRV), an indirect indicator of cardiac autonomic tone, and electrocardiogram (ECG) parameters (Watanabe *et al*., 2001). Recently, exercise stress was used to demonstrate that exposure to diesel exhaust during exercise

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in men with coronary artery disease caused myocardial ischemia (Mills *et al*., 2007). Analogous effects, including increases in cardiac arrhythmia and myocardial ischemia, were demonstrated in rats infused with the sympathomimetic dobutamine 1 day after exposure to diesel exhaust (Hazari *et al*., 2012). In addition, rats exposed to particulate matter or acrolein had exaggerated sensitivity to aconitine, an agent that causes myocardial calcium loading (Hazari *et al*., 2009). These results are consistent with epidemiological evidence for a time lag in responses and highlight the value of stress tests in unmasking latent responses.

We have previously demonstrated that exposure to both particulate and gaseous air pollutants, including acrolein, causes exaggerated cardiovascular effects in rat models of hypertension and heart failure (Carll *et al*., 2012; Farraj *et al*., 2011; Hazari *et al*., 2009; Lamb *et al*., 2012). Whereas the mechanisms responsible for these effects are unclear, recent work by Wang *et al*. (2012) demonstrated that cardiac arrhythmias in particulate-exposed heart failure mice were in part due to altered sensitivity of the carotid body, a key organ involved in oxygen sensing and initiating reflex cardiopulmonary changes to maintain homeostasis. Furthermore, exposures to the air pollutants, tobacco smoke (Adgent, 2006), sulfur dioxide, and nitrogen dioxide (Hoppenbrouwers *et al*., 1981), have been linked to abnormal cardiopulmonary sensitivity responses to hypoxia. These findings suggest that the response to hypoxia may be useful in unmasking the latent effects of air pollution. The purpose of this study was to (1) examine the utility of a novel hypoxia stress test in revealing the potential latent effects of air pollution and (2) test the hypothesis that acrolein exposure will modify the response to hypoxia in hypertensive rats, but not in normal rats, given that hypertensive individuals have exaggerated sensitivity to low atmospheric oxygen (Yu *et al*., 1999). HR, blood pressure (BP), ECG, and HRV were measured during acrolein exposure and 1 day later during hypoxia challenge.

MATERIALS AND METHODS

Animals. Twelve-week-old male spontaneously hypertensive (SH) ($n = 6$) and Wistar Kyoto (WKY) normotensive ($n = 6$) rats (Charles River, Raleigh, NC) were housed in plastic cages (one per cage), maintained on a 12-h light/dark cycle at approximately 22°C and 50% relative humidity in our Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, and held for a minimum of 1 week before exposure. Food (Prolab RMH 3000; PMI Nutrition International, St Louis, MO) and water were provided *ad libitum*, and all rats were randomized by weight. After all experiments were performed, rats were deeply anesthetized with an ip injection of Euthasol (200 mg/kg Na pentobarbital and 25 mg/kg phenytoin; Virbac Animal Health, Fort Worth, TX) and euthanized by exsanguination. The Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency (U.S. EPA) approved all protocols.

Telemeter implantation. Animals were implanted with radiotelemetry transmitters to monitor HR, BP, and heart rhythms and electrocardiographic intervals (Model TL11M2-C50-PXT; Data Science International, Inc., St Paul, MN). Charles River Laboratories performed all telemetry implantation surgeries in accordance with methods specified by the vendor (Charles River Laboratories, 2005). Animals were shipped to the U.S. EPA 10 days after implantation surgeries, and animals were allowed to recover for 1 week at the U.S. EPA before exposures began.

A separate cohort of SH ($n = 5$) and WKY ($n = 5$) rats were implanted with femoral artery catheters in accordance with methods specified by the vendor (Charles River Laboratories, 2005). Animals were shipped to the U.S. EPA within 1 week of surgery. The catheters were flushed with saline, locked with heparin, and plugged immediately upon arrival and every 2 days thereafter until exposure began.

Acrolein exposure and hypoxia stress test. A paired design was utilized to control for baseline differences in HR, BP, and ECG rhythms between rats. SH and WKY rats were first exposed to air control for 3 h and then underwent a hypoxia stress test 24 h later. The hypoxia stress test was performed 24 h after acrolein exposure to uncover latent responses consistent with the delay between exposure and adverse health effects observed in some epidemiological studies (Peters *et al*., 2000, 2004). Exposure to subatmospheric oxygen has been used as a stress test in humans with hypertension to demonstrate exaggerated cardiorespiratory responses to hypoxia (Ledderhos *et al*., 2002). After 5 days, these same rats were exposed to 3 ppm acrolein for 3 h and then underwent a second hypoxia stress test 24 h later (please refer to Fig. 1A). We assumed that a period of 5 days between the initial hypoxia stress test and the subsequent exposures would allow residual effects, if any, of the initial hypoxia stress test to lapse. This was confirmed by the absence of any differences in air-exposed rats at baseline relative to the previous air and hypoxia exposures.

All exposures (air and acrolein) and the hypoxia stress test took place in whole-body plethysmography (WBP) chambers (Model PLY3213, Buxco Electronics, Inc., Wilmington, NC), which continuously and noninvasively monitor ventilatory parameters in conscious animals. All rats were acclimated to the chambers for 1 h two days prior to both air and acrolein exposure. On exposure days, rats were allowed to acclimate to exposure chambers for 30 min, and then baseline data were recorded for the next 30 min. For air exposure, rats were exposed to filtered air in WBP chambers for 3 h. HR, systolic and diastolic BP, ECG waveforms, and ventilatory data were collected during the entire exposure period. After exposure, animals were returned to their home cages. Twenty-four hours after air exposure, animals underwent a hypoxia stress test. Animals were placed in WBP chambers and allowed to acclimate for 30 min. A 30-min baseline was then recorded. After baseline measurements, animals were kept at 20% oxygen concentration (ambient O_2 concentration) for 5 min before the hypoxia stress test. To reduce the PO_2 in the chamber, 100% nitrogen (N_2) gas was delivered to a glass mixing chamber where the gas was mixed with dry filtered air to achieve a fraction of inspired O_2 (FIO_2) of 10% at a flow rate of 2 L/min. Oxygen levels were monitored continuously with an O_2 sensor attached directly to the chamber, and an FIO_2 of 10% was maintained for 10 min. Nitrogen was then turned off, and the FIO_2 oxygen saturation rose to 20% (Fig. 1B). Rats were then kept in the chambers for an additional 15 min. HR, systolic and diastolic BP, ECG waveforms, and ventilatory data were collected continuously during the stress test.

Five days after air exposure and the hypoxia stress test when all latent preconditioning was absent, these same rats were exposed to 3 ppm acrolein for 3 h in the same WBP chambers. The concentration of acrolein (3 ppm) is higher than ambient levels, but it may represent concentrations in high combustion areas (Hazari *et al*., 2008). In addition, cigarette smoke contains up to 90 ppm acrolein (Esterbauer *et al*., 1991), and acrolein levels in sidestream tobacco smoke are as high as 10 ppm (Esterbauer *et al*., 1991). Acrolein gas was metered from a 1000 ppm cylinder into a glass mixing chamber where the gas was mixed with dry filtered dilution air to achieve a final concentration of 3 ppm of acrolein with a total flow of 6 L/min. The actual chamber concentration was measured using an HP5890 gas chromatograph (GMI Inc., Ramsey, MN) equipped with manual injection, a flame ionization detector, and a DB-VRX capillary column. The plethysmograph pressure was monitored using Biosystems XA software (Buxco Electronics, Inc.). Using respiratory-induced fluctuations in ambient pressure, respiratory parameters including tidal volume, breathing frequency, inspiratory time, and expiratory time were

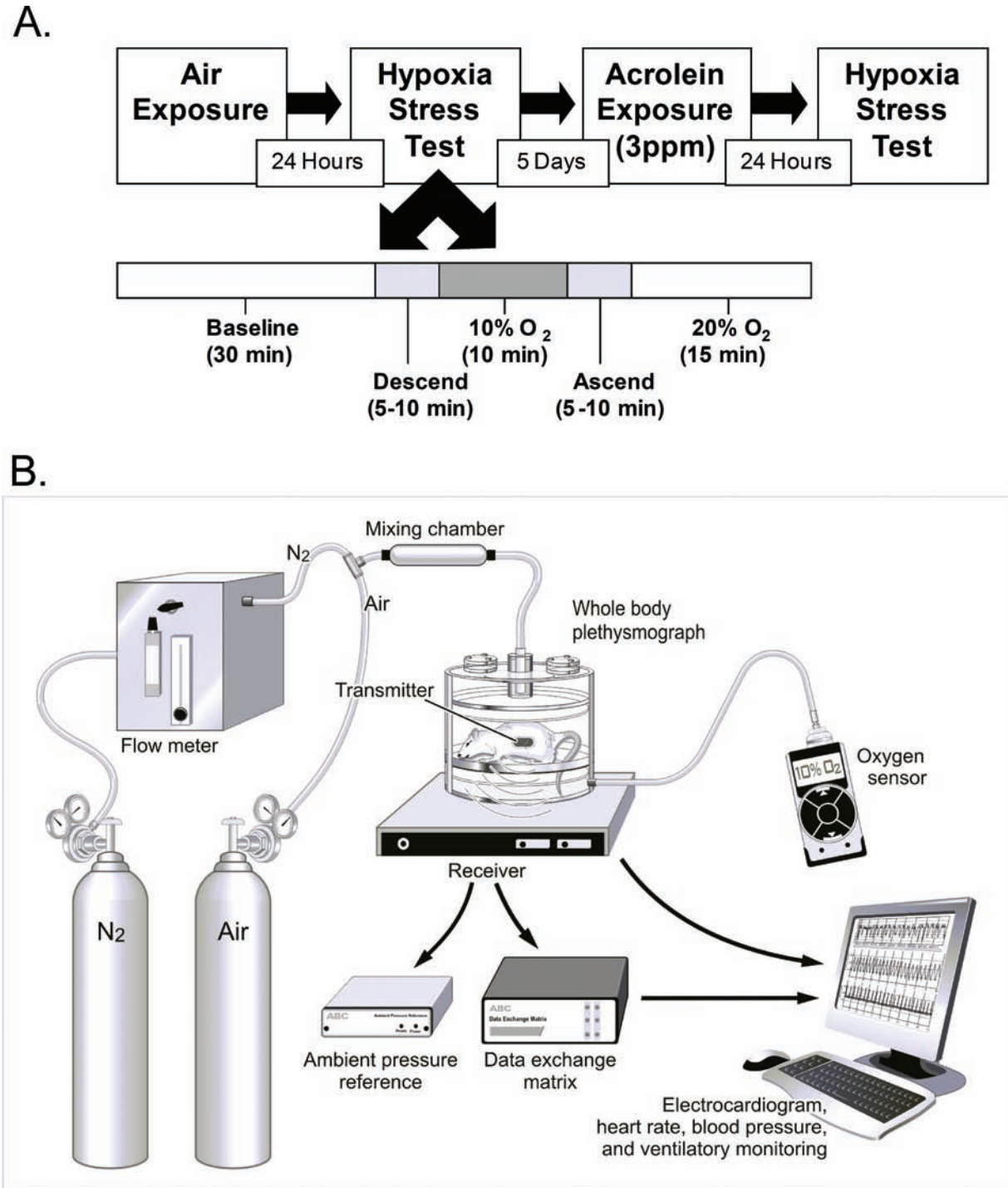

FIG. 1. A novel hypoxia stress test may reveal latent sensitivity to a gaseous pollutant. Panel (A) describes the experimental design for the study. Rats were exposed to air for 3 h on day 1 and underwent a hypoxia stress test 24 h after air exposure. These same rats were given a 5-day washout period and then exposed to 3 ppm acrolein for 3 h followed by a hypoxia stress test 24 h after acrolein exposure. The hypoxia stress test consists of a 30-min baseline, the descend period (time it takes for FIO_2 to decrease from 20 to 10%), a 10-min hypoxia period (10% FIO_2, an ascend period—time it takes for FIO_2 to increase from 10 to 20%), and a 15-min period after the return to normal FIO_2 (20%). Panel (B) represents the hypoxia stress test experimental setup. The rat is placed in a Buxco plethysmograph that sits over a radiotelemetry receiver. Ventilatory, ECG, HR, and BP data are captured during the entire stress test. For hypoxia, nitrogen is metered into the plethysmograph, and an oxygen sensor attached to the plethysmograph records FIO_2 in real time.

calculated and recorded on a breath-by-breath basis and averaged over 10-s intervals. HR, systolic and diastolic BP, ECG waveforms, and ventilatory data were collected during the exposure, and animals were returned to their home cages after exposure. Rats underwent a hypoxia stress test 24 h later as previously described. Because the data collected were repeated measures, the data during acrolein exposure and the subsequent hypoxia stress test are presented as % change from air exposure and the initial hypoxia stress test, respectively.

Validation of hypoxia stress test. The hypoxia stress test was validated by measuring arterial blood O_2 saturation (SaO_2) during hypoxia challenge in a separate cohort of rats. Rats implanted with femoral artery catheters were acclimated to WBP chambers (Model PLY3213, Buxco Electronics, Inc.) for 1 h two days prior to hypoxia validation. On the day of validation, the exteriorized catheter was extended with a 1-ml PE50 catheter attached to a 23-gauge adapter and run through a premeasured hole in the wall of the plethysmograph to allow for blood draws outside of the sealed exposure chamber. Rats were acclimated to the exposure chambers for 30 min and then underwent a 250-µl blood draw while conscious and unrestrained within the chamber. This served as the baseline blood sample. Nitrogen was metered into the chamber to reduce the FIO_2 to 10%, and a second 250-µl blood sample was taken after the FIO_2 stabilized at 10%. The addition of N_2 was discontinued, and a final 250-µl blood sample was taken 15 min after the oxygen content of the chamber stabilized at an FIO_2 of 20%. For each blood draw, the pin was removed from the tip of the catheter, and the heparin was allowed to exit the catheter. When only blood remained, a 23-gauge adapter attached to a 1-ml syringe was placed at the end of the catheter and used to draw a 250-µl sample. The blood sample was immediately read on an OPTI CCA-TS Blood Analyzer (OPTI Medical Systems, Inc.) using the OPTI Cassette E-Ca, which measures blood pH, PCO_2, PO_2, Na^+, K^+, Ca^{2+}, total hemoglobin, oxygen saturation (SaO_2), and hematocrit. The catheter was flushed with saline and locked with heparin after each blood draw.

WBP data acquisition and analysis. All exposures were performed in WBP chambers (Buxco Electronics, Sharon, CT). The plethysmography methodology permitted continuous monitoring of breathing frequency, tidal volume, minute volume, inspiratory time, and expiratory time. Animals were acclimated to plethysmographs for 1 h each day two days prior to exposure before both air and acrolein exposure. Plethysmography chambers (model PLY3213; Buxco Electronics) were calibrated each day before every animal loading. A bias flow regulator delivered fresh air (1.8 L/min) to each cylindrical chamber, preventing CO_2 buildup within the chamber. Unrestrained animals were placed in individual cylindrical plethysmograph boxes containing a built-in reference chamber for measuring respiration-induced pressure fluctuations. Data were channeled to computer software (BioSystem XA; Buxco Electronics) that calculated respiratory parameters. Data were collected continuously for each parameter, and automated breath-by-breath analyses were performed using a rejection algorithm to eliminate breaths that were outside a given range. On exposure day, animals were acclimated to the chambers for 30 min before any readings were taken. After acclimation, 30 min of baseline data were taken, and animals were exposed to either control air or 3 ppm acrolein for 3 h. After exposure, rats were removed and returned to home cages.

Radiotelemetry data acquisition. Radiotelemetry methodology (Data Sciences International, Inc.) enabled constant monitoring of ECG data in unrestrained, unanesthetized rats from implantation until euthanasia. Remote receivers (DataART3.01; Data Sciences International, Inc.) were positioned under the home cages and under the plethysmographs during exposure, which collected the ECG data and transferred them to the computer for storage. In home cages, sixty-second segments of ECG waveforms were acquired and saved at 15-min intervals from surgical recovery through euthanasia not including the exposure period. Pre-exposure baseline data were collected from home cages and a 30-min baseline in exposure cages after a 30-min acclimation period. During the 3-h exposure, sixty-second segments were acquired and saved at 5-min intervals. After exposure, rats were monitored in home cages until euthanasia, approximately 18 h after the end of the final hypoxia challenge. HR and BP were automatically obtained from the ECG and BP waveforms, respectively, with data acquisition software (DataART3.01; Data Sciences International, Inc.).

ECG, arrhythmia identification, and HRV analysis. ECGAuto software (EMKA Technologies, Falls Church, VA) was used for automated analyses of ECG wave amplitudes and segment durations and areas, as well as for the visual identification and enumeration of cardiac arrhythmias. Several parameters were determined for each ECG waveform: PR interval; R amplitude and duration; QRS area; ST interval, amplitude, and area; and T-wave amplitude and area; QT interval; and heart rate–corrected QT interval. ECG parameters during exposure were analyzed in terms of baseline (30 min recordings while in the exposure chambers immediately before the beginning of exposure) and hours 1–3 (constituting the entire exposure period between 8:00 a.m. and 11:00 a.m.).

Cardiac arrhythmic events were identified in part by using the Lambeth conventions (Walker *et al*., 1988) as a guideline for the identification of arrhythmias in rats. Arrhythmias were identified as atrial premature beats, ventricular premature beats, sinoatrial blocks, atrioventricular blocks, or ventricular tachycardia. Arrhythmias were quantified, counted, and totaled over an 18-h period prior to exposure (this corresponded to the same times assessed after exposure), during the 4-h exposure period, or during the 18-h period beginning immediately after exposure. Total arrhythmia counts during exposure were quantified (total of forty-eight 2-min segments during 3-h exposure period) and expressed as counts per minute.

For the analysis of HRV, thorough visual inspection was conducted to identify and exclude arrhythmias, artifacts, and 1-min ECG waveforms lacking distinguishable R waves for more than 30 s. The analysis of HRV generated HR and time-domain measures, including mean time between adjacent QRS complex peaks (the RR interval), a standard deviation of the RR interval (SDNN), SDNN normalized for the effects of HR (SDNN/[RR interval × 100]), and the square root of the mean of squared differences of adjacent RR intervals (RMSSD). The SDNN represents overall HRV, whereas RMSSD represents parasympathetic influence over HR. The analysis of HRV also calculated frequency domain parameters, particularly low frequency (LF) and high frequency (HF), and the ratio of these two frequency domains (LF/HF). LF is generally believed to represent a mixture of sympathetic and parasympathetic tone, whereas HF indicates cardiac parasympathetic (vagal) tone, and LF/HF serves as an index of sympathovagal balance.

Statistics. The statistical analyses of the Buxco, ECG, and HRV data in this study used SAS version 9.2 software (SAS Institute Inc., Cary, NC). We used PROC MIXED of SAS because it offers greater flexibility for the modeling of repeated measures data than PROC GLM. It is also suitable for analysis of large, unbalanced data with missing data at random. A linear mixed model with restricted maximum-likelihood estimation analysis, least squares means, and repeated measures ANOVA was used to determine which TIME × TRT interactions were statistically significant between baseline and exposure. Multiple comparison adjustment for the p values and confidence limits for the differences between the least squares means was done using adjust = Tukey HSD test. All blood gas data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) with a one-way ANOVA model examining the main effects of each model. All strain comparisons were performed using GraphPad Prism (GraphPad Software, Inc.) with a Student t-test. Trend analysis of SDNN data during exposure and HR data during hypoxia challenge was performed using GraphPad Prism (GraphPad Software, Inc.) with a one-way ANOVA followed by a *post hoc* test for linear trend between mean and column number. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Acrolein on HR, BP, and HRV

HRs increased in SH rats during all 3 h of acrolein exposure (Fig. 2A). At hour 1, mean heart rates increased from baseline levels of 294.4 ± 5.4 beats per minute (bpm) to 313.3 ± 15.3

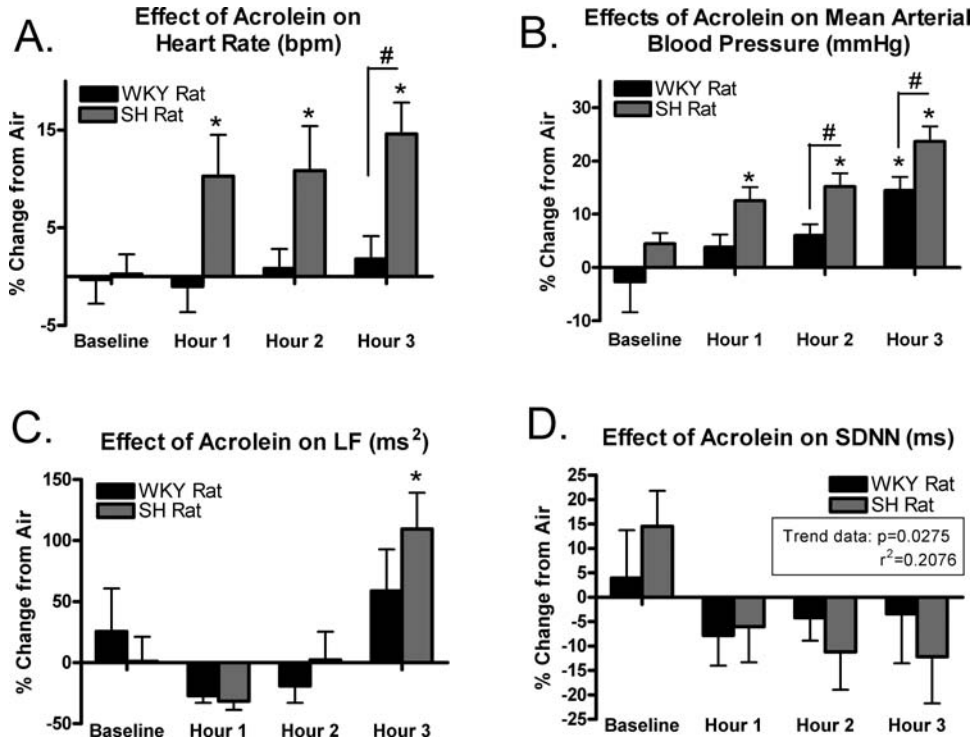

FIG. 2. Acrolein exposure causes increases in HR, mean arterial BP, and the LF component of HRV in SH rats. Panels (A), (B), (C), and (D) relate to HR, BP, LF, and SDNN values at baseline and all 3 h of acrolein exposure, respectively. Panels (A), (B), (C), and (D) are presented as % change from air. Percent change baseline values are compared with the average % change values during each hour of exposure. Means and standard errors are reported. Significant differences from baseline percent change values ($p < 0.05$) are denoted with *. Significant differences ($p < 0.05$) between SH and WKY strains at each hour are denoted with #. There was a significant negative trend in the SDNN data ($p = 0.0275$; $r^2 = 0.2076$).

bpm. At hour 2, mean heart rates increased from baseline levels of 269.2 ± 3.9 bpm to 297.7 ± 16.0 bpm. At hour 3, mean heart rates increased from baseline levels of 274.1 ± 6.7 bpm to 305 ± 16.7 bpm. When each acrolein-exposed rat is compared with itself during air exposure, these changes constituted a 10.3 ± 4.22% increase from air at hour 1, a 10.8 ± 4.57% increase from air at hour 2, and a 14.6 ± 3.17% increase from air at hour 3 ($p < 0.05$; Fig. 2A). HR did not change in WKY rats during acrolein exposure.

Before exposures commenced, the baseline BP values for all rats were calculated, and it was determined that SH rats had an average of 44 mmHg higher baseline mean arterial BP level than WKY rats (162.46 ± 3.71 mmHg vs. 118.57 ± 4.26 mmHg, respectively), consistent with their hypertensive phenotype. Mean arterial BP increased during hour 2 of acrolein exposure in SH rats and during hour 3 of acrolein exposure in both SH and WKY rats (Fig. 2B). In SH rats, mean arterial BP increased from baseline levels of 150.20 ± 2.35 mmHg to 169.65 ± 4.04 mmHg at hour 2, constituting a 15.19 ± 2.51% increase from air ($p < 0.05$; Fig. 2B). At hour 3, mean arterial BP increased from baseline levels of 154.14 ± 2.07 mmHg to 189.32 ± 4.06 mmHg, constituting a 23.67 ± 2.79% increase from air in SH rats ($p < 0.05$; Fig. 2B). The increases in mean arterial BP at hour 3 were accompanied by 22.86 ± 3.38% and 23.31 ± 3.30% increases in systolic and diastolic BP, respectively. In WKY rats, BP significantly increased from baseline levels of 120.15 ± 2.74 mmHg to 130.24 ± 5.38 mmHg at hour 3, constituting a 14.44 ± 2.55% increase from air ($p < 0.05$; Fig. 2B).

SH rats also had a significant increase in the LF HRV parameter during hour 3 of acrolein exposure (Fig. 2C). LF increased from baseline levels of 1.55 ± 0.37 to 3.07 ± 0.51 ms^2, constituting a 109.39 ± 29.74% increase from air ($p < 0.05$; Fig. 2C). In addition, SH rats had a nonsignificant decrease in SDNN during all hours of acrolein exposure, but there was a significant negative trend in the data ($p = 0.0275$; $r^2 = 0.2076$) (Fig. 2D). There were no significant changes in RMSSD, HF, and LF/HF in SH rats, and there were no significant changes in any HRV parameter in WKY rats.

Ventilatory Changes During Acrolein Exposure

SH rats had a significant increase in breathing frequency and minute volume in the third hour of acrolein exposure (Figs. 3A and B). Breathing frequency increased from baseline levels of 101.33 ± 8.86 breaths per minute at baseline to 122.95 ± 12.96 breaths per minute at hour 3, constituting a 34 ± 8.61% increase from air ($p < 0.05$; Fig. 3A). Minute volume increased from 149.87 ± 11.4 ml at baseline to 181.4 ± 20.85 ml at hour 3 of acrolein exposure, constituting an increase of 25 ± 4.27% from

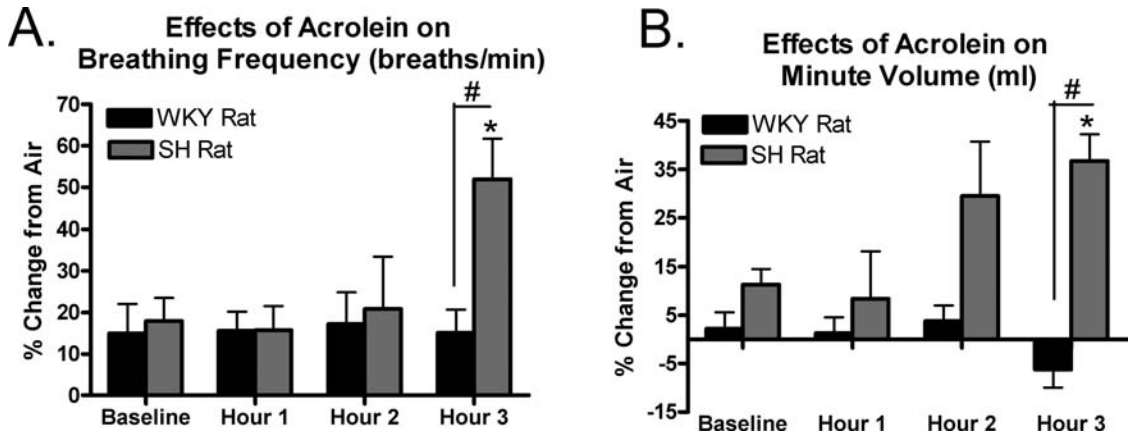

FIG. 3. Acrolein exposure causes significant increases in breathing frequency and minute volume in SH rats. Panels (A) and (B) relate to breathing frequency and minute volume, respectively, measured at baseline, 1, 2, and 3 h during acrolein exposure. All values are presented as % change from air. Percent change baseline values are compared with the average % change values during each hour of exposure. Significant differences from baseline percent change values ($p < 0.05$) are denoted with *. Significant differences ($p < 0.05$) between SH and WKY strains at each hour are denoted with #.

TABLE 1
Arterial Blood Parameters During the Hypoxia Stress Test

Exposure	Timing	pO_2 (mmHg)	pCO_2 (mmHg)	SaO_2 (%)	pH	K^+ (mmol/l)
SH rat	Baseline	98.60 ± 2.42	37.00 ± 1.00	93.60 ± 0.51	7.31 ± 0.033	7.66 ± 1.10
	10% chamber O_2	50.20 ± 1.71*	31.00 ± 0.84*	79.40 ± 0.68*	7.47 ± 0.044	7.36 ± 0.81
	20% chamber O_2	94.60 ± 4.53	38.20 ± 1.80	94.20 ± 0.37	7.39 ± 0.048	6.70 ± 0.72
WKY rat	Baseline	99.80 ± 5.16	41.40 ± 2.86	94.60 ± 0.68	7.41 ± 0.045	5.70 ± 0.49
	10% chamber O_2	54.80 ± 2.60*	28.60 ± 1.25*	82.40 ± 0.68*	7.43 ± 0.070	5.80 ± 0.87
	20% chamber O_2	95.40 ± 1.89	41.80 ± 1.80	93.40 ± 0.40	7.40 ± 0.023	5.76 ± 0.69

Notes. The hypoxia stress test successfully decreases pO_2, pCO_2, and blood SaO_2 in arterial blood in SH and WKY rats. pH and K^+ concentrations are also reported. Means and standard errors are reported.
Significant differences ($p < 0.05$) are denoted with *.

air ($p < 0.05$; Fig. 3B). There were no significant changes in ventilatory parameters during acrolein exposure in WKY rats.

Electrocardiographic Changes and Arrhythmogenesis During Acrolein Exposure

There were no significant changes in PR interval, QRS interval or area, and ST- or T-wave area in SH and WKY rats exposed to acrolein (Supplementary table I). WKY rats did have a significant increase in the ST segment, but this change occurred during exposure to air and is not related to acrolein exposure (Supplementary table I). There were no significant changes in ECG, HRV, or arrhythmia after either hypoxia challenge. There were no significant changes in the frequency of spontaneous arrhythmia during or after acrolein exposure.

Validation of the Hypoxia Stress Test

All data for hypoxia validation are presented in Table I. A separate cohort of SH and WKY rats implanted with femoral artery catheters underwent the hypoxia stress test, and blood samples were analyzed before exposure to the 10% chamber O_2 atmosphere, during exposure to the 10% chamber O_2 atmosphere, and after the return to normal 20% chamber O_2 concentration. Exposure to 10% chamber O_2 atmosphere caused decreases in pO_2, pCO_2, and arterial blood O_2 saturation in both strains of rats. In SH rats, pO_2 levels decreased from 98.6 ± 2.42 mmHg at baseline to 50.20 ± 1.71 mmHg during exposure to 10% chamber O_2 and increased again to 94.60 ± 4.53 mmHg after O_2 levels were returned to 20% ($p < 0.05$; Table I). In WKY rats, pO_2 levels decreased from 99.80 ± 5.16 mmHg at baseline to 54.80 ± 2.60 mmHg during exposure to 10% chamber O_2 and increased again to 95.40 ± 1.89 mmHg after O_2 levels were returned to 20% ($p < 0.05$; Table I).

In SH rats, pCO_2 levels decreased from 37.00 ± 1.00 mmHg at baseline to 31.00 ± 0.84 mmHg during exposure to 10% chamber O_2 and increased again to 38.20 ± 1.80 mmHg after O_2 levels were returned to 20% ($p < 0.05$; Table I). In WKY rats, pCO_2 levels decreased from 41.40 ± 2.86 mmHg at baseline to 28.60 ± 1.25 mmHg during exposure to 10% chamber O_2 and increased again to 41.80 ± 1.80 mmHg after oxygen levels were returned to 20% ($p < 0.05$; Table I).

In SH rats, SaO_2 levels decreased from 93.60 ± 0.51% at baseline to 79.40 ± 0.68% during exposure to 10% chamber O_2 and increased again to 94.20 ± 0.37% after O_2 levels were returned to 20% ($p < 0.05$; Table I). In WKY rats, O_2 saturation levels

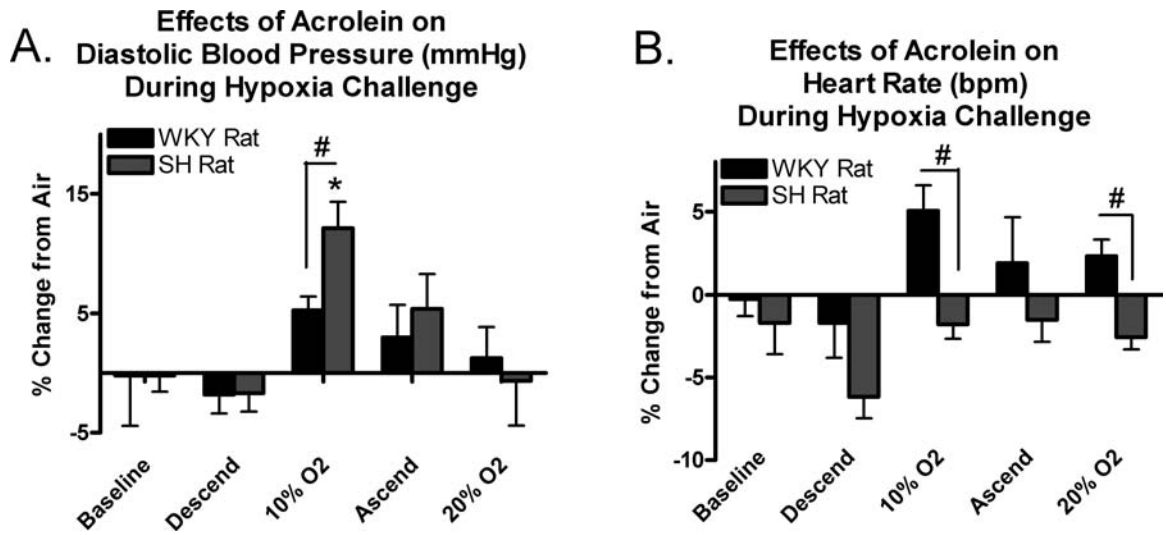

FIG. 4. Hypoxia stress test after acrolein exposure causes significant increases in diastolic BP in SH rats. Panels (A) and (B) refer to diastolic BP and HR, respectively, measured at baseline, the descend period, 10% FIO_2, the ascend period, and the return to 20% FIO_2. All values are presented as % change from hypoxia challenge after air exposure. Percent change baseline values are compared with the average % change values during the different segments of the hypoxia challenge. Means and standard errors are reported. Significant differences from baseline percent change values ($p < 0.05$) are denoted with *. Significant differences ($p < 0.05$) between SH and WKY strains at each hour are denoted with #.

decreased from 94.60 ± 0.68% at baseline to 82.40 ± 0.68% during exposure to 10% chamber O_2 and increased again to 93.40 ± 0.40% after O_2 levels were returned to 20% ($p < 0.05$; Table I).

BP and HR Changes During Hypoxia Stress Test

Hypoxia stress test was initially performed 24 h after a 3-h exposure to air. The 10% chamber O_2 concentration caused similar increases in HR in both the SH and WKY rats during the hypoxia period but did not affect systolic BP in either strain (data not shown).

Hypoxia stress test after acrolein exposure caused a significant increase in diastolic BP during exposure to 10% chamber O_2 in SH rats ($p < 0.05$; Fig. 4A). Diastolic BP increased from 124.99 ± 5.16 mmHg at baseline to 135.99 ± 2.51 mmHg during exposure to 10% chamber O_2, constituting a 12.11 ± 2.23% increase from air ($p < 0.05$; Fig. 4A). The hypoxia stress test caused a nonsignificant decrease in HR during the descend period, the time during which chamber O_2 saturation decreases from 20 to 10%, in the SH rat and a nonsignificant increase in HR during exposure to 10% chamber O_2 in WKY rats (Fig. 4B). In addition, SH rats displayed a higher baseline BP than WKY rats during the entire hypoxia stress test.

Respiratory Changes During Hypoxia Stress Test

Hypoxia stress test following air exposure increased breathing frequency and minute volume in SH and WKY rats during the descent and hypoxia period of the stress test and decreased expiratory time in SH and WKY rats during the descend period (data not shown). The magnitudes of these changes were similar between the two strains.

WKY rats, but not SH rats, showed significant increases in minute volume and expiratory time during hypoxia stress test performed after acrolein exposure. Minute volume increased from baseline levels of 125.00 ± 11.74 to 187.36 ± 9.49 ml, constituting a 57.93 ± 23.8% increase from air ($p < 0.05$; Fig. 5A). Expiratory time increased from baseline levels of 0.30 ± 0.02 to 0.72 ± 0.03 s, constituting a 130.02 ± 31.60% change from air during the ascend period ($p < 0.05$; Fig. 5B).

Arrhythmogenesis, Electrocardiographic, and HRV Changes During Hypoxia Stress Test

There were no significant changes in frequency of spontaneous arrhythmia, electrocardiographic, or HRV parameters during the hypoxia stress test after acrolein exposure in SH or WKY rats (Supplementary tables II and III, respectively).

DISCUSSION

This study demonstrates that acrolein exposure alone increases HR, mean arterial BP, breathing frequency, and minute volume in the hypertensive SH rat, with limited effects in the normotensive WKY rat. The cardiovascular responses in the SH rat during acrolein exposure were coupled with increases in the HRV parameter LF indicative of altered autonomic tone. The hypoxia stress test performed after acrolein exposure revealed additional strain-dependent cardiopulmonary responses including increased diastolic BP only in the SH rat and increased minute volume and expiratory time only in WKY rats.

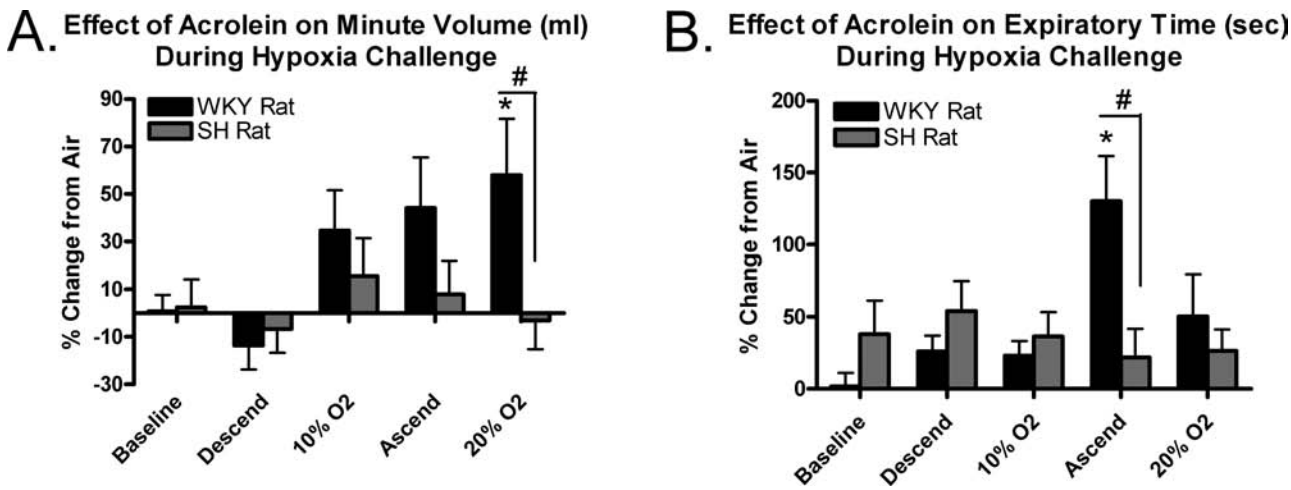

FIG. 5. Hypoxia stress test after acrolein exposure causes significant increases in minute volume and expiratory time in WKY rats. Panels (A) and (B) relate to minute volume and expiratory time, respectively, measured at baseline, the descend period, 10% FIO_2, the ascend period, and the return to 20% FIO_2. All values are presented as % change from hypoxia challenge after air exposure. Percent change baseline values are compared with the average % change values during the different segments of the hypoxia challenge. Means and standard errors are reported. Significant differences from baseline percent change values ($p < 0.05$) are denoted with *. Significant differences ($p < 0.05$) between SH and WKY strains at each hour are denoted with #.

The increases in HR and BP during acrolein exposure in the hypertensive rat may be related to altered autonomic tone during exposure. Chronic exposure to acrolein in mice has been linked to left ventricular dilation (Ismahil *et al.*, 2011) and progression of atherosclerosis (Srivastava *et al.*, 2011), whereas acute exposure to 3 ppm acrolein in this study caused significant increases in HR in SH rats during all 3 h of exposure. This was accompanied by increases in BP and the LF component of HRV and, although not statistically significant, a decrease in SDNN. Although the implications of these changes in rats are unclear, similar changes in humans are consistent with elevated sympathetic tone and an increased risk of heart disease (Pope *et al.*, 2001). In addition, iv injection of acrolein causes significant increases in BP that are reversed with guanethidine, a sympatholytic drug (Green and Egle, 1983). Despite these findings, there were no significant changes in LF/HF, a measure of sympathetic and parasympathetic balance. Furthermore, LF alone may not be a reliable indicator of sympathetic tone and may instead reflect an interaction of the sympathetic and parasympathetic nervous systems (Houle and Billman, 1999). Thus, further work is required to confirm linkages with altered autonomic tone. Although WKY rats had a significant increase in BP in the third hour of acrolein exposure, this response was not accompanied by increases in HR or any significant changes in HRV.

The hypoxia stress test revealed exaggerated cardiovascular sensitivity, characterized by increased diastolic BP, only in acrolein-exposed SH rats. This response may be due to increased vascular resistance as *in vivo* acrolein exposure in SH rats has been shown to increase *ex vivo* aortic reactivity (Conklin *et al.*, 2006). Hypoxia has been shown to heighten sympathetic activation in healthy men during periods of submaximal exercise (Al Haddad *et al.*, 2012). In addition, chronic intermittent hypoxia has been shown to increase arterial BP and impair vasodilation through increased levels of angiotensin II (Marcus *et al.*, 2012). Carotid body–mediated sensation of low blood SaO_2 levels triggers reflex cardiovascular responses including tachycardia, hypertension, and increased cardiac output (Downing *et al.*, 1962). SH rats have larger carotid bodies (Habeck *et al.*, 1985) and are more sensitive to the effects of hypoxia as evidenced by increased carotid sinus nerve activity and intracellular Ca^{2+} changes (Weil *et al.*, 1998). Thus, differential carotid body sensitivity may in part underlie the differences in responses to acrolein and hypoxia in normotensive and hypertensive rats. Recent work by Wang *et al.* (2012) demonstrated that particulate matter exposure–induced cardiac arrhythmias in mice with heart failure were in part due to dysregulation of carotid body sensitivity. Further research is required to examine the impact of carotid body sensitivity and the potential effects of air pollutant exposure on carotid body–mediated cardiovascular responses. Although the ventilatory responses to hypoxia after the mock air exposure were similar in SH and WKY rats, only WKY rats exposed to acrolein had significant increases in minute volume and expiratory time during the hypoxia stress test. It is unclear exactly why these ventilatory responses occurred only in WKY rats and not in SH rats, but it may relate to the increased respiratory sensitivity to air pollutant inhalation in the SH rat compared with the WKY rat. Previous studies (Farraj *et al.*, 2011) have shown that SH rats respond with greater airway inflammation and injury after air pollution exposure than WKY rats, and underlying susceptibility in the SH rat may result in the nerve fibers being overloaded and unable to illicit a normal response to acrolein.

An acrolein-induced airway lesion in the SH rat may have precluded a normal ventilatory response to hypoxia during the hypoxia stress test (Leikauf *et al*., 1989).

These findings demonstrate that the hypoxia stress test is a novel approach to reveal latent responses to air pollutants. Researchers are becoming increasingly interested in latent responses to air pollution exposure, and stress tests provide a useful model to study latent effects that would be missed in an acute exposure setting. Traditionally, epidemiological studies have estimated morbidity and mortality within 24 h of air pollution events. Although this time frame reveals significant increases in cardiovascular events after air pollution exposure, it may miss equally severe delayed responses that can occur up to 3 days (Faustini *et al*., 2011) and even 2 weeks after exposure (Goodman *et al*., 2004). Several stress tests have been used to determine enhanced sensitivity to air pollutants including exercise stress test in humans (Gold *et al*., 2005), dobutamine stress test in rats (Hazari *et al*., 2012), and isoproterenol cardiac stress test in rats (André *et al*., 2011). Intermittent hypoxia has been used to model nocturnal desaturation in sleep apnea patients (Norton *et al*., 2011). We designed a new hypoxia stress test with one prolonged (10 min) acute session of hypoxia followed by a return to normoxic conditions. The effects of the 10% O_2 atmosphere were validated by measuring blood O_2 saturation in arterial blood drawn before, during, and after hypoxia challenge. Arterial O_2 saturation, pO_2, and pCO_2 decreased significantly during hypoxia in both strains of rats. In addition, pH was not significantly affected by hypoxia challenge, demonstrating that the rat completely compensates for the drop in pCO_2, suggesting that the effects are not due to metabolic acidosis. Normal pO_2 levels in humans range from 80 to 100 mmHg, and a drop in partial pressure below 60 mmHg is required for hypoxemia. Normal pCO_2 levels in the humans range from 35–45 mmHg, and the hypoxia stress test reduced pCO_2 levels well below this level. Although the stress test was used in this context to examine susceptibility after air pollution exposure, the data suggest that it can easily be applied to different models to examine overall cardiovascular sensitivity.

The exaggerated effects of acrolein in the SH rats in this study are consistent with our previous studies with other air pollutants indicating enhanced sensitivity in this strain (Farraj *et al*., 2011; Lamb *et al*., 2012). In this study, SH rats had an average of 44 mmHg higher BP than WKY rats. It has long been known that hypertension increases the risk of cardiovascular disease and stroke (Brook *et al*., 2010). In addition to higher baseline BP, SH rats have increased arterial wall thickness (Mulvany and Halpern, 1977) and undergo left ventricular remodeling characterized by hypertrophy, fibrosis, and changes in membrane channels, cellular energetics, and ion regulation that combine to heighten myocardial sensitivity (Bernardo *et al*., 2010). SH rats were not only more susceptible to HR and BP increases during acrolein exposure, they also had significant increases in breathing frequency and minute volume in the third hour of exposure. Previous studies have found that acrolein, like other pulmonary irritants, causes decreases in respiratory rate (Hazari *et al*., 2008). It is unclear why SH rats had increased breathing frequency at hour 3 of acrolein exposure, but it is possible that these responses were secondary to the significant increases in HR and BP that were initiated during hours 1 and 2 of acrolein exposure. Minute volume tends to increase with exercise or stress, and it has been shown to increase with acrolein exposures (Linhart *et al*., 1996). Taken together, the results demonstrate that the SH rat has increased susceptibility to the adverse effects of acrolein exposure, including cardiovascular effects that appear directly and immediately and priming effects through homeostatic control pathways perhaps involving autonomic reflexes that set the stage for latent responsiveness to various stimuli.

The present findings demonstrate that acrolein-induced cardiovascular responses may result from modulation of autonomic tone, specifically increased sympathetic input to the heart. These findings also highlight the utility of the hypoxia stress test as a tool to unmask the potential for latent effects of air pollution via less appreciated mechanisms that mediate cardiovascular dysfunction associated with exposure to air pollution. To further examine the utility of this hypoxia stress test, we will assess the effects of exposure to other gaseous and particulate air pollutants (e.g., ozone and diesel exhaust) in future studies. Finally, the results further show that pre-existing cardiovascular disease confers exaggerated sensitivity to the effects of a model irritant, acrolein, that are consistent with the temporal relationship between exposure and clinical outcomes observed in epidemiological studies showing "delayed or latent" outcomes of air pollutant exposures.

SUPPLEMENTARY DATA

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

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REFERENCES

Adgent, M. A. (2006). Environmental tobacco smoke and sudden infant death syndrome: A review. *Birth Defects Res. B Dev. Reprod. Toxicol.* **77,** 69–85.

Al Haddad, H., Mendez-Villanueva, A., Bourdon, P. C., and Buchheit, M. (2012). Effect of acute hypoxia on post-exercise parasympathetic reactivation in healthy men. *Front. Physiol.* **3,** 289.

André, L., Gouzi, F., Thireau, J., Meyer, G., Boissiere, J., Delage, M., Abdellaoui, A., Feillet-Coudray, C., Fouret, G., Cristol, J. P., *et al.* (2011). Carbon monoxide exposure enhances arrhythmia after cardiac stress: Involvement of oxidative stress. *Basic Res. Cardiol.* **106,** 1235–1246.

Bernardo, B. C., Weeks, K. L., Pretorius, L., and McMullen, J. R. (2010). Molecular distinction between physiological and pathological cardiac hypertrophy: Experimental findings and therapeutic strategies. *Pharmacol. Ther.* **128,** 191–227.

Brook, R. D., Rajagopalan, S., Pope, C. A., 3rd, Brook, J. R., Bhatnagar, A., Diez-Roux, A. V., Holguin, F., Hong, Y., Luepker, R. V., Mittleman, M. A., *et al.*, American Heart Association Council on Epidemiology and Prevention, Council on the Kidney in Cardiovascular Disease, and Council on Nutrition, Physical Activity and Metabolism. (2010). Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. *Circulation* **121,** 2331–2378.

Carll, A. P., Hazari, M. S., Perez, C. M., Krantz, Q. T., King, C. J., Winsett, D. W., Costa, D. L., and Farraj, A. K. (2012). Whole and particle-free diesel exhausts differentially affect cardiac electrophysiology, blood pressure, and autonomic balance in heart failure-prone rats. *Toxicol. Sci.* **128,** 490–499.

Charles River Laboratories. (2005). Charles River Surgical Capabilities Reference Paper. **13,** 1.

Conklin, D. J., Bhatnagar, A., Cowley, H. R., Johnson, G. H., Wiechmann, R. J., Sayre, L. M., Trent, M. B., and Boor, P. J. (2006). Acrolein generation stimulates hypercontraction in isolated human blood vessels. *Toxicol. Appl. Pharmacol.* **217,** 277–288.

Downing, S. E., Remensnyder, J. P., and Mitchell, J. H. (1962). Cardiovascular responses to hypoxic stimulation of the carotid bodies. *Circ. Res.* **10,** 676–685.

Esterbauer, H., Schaur, R. J., and Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* **11,** 81–128.

Farraj, A. K., Hazari, M. S., Haykal-Coates, N., Lamb, C., Winsett, D. W., Ge, Y., Ledbetter, A. D., Carll, A. P., Bruno, M., Ghio, A., *et al.* (2011). ST depression, arrhythmia, vagal dominance, and reduced cardiac micro-RNA in particulate-exposed rats. *Am. J. Respir. Cell Mol. Biol.* **44,** 185–196.

Faustini, A., Stafoggia, M., Berti, G., Bisanti, L., Chiusolo, M., Cernigliaro, A., Mallone, S., Primerano, R., Scarnato, C., Simonato, L., *et al.*, EpiAir Collaborative Group. (2011). The relationship between ambient particulate matter and respiratory mortality: A multi-city study in Italy. *Eur. Respir. J.* **38,** 538–547.

Gold, D. R., Litonjua, A. A., Zanobetti, A., Coull, B. A., Schwartz, J., MacCallum, G., Verrier, R. L., Nearing, B. D., Canner, M. J., Suh, H., *et al.* (2005). Air pollution and ST-segment depression in elderly subjects. *Environ. Health Perspect.* **113,** 883–887.

Goldberger, J. J., Le, F. K., Lahiri, M., Kannankeril, P. J., Ng, J., and Kadish, A. H. (2006). Assessment of parasympathetic reactivation after exercise. *Am. J. Physiol. Heart Circ. Physiol.* **290,** H2446–H2452.

Goodman, P. G., Dockery, D. W., and Clancy, L. (2004). Cause-specific mortality and the extended effects of particulate pollution and temperature exposure. *Environ. Health Perspect.* **112,** 179–185.

Green, M. A., and Egle, J. L., Jr. (1983). Effects of intravenous acetaldehyde, acrolein, formaldehyde and propionaldehyde on arterial blood pressure following acute guanethidine treatment. *Res. Commun. Chem. Pathol. Pharmacol.* **40,** 337–340.

Habeck, J. O., Huckstorf, C., and Honig, A. (1985). Influence of age on the carotid bodies of spontaneously hypertensive (SHR) and normotensive rats. II. Alterations of the vascular wall. *Exp. Pathol.* **27,** 79–89.

Hazari, M. S., Callaway, J., Winsett, D. W., Lamb, C., Haykal-Coates, N., Krantz, Q. T., King, C., Costa, D. L., and Farraj, A. K. (2012). Dobutamine "stress" test and latent cardiac susceptibility to inhaled diesel exhaust in normal and hypertensive rats. *Environ. Health Perspect.* **120,** 1088–1093.

Hazari, M. S., Haykal-Coates, N., Winsett, D. W., Costa, D. L., and Farraj, A. K. (2009). A single exposure to particulate or gaseous air pollution increases the risk of aconitine-induced cardiac arrhythmia in hypertensive rats. *Toxicol. Sci.* **112,** 532–542.

Hazari, M. S., Rowan, W. H., Winsett, D. W., Ledbetter, A. D., Haykal-Coates, N., Watkinson, W. P., and Costa, D. L. (2008). Potentiation of pulmonary reflex response to capsaicin 24h following whole-body acrolein exposure is mediated by TRPV1. *Respir. Physiol. Neurobiol.* **160,** 160–171.

Hoppenbrouwers, T., Calub, M., Arakawa, K., and Hodgman, J. E. (1981). Seasonal relationship of sudden infant death syndrome and environmental pollutants. *Am. J. Epidemiol.* **113,** 623–635.

Houle, M. S., and Billman, G. E. (1999). Low-frequency component of the heart rate variability spectrum: A poor marker of sympathetic activity. *Am. J. Physiol.* **276**(1 Pt 2), H215–H223.

Ismahil, M. A., Hamid, T., Haberzettl, P., Gu, Y., Chandrasekar, B., Srivastava, S., Bhatnagar, A., and Prabhu, S. D. (2011). Chronic oral exposure to the aldehyde pollutant acrolein induces dilated cardiomyopathy. *Am. J. Physiol. Heart Circ. Physiol.* **301,** 2050–2060.

Lamb, C. M., Hazari, M. S., Haykal-Coates, N., Carll, A. P., Krantz, Q. T., King, C., Winsett, D. W., Cascio, W. E., Costa, D. L., and Farraj, A. K. (2012). Divergent electrocardiographic responses to whole and particle-free diesel exhaust inhalation in spontaneously hypertensive rats. *Toxicol. Sci.* **125,** 558–568.

Ledderhos, C., Pongratz, H., Exner, J., Gens, A., Roloff, D., and Honig, A. (2002). Reduced tolerance of simulated altitude (4200 m) in young men with borderline hypertension. *Aviat. Space. Environ. Med.* **73,** 1063–1066.

Leikauf, G. D., Leming, L. M., O'Donnell, J. R., and Doupnik, C. A. (1989). Bronchial responsiveness and inflammation in guinea pigs exposed to acrolein. *J. Appl. Physiol.* **66,** 171–178.

Linhart, I., Frantík, E., Vodicková, L., Vosmanská, M., Smejkal, J., and Mitera, J. (1996). Biotransformation of acrolein in rat: Excretion of mercapturic acids after inhalation and intraperitoneal injection. *Toxicol. Appl. Pharmacol.* **136,** 155–160.

Marcus, N. J., Philippi, N. R., Bird, C. E., Li, Y. L., Schultz, H. D., and Morgan, B. J. (2012). Effect of AT1 receptor blockade on intermittent hypoxia-induced endothelial dysfunction. *Respir. Physiol. Neurobiol.* **183,** 67–74.

Mills, N. L., Törnqvist, H., Gonzalez, M. C., Vink, E., Robinson, S. D., Söderberg, S., Boon, N. A., Donaldson, K., Sandström, T., Blomberg, A., *et al.* (2007). Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. *N. Engl. J. Med.* **357,** 1075–1082.

Mulvany, M. J., and Halpern, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.* **41,** 19–26.

Norton, C. E., Jernigan, N. L., Kanagy, N. L., Walker, B. R., and Resta, T. C. (2011). Intermittent hypoxia augments pulmonary vascular smooth muscle reactivity to NO: Regulation by reactive oxygen species. *J. Appl. Physiol.* **111,** 980–988.

Peters, A., Liu, E., Verrier, R. L., Schwartz, J., Gold, D. R., Mittleman, M., Baliff, J., Oh, J. A., Allen, G., Monahan, K., *et al.* (2000). Air pollution and incidence of cardiac arrhythmia. *Epidemiology* **11,** 11–17.

Peters, A., von Klot, S., Heier, M., Trentinaglia, I., Hörmann, A., Wichmann, H. E., and Löwel, H., Cooperative Health Research in the Region of Augsburg Study Group. (2004). Exposure to traffic and the onset of myocardial infarction. *N. Engl. J. Med.* **351,** 1721–1730.

Pope, C. A., 3rd, Eatough, D. J., Gold, D. R., Pang, Y., Nielsen, K. R., Nath, P., Verrier, R. L., and Kanner, R. E. (2001). Acute exposure to environmental tobacco smoke and heart rate variability. *Environ. Health Perspect.* **109,** 711–716.

Srivastava, S., Sithu, S. D., Vladykovskaya, E., Haberzettl, P., Hoetker, D. J., Siddiqui, M. A., Conklin, D. J., D'Souza, S. E., and Bhatnagar, A. (2011). Oral exposure to acrolein exacerbates atherosclerosis in apoE-null mice. *Atherosclerosis* **215,** 301–308.

Walker, M. J., Curtis, M. J., Hearse, D. J., Campbell, R. W., Janse, M. J., Yellon, D. M., Cobbe, S. M., Coker, S. J., Harness, J. B., and Harron, D. W. (1988). The Lambeth Conventions: Guidelines for the study of arrhythmias in ischaemia infarction, and reperfusion. *Cardiovasc. Res.* **22,** 447–455.

Wang, T., Lang, G. D., Moreno-Vinasco, L., Huang, Y., Goonewardena, S. N., Peng, Y. J., Svensson, E. C., Natarajan, V., Lang, R. M., Linares, J. D., *et al.* (2012). Particulate matter induces cardiac arrhythmias via dysregulation of carotid body sensitivity and cardiac sodium channels. *Am. J. Respir. Cell Mol. Biol.* **46,** 524–531.

Watanabe, J., Thamilarasan, M., Blackstone, E. H., Thomas, J. D., and Lauer, M. S. (2001). Heart rate recovery immediately after treadmill exercise and left ventricular systolic dysfunction as predictors of mortality: The case of stress echocardiography. *Circulation* **104,** 1911–1916.

Weil, J. V., Stevens, T., Pickett, C. K., Tatsumi, K., Dickinson, M. G., Jacoby, C. R., and Rodman, D. M. (1998). Strain-associated differences in hypoxic chemosensitivity of the carotid body in rats. *Am. J. Physiol.* **274**(5 Pt 1), L767–L774.

Yu, B. H., Mills, P. J., Ziegler, M. G., and Dimsdale, J. E. (1999). Sympathetic and respiratory responses to hypoxia in essential hypertension. *Clin. Exp. Hypertens.* **21,** 249–262.