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Comparative cardiopulmonary toxicity of exhausts from soy-based biofuels and diesel in healthy and hypertensive rats

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Abstract

Increased use of renewable energy sources raise concerns about health effects of new emissions. We analyzed relative cardiopulmonary health effects of exhausts from (1) 100% soy biofuel (B100), (2) 20% soy biofuel + 80% low sulfur petroleum diesel (B20), and (3) 100% petroleum diesel (B0) in rats. Normotensive Wistar–Kyoto (WKY) and spontaneously hypertensive rats were exposed to these three exhausts at 0, 50, 150 and 500 μg/m^3, 4 h/day for 2 days or 4 weeks (5 days/week). In addition, WKY rats were exposed for 1 day and responses were analyzed 0 h, 1 day or 4 days later for time-course assessment. Hematological parameters, *in vitro* platelet aggregation, bronchoalveolar lavage fluid (BALF) markers of pulmonary injury and inflammation, *ex vivo* aortic ring constriction, heart and aorta mRNA markers of vasoconstriction, thrombosis and atherogenesis were analyzed. The presence of pigmented macrophages in the lung alveoli was clearly evident with all three exhausts without apparent pathology. Overall, exposure to all three exhausts produced only modest effects in most endpoints analyzed in both strains. BALF γ-glutamyl transferase (GGT) activity was the most consistent marker and was increased in both strains, primarily with B0 (B0>B100>B20). This increase was associated with only modest increases in BALF neutrophils. Small and very acute increases occurred in aorta mRNA markers of vasoconstriction and thrombosis with B100 but not B0 in WKY rats. Our comparative

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Supplementary material available online

Supplementary Tables S1–S12

Declaration of interest

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evaluations show modest cardiovascular and pulmonary effects at low concentrations of all exhausts: B0 causing more pulmonary injury and B100 more acute vascular effects. BALF GGT activity could serve as a sensitive biomarker of inhaled pollutants.

Keywords

Biodiesel; biomarkers; hypertensive rats; pulmonary toxicity; toxicity

Introduction

Inhaled pollutants have been linked to adverse pulmonary and extra-pulmonary effects (physiological, arrhythmogenic, ischemic, thrombogenic, stroke-related, atherogenic and more recently diabetogenic) in a number of epidemiological studies (Gold & Mittleman, 2013). Numerous toxicological studies have examined cardiopulmonary health effects of a variety of ambient gas and particulate pollutants such as ozone, real-time ambient particulate matter (PM) and source-specific pollutants, including power plant combustion collected PM, diesel exhaust (DE), nano materials, asbestos and even biofuel exhausts (Brito et al., 2010; Golomb at al., 2012; Kodavanti et al., 2000, 2005, 2011, 2013; Shannahan et al., 2012; Tong et al., 2009). Pulmonary injury from inhalation of these pollutants could be influenced by chemical composition, physical characteristics, surface reactivity, the level of exposure, the method of exposure, the lung deposition of specific pollutant, longevity of exposure and more importantly the animal species and strain related factors. As many sources contribute to air pollution and the atmospheric chemistry changes in a dynamic manner, it is challenging to attribute any specific health effects to a given characteristic of a pollutant unless controlled exposures are conducted in laboratory animals. Comparative toxicological studies done using source specific pollutants provide insights into the chemical signature of pollutants responsible for adverse health outcomes.

Vehicular exhausts, including diesel emissions contribute significantly to the ambient pollution. More importantly, vehicular emissions have been linked to a variety of adverse pulmonary and cardiovascular health effects in humans, especially those with underlying cardiovascular diseases (CVD). These include altered endothelial function (Wauters et al., 2013), systemic hematological and coagulative changes (Krishnan et al., 2013; Lucking et al., 2011), atherosclerosis (Rivera et al., 2013); increased blood pressure (Cosselman et al., 2012); decreased heart rate variability (Adam et al., 2012), and ischemia (Mills et al., 2007). A large number of animal toxicological studies, including our own, have supported the causal role of DE in adverse pulmonary and cardiovascular health effects. We have shown that inhalation of whole DE at high concentrations causes acute pulmonary inflammation, cardiac mitochondrial functional impairment and changes in expression of genes involved in myocardial contractility and antioxidant homeostasis in healthy rats (Gottipolu et al., 2009). We have further shown that longer exposure of rats to whole DE (gas phase plus particles) or bulk collected DE particles induces vascular gene expression changes reflecting increased oxidative stress, inflammation, vasoconstriction and thrombosis in healthy and hypertensive rats (Kodavanti et al., 2011, 2013). Further, we noted that the DE-induced pulmonary protein leakage and related vascular changes in hypertensive rats are reversed if the

hypertension was pharmacologically reversed, suggesting the contribution of underlying disease in exacerbation of DE vascular impairments (Kodavanti et al., 2013). A number of other studies have also shown NO-mediated vasodilation impairment by DE in laboratory rodents (Miller et al., 2009). Only a few studies have been conducted to determine relative pulmonary and cardiovascular toxicity of inhaled oil-based fuel exhausts (Brito et al., 2010; Yanamala et al., 2013).

The increased use of renewable energy sources is likely to change atmospheric pollution profile and subsequently, the human health impact. The use of plant oils has been experimented with for a number of decades on a local basis, but is not extensively implemented as of yet. Limited evidence suggests that the exhausts from biofuel methyl esters of rape seed oil and soy oils have generated lesser emissions of volatile organic constituents and particle mass when compared to conventional DE (Hemmingsen et al., 2011; McDonald et al., 1997). However, it has been shown that atmospheric aging of biodiesel exhausts generates more ozone than aging of DE (Schröder et al., 1999). A number of *in vitro* mutagenicity and toxicity assays have shown variable findings regarding relative potencies of biodiesel versus DE extracts (Bünger et al., 1998, 2000, 2007). No systematic toxicological studies have been conducted in laboratory rodents on relative cardiopulmonary health effects of DE versus exhausts from different biofuel blends.

It is likely that the fatty acid compositions of these oils vary significantly and likewise, the exhaust composition might differ (Madden et al., 2011). Methyl esters of different oils from soybean, canola seed, sunflower seed, cotton seed and peanuts, have the potential for use in energy production. However, at this time, primarily soybean and rape seed oils are being used in the US and Europe, respectively. Particularly, these oils are mixed with conventional diesel to produce different blends of biofuel and petroleum diesel, since the availability of biofuels is still limited. It has been shown that the chemical species generated by biodiesel plus diesel blend exhaust vary significantly from those produced by diesel or soybeans alone (Brito et al., 2010). However, no studies have been conducted to determine relative toxicity of diesel, biodiesel and diesel plus biodiesel blends. The goal of this study was to systematically examine cardiopulmonary health effects of acute and 4-week exposure to exhaust from soy-methyl esters (100%; B100), petroleum diesel plus soy methyl ester blend (80%:20%; B20) and petroleum diesel (0%; B0) in healthy Wistar–Kyoto (WKY) and susceptible spontaneously hypertensive (SH) rats. Identical engine operation and exposure conditions were used to generate and direct these exhausts to the exposure chambers to minimize differences in the chemical composition due to varying engine operating conditions. Exposures were conducted at relatively low particulate mass concentration to simulate real environmental situations. A variety of biological endpoints were assessed to determine relative toxicity to the cardiovascular system. We believed that this assessment of comparative toxicity would provide critical information on health hazards associated with exhausts of biofuel blends relative to DE.

Materials and methods

Animals

Male WKY and SH rats (10–11 weeks old) were purchased from Charles River Laboratories Inc., Raleigh, NC. All rats were maintained in an isolated animal room [Association for Assessment and Accreditation of Laboratory Animal Care (AALAC) approved] at 21 ± 1 °C, 50 ± 5% relative humidity and 12 h light/dark cycle. Rats were housed (2/cage) in polycarbonate cages containing hardwood (Beta Chip®, Warrensburg, NY) bedding during a 1 week acclimation. After acclimation, animals were transported to a nearby satellite animal holding room and were single-housed in polycarbonate individually ventilated cages with Beta Chip bedding during non-exposure periods. Animals received standard (5001) Purina rat chow (Brentwood, MO) and water *ad libitum*, except during exposures. The US EPA NHEERL Institutional Animal Care and Use Committee (IACUC) approved the protocol.

Biodiesel and diesel exhausts (B0, B20 and B100) generation and animal exposures

For these experiments, an ultra-low sulfur (<15 ppm) petroleum diesel fuel (B0) was purchased from Red Star Oil (Raleigh, NC), and a 100% soy biodiesel fuel (B100) was purchased from Piedmont Biofuels (Pittsboro, NC). The B20 blend was made by splash blending 20% biodiesel and 80% petroleum diesel by volume. B100, B20 and B0 were used with a Yanmar L70 diesel engine (Adairville, GA) and Pramac E3750 generator (Marietta, GA). This single cylinder, air-cooled, direct injection, 320-cm^3 engine ran at 5.8 hp (4.3 kW) continuous load, 3600 rpm. This diesel genset system was used because it is inexpensive and quickly replaceable in case of mechanical issues during exposure studies. Such small diesel systems have been used in other toxicological studies involving DE as explained in a companion paper by Mutlu et al. (2015). The engine air-inlet flow rate, fuel-consumption rate, generator voltage and generator current are detailed for all fuel types in the companion paper by Mutlu et al. (2015). The engine exhaust (~85 L/min) was diluted with HEPA and charcoal filtered room air (~7:1 dilution) and was directed to three 984 L Hazelton model 1000 inhalation exposure chambers located in an adjacent isolated satellite animal holding/inhalation exposure laboratory. Additional HEPA filtered room air was introduced for secondary dilution to the desired exposure concentrations. The aerosol concentration in each chamber was continuously monitored using tapered element oscillating microbalances (TEOM, Rupprecht and Patashnick Co., series 1400, Albany, NY). The sampling port for PM and gas measurements were located at the level of animal breathing zone in the chamber. Particle-size distributions were measured using a scanning mobility particle sizer (SMPS, TSI Inc., model 3080, St. Paul, MN) for all chambers. Target exposure concentrations of 50, 150 and 500 μg/m^3 were achieved concurrently in the three chambers by manually adjusting the secondary dilution according to the TEOM determined mass concentration. The fourth Hazelton chamber was used as a control, receiving only HEPA/charcoal filtered room air. Inhalation chamber temperatures, pressures and relative humidity were measured hourly during exposure. The flow rate of 283 L/min (10 ft^3/min) for all chambers was maintained, which is sufficient for approximately 17 air changes per hour. Also, all chambers were continuously monitored for oxygen (O_2, California Analytical Instruments, model 200, Orange, CA), carbon monoxide (CO, Thermo Electron Instruments, model 48, Fitchburg, WI), sulfur dioxide (SO_2, Thermo Environmental Instruments, model

43C, Franklin, MA), nitric oxide and total nitrogen oxides (NO and NO_x, Thermo Scientific, model 42i-HL, West Palm Beach, FL). Nitrogen dioxide (NO_2) levels were obtained by subtracting NO values from total NO_x. Further details are provided in the companion paper by Mutlu et al. (2015). The average chamber gasses and PM mass concentrations achieved during staggered 5–6 weeks exposures are given in Table 1. Separate tables for 2-day and 1-day exposure are not provided since those exposures were conducted along with the exposures of 4 week animals.

Three exposure experiments were conducted with each fuel exhaust: (1) male WKY rats (n = 8) exposed 4 h to a given biofuel and examined at 0, 24 and 96 h post-exposure. (2) Male WKY (n = 8) and SH rats (n = 6) exposed to a given biofuel 4 h/day × 2 days and toxicology endpoints were assessed 1 day after final exposure and (3) WKY (n = 8) and SH rats (n = 6) exposed to a given biofuel 4 h/day × 5 days/week × 4 weeks and toxicology endpoints were assessed 1 day after final exposure (Table 3). Each day, exposure duration was fixed for 4 h to minimize animal stress associated with being in the chamber, and to assure constant exhaust generation and chamber operation once exposure began.

Glucose tolerance testing (GTT)

For animals undergoing 4-week exposure in the third study, GTT was performed to examine potential metabolic effects at three time points: (1) prior to the start of exposure to B0, (2) immediately after 2 days of exposure (4 h/day) and (3) immediately after the 4th week final exposure. Based on our recent observation that exposure to ozone induces profound systemic metabolic alterations including glucose intolerance (Bass et al., 2013), we presumed that exposure to combustion exhausts might produce similar effects. Since animals were fasted during exposure, additional fasting was not needed prior to performing GTT. Prior to glucose injections, baseline glucose levels were measured by pricking the distal surface of rats' tails with a sterile needle, to obtain ~1 μL of blood. A Bayer Contour glucose meter was used to determine blood glucose levels, using test strips, which require 0.6 μL whole blood. After the first measurement, rats were given an intraperitoneal injection of glucose solution with a dose of 2 g/kg/10mL (20% D-glucose; 10 mL/kg). Measurement with the glucose meter was repeated every 30 min over the course of 2 h.

Necropsy and sample collection and bronchoalveolar lavage (BAL)

At designated time points (Table 2), rats were weighed and anesthetized with an overdose of sodium pentobarbital (Virbac AH, Inc., Fort Worth, TX; 50–100 mg/kg, ip). Blood was collected through abdominal aortic puncture directly into vaccutainers containing citrate for platelet aggregation assay, EDTA for complete blood counts. Left ventricular tissues were quick frozen in liquid nitrogen for later RNA analysis. The right lung was lavaged using a volume of Ca^{2+}/Mg^{2+} free phosphate buffered saline (pH 7.4) equal to 28 mL/kg body weight (total lung capacity) × 0.6 (right lung is 60% of total lung weight). Three washes were performed with the same buffer aliquot. BAL fluid (BALF) was stored in 15mL sterile tubes on ice. The left lung was inflated with 10% formalin and processed for histopathology as described below. Thoracic aortas were isolated from surrounding connective tissues and quick frozen in liquid nitrogen for later RNA isolation and PCR.

Lung histology

Tracheally fixed left lung was washed in distilled water, dehydrated in a graded ethanol series and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin (H&E). All tissue sections were examined by a certified veterinary pathologist using light microscopy. Semi-quantitative scoring of a variety of pathological indices used severity scores: 0 = not present, 1 = minimal (<10% of examined area), 2 = mild (11–40%), 3 = moderate (41–80%); 4 = marked (81–100%).

Platelet aggregation *in vitro* and complete blood count

Citrated blood was centrifuged at 200 × *g* for 30 s, an aliquot of the platelet-rich plasma was collected and the remaining sample was centrifuged at 2000 × *g* for 120 s to collect a platelet-poor fraction of plasma. Adenosine diphosphate (ADP)-induced primary aggregation, and rates of aggregation and disaggregation were measured by adding 25 μL of ADP (1×10^{-4} M) to the platelet-rich plasma fraction at 37 °C using a platelet aggregation profiler (Platelet Aggregation Profiler Model PAP-8E, Bio/Data Corp, Horsham, PA). The light transmission set by the absorbance of the platelet-poor plasma fraction was used to blank each individual sample. Plasma samples from each individual animal were run in duplicate and an average of these duplicates was used. Complete blood counts of EDTA containing blood tubes were performed using a Beckman-Coulter AcT blood analyzer (Beckman-Coulter Inc., Fullerton, CA).

Cell differential and BALF analysis

Aliquots of BALF were used to determine total cell counts with a Z1 Coulter Counter (Coulter, Inc., Miami, FL) and cell differentials according to previously described procedures (Gordon et al., 2013). The cell free BALF was used to analyze lung injury biomarkers [total protein, albumin lactate dehydrogenase (LDH) activity, N-acetyl glucosaminidase (NAG) activity and γ-glutamyl transferase (GGT)] activity as described earlier (Gordon et al., 2013).

RNA isolation and real time reverse transcriptase PCR (RT-PCR)

Total thoracic aorta RNA was isolated (and frozen at −80 °C) with a commercially available RNeasy mini kit (Qiagen, Valencia, CA) using silica gel membrane purification and RNA yield was determined spectrophotometrically on a NanoDrop 1000 (Thermo Scientific, Wilmington, DE). RT-PCR was conducted on an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA) as described previously (Gordon et al., 2013). Primers were purchased from ABI as inventoried TaqMan Gene Expression Assays, each containing a 6-carboxy-fluorescein (FAM dye) label at the 5′-end. Data were analyzed using ABI sequence detection software (SDS version 2.2, Applied Biosystems, Foster City, CA). For each PCR plate, cycle threshold (cT) was set to an order of magnitude above background. For each individual sample, target gene cT was normalized to a control gene cT, 18S ribosomal RNA (18S), to account for variability in the starting RNA amount. Expression of each exposure group was quantified as fold difference over air control, at the corresponding time point.

Statistical analysis

The data for each experiment in each biofuel were analyzed using Sigma Stat 3.5 (Jandel Scientific Software, San Jose, CA, $n = 8$ for WKY and $n = 6$ for SH). GT data for each time point were analyzed using two way analysis of variance (ANOVA) with strain and exposure as two factors followed by Tukey's test. Two-way ANOVA tests were also followed by Tukey's *post hoc* comparison for BALF, hematological and mRNA expression data. Time and exposure were considered as two factors for the time course studies. Rat strain and exposure were considered as two factors for 2 days and 4 weeks exposure studies. The nominal Type I error rate (α) was set at 0.05. No adjustments were made for multiple comparisons.

Results

Inhalation exposure conditions for B0, B20 and B100 exhausts

For comparative assessment of toxicity of B0, B20 and B100, the engine operation conditions and fuel types (petroleum diesel and soy methyl esters) were kept identical between studies. A detailed description of aerosol characterization is given in a companion paper by Mutlu et al. (2015). Since fresh exhausts generated by fuel combustion can produce a mixture of particulate and gaseous pollutants, and we have observed that gas components of DE contribute to cardiopulmonary health effects, we summarized the mass based PM levels and gas phase components such as CO, NO, NO_x and SO_2 during exposure of rats over the entire period of 5–6 weeks that accommodated 1 day, 2 days and 4 weeks exposures (Table 1).

The chamber concentrations of CO increased as the mass PM concentration in each of the fuel exhaust. The B0 (100% petroleum diesel) produced the highest levels of CO followed by B20 and B100 (Table 1). However, in general, NO and NO_x concentrations were similar in B0 and B20 chambers with 500 $\mu g/m^3$ (high) concentration. The B100 chamber NO_x were only slightly lower than other two exhaust types. SO_2 concentrations were within 1 ppm for all three exhausts and concentrations. The actual mass concentrations achieved for each exposure condition was within 5% of the desired concentrations for each type of exhaust (Table 1). These conditions were in general agreement with all other exposures conducted involving mice.

Metabolic effects

The glucose tolerance tests were performed to determine metabolic impairment prior to the start of exposure, immediately after second exposure and immediately following 4-week exposure of rats to B0. Since no exposure related effects were noted in glucose tolerance testing (GTT), no further analyses were performed to determine metabolic impairment. It was interesting to note that SH rats with underlying hypertension exhibited hypoglycemia (measurements taken from fasting blood glucose prior to injecting glucose for GTT), as compared to WKY rats and had more rapid clearance of glucose than healthy WKY rats (Figure 1). No significant exposure-related differences were observed when the area under the curve was calculated for GTT (not shown).

Lung injury and inflammation

In order to comprehensively assess toxicity, three experiments were conducted for each type of exhaust (Table 2): (1) time course study to determine the time course of cardiopulmonary effects; (2) acute study to determine acute 2-day exposure effects; and (3) 4-week study to determine cumulative effects over 4 weeks. A number of lung injury markers including total protein, albumin, LDH activity, NAG activity and GGT activity were assessed in cell free BALF for each study and exhaust type (Figures 2–4; Supplemental Tables S1–S3). In general, most responses were modest to mild even at high concentration of exhausts. No consistent exposure related increases were noted in protein, albumin, LDH activity or NAG activity in any study or exhaust type. The activity of BALF GGT was increased after exposure, specifically to B0, in all studies: the time course, acute and 4 week studies (Figures 2–4). Four-week exposure to B100 also caused a small but significant increase in GGT in WKY rats (Figure 4). In general, WKY rats responded to a greater degree than SH rats in each study involving B0. GGT activity was increased in SH rats exposed to B20 for 2 days (Figure 3). Rat strain related differences were noted in other BALF markers of injury such as protein, albumin, LDH and NAG (Supplemental Tables S1–S3). Rat strain related differences in baseline levels of lung injury markers have been noted in our earlier studies involving PM and DE exposures (Kodavanti et al., 2005, 2013).

Neutrophilic inflammation as determined by analysis of BALF was in general variable but reached significance at the highest concentration of B0 in the time course study involving WKY rats (Figure 5). Exposure to B100 actually resulted in a reduced number of neutrophils in BALF at the highest concentration in the time course study. Neutrophil numbers were increased in SH rats exposed to B20 for 2 days, however this response was not concentration dependent as all exposed groups were significant relative to air controls (Figure 6). No increases were noted in BALF neutrophils in rats exposed for 4 weeks to any of the exhaust types (Figure 7). BALF macrophages did not change in a consistent manner in any of the studies or exhaust types (Supplemental Tables S1–S3) but rat strain related differences were noted (Supplemental Tables S2 and S3).

Lung pathology

The histological examination of lung tissues stained with hematoxylin and eosin and observed under light microscopy indicated the presence of pigmented macrophages, which were clearly evident in a concentration dependent manner. The presence of these dark stained granules within the cytoplasm of macrophages was not associated with any inflammatory or fibrotic reaction in the lungs. The particle laden macrophages at higher concentration and 4-week time point achieved a pathology score of 1 (minimal) suggesting that exhaust particle accumulation in macrophages was not near saturation (data not shown). To demonstrate that other than particle-laden macrophages, no histological changes were apparent, representative sections of lung tissues only from WKY and SH rats exposed to air or B20 for 4 weeks are shown in Figure 8 to demonstrate presence of particles in macrophages.

Systemic hematological effects and platelet aggregation *in vitro*

Systemic hematological, inflammatory and *in vitro* platelet aggregation effects were examined based on the presumption that increased levels of incompletely combusted fatty methyl esters might readily be absorbed through the pulmonary vasculature and induce systemic effects. No consistent exposure effects on red blood cells, hemoglobin, hematocrit or MCHC were noted in WKYor SH rats in any of the studies (Supplemental Tables S4–S6). In the time course study, the levels of hematocrit and MCHC were decreased at the highest concentration and all times after the B100 exposure (Supplemental Table S4); however, this change was not apparent in the 2 day and 4 week studies with B100 or any other fuel types (Supplementary Tables S5 and S6). Strain related differences seen in our earlier studies were readily apparent in this study too. SH rats had higher levels of red blood cells, hemoglobin, hematocrit and platelets than WKY rats (Supplemental Tables S4–S6). Circulating white blood cells and lymphocytes were not consistently changed with any of the exhaust types except for a trend of an increase at the high concentration of B100 in the time course study (Supplemental Table S4).

Platelet aggregation *in vitro* can be impacted if activation occurred *in vivo* during exposure to exhausts from diesel and biofuels. We determined platelet aggregation in each of the studies involving B0, B20 and B100. No consistent exposure-related changes were noted with any of the biofuels or exposure scenarios, suggesting that inhalation of fresh biofuel exhaust up to 500 $\mu g/m^3$ does not impair platelet aggregation (Supplemental Tables S7–S9). However, rat strain-related differences were clearly apparent with regards to primary aggregation and disaggregation, the time to reach maximum aggregation (primary slop) or maximum aggregation. The SH rats showed lower platelet activity to adenosine diphosphate *in vitro* (Supplemental Tables S8 and S9), suggesting that their platelets remain in an activated state *in vivo* and thus are not able to re-respond to adenosine diphosphate.

Vascular aorta and left ventricular effects at mRNA level on markers of contractility, thrombosis and atherosclerosis

Aorta samples from rats exposed to air or 500 $\mu g/m^3$ exhausts were selected from the time course study and 4-week exposure study. Relatively small changes were noted in a few markers in rats exposed to B0 (DE). Expression of ET-1 and tPA were significantly increased in WKY rats in time course study after 4 days of B0 exposure (1.6 fold and 1.4 fold, respectively) relative to air exposed rats (Supplemental Table S10). These increases were not observed in the 4-week study in WKY or SH rats exposed to B0 (Supplemental Table S11). B100 increased expression of tPA, TF, TNF-α and RAGE immediately after exposure in the time course study (Supplemental Table S10). In a 4-week study, exposure to B100 was associated with increased expression of TF and ET-1 in SH rats but not in WKY (Supplemental Table S11). B20 exposure caused only modest increases in tPA and iNOS expression in the time course study (Supplemental Table S10). Thus, overall this data set suggests that some vascular contractile and prothrombotic effects at the mRNA level can be induced acutely in rats and it appears that B100 might induce a slightly greater response than B20 or B0.

It was presumed that myocardial tissue might experience acute changes in contractility and vasoconstriction from exposure to DE or biofuel exhausts. Therefore, mRNA expression of contractility, vascular response and thrombosis were examined in the left ventricles at the 0-h time point in WKY rats exposed for 4 h to air, B0, B20 or B100 (time course study). No exposure related changes in any of the selected markers were noted (Supplemental Table S12).

Discussion

The purpose of this study was to examine pulmonary, systemic and cardiovascular effects of B0, B20 and B100 exhausts equated to the same mass concentration of PM such that we are able to compare and contrast health outcomes in healthy and hypertensive rats. A number of biological endpoints were examined (Table 2) with varied exposure scenarios to determine time related, acute or 4 week effects in healthy and hypertensive rats. Overall, we noted modest to mild and variable degrees of pulmonary injury/inflammation, and effects on expression of markers of vasoconstriction and thrombosis in the aorta following exposure to high concentrations of selected exhausts. B0 appeared to induce greater pulmonary injury as determined by increased BALF GGT activity (apparently the most sensitive BALF biomarker of all those examined) than B20 or B100. WKY rats had a larger response than SH in regards to GGT activity. Mild neutrophilic inflammation was also noted with B0 in WKY but not with B20 or B100 in the time course study. Modest increases in BALF neutrophils were observed in SH rats exposed for 2 days to B20. Vascular response, as determined by mRNA expression changes in selected aorta markers seemed to occur more readily with B100 than B0 or B20 immediately after a single exposure. No pathological findings were noted in the lungs, and the systemic changes in hematological parameters and platelet aggregation were minimal with all exhausts. The baseline strain related differences between WKY and SH rats were evident in pulmonary injury markers, hematological parameters, platelet aggregation and in vascular mRNA expression as noted in our previous studies (Kodavanti et al., 2011, 2013).

Previous *in vitro* toxicity and mutagenicity assays comparing the toxic potency of B0 versus B100 do not provide consistent findings (Ackland et al., 2007; Bünger et al., 2000; Kooter et al., 2011), perhaps largely due to the differences in methodologies used for generating exhausts and preparing samples for in vitro assays. Likewise, limited *in vivo* animal toxicity studies also provide inconsistent findings about the relative pulmonary toxicities of B0 versus B100 (Brito et al., 2010; Finch et al., 2002; Swanson et al., 2009; Yanamala et al., 2013). Therefore, we performed comparative assessment of pulmonary, systemic and cardiovascular effects such that compositional differences between B100 and B0 could help us in delineating observed differences in health effects.

The composition analysis of B100 versus B0 indicated that, as expected, organic carbon, especially long chain hydrocarbons were enriched in B100, however it contained half the PAHs found in B0 (Mutlu et al., 2015). It was also evident that the mean particle size for B0 was smaller than that of B100 in our study; both in ultrafine range (Mutlu et al., 2015). Since the components of B100 are likely to be more soluble than B0 in physiological fluid such as airway lining fluid (Schröder et al., 1999), it is possible that once deposited in the

lung, the greater proportion of B0 components translocate systemically. It is important to note that the concentration of CO varied significantly between B0 and B100 (B0>B100). CO at concentrations found in the B0 chamber can produce clinically significant biological effects (Bye et al., 2008) and therefore, we presumed that CO might contribute to acute systemic and vascular effects of B0 once inhaled.

The exposure concentrations we used for each exhaust were within the range that humans are likely to be exposed near the tail pipe (Buzzard et al., 2009) and thus, environmentally relevant. Since studies using lower concentrations of vehicular emissions have found profound systemic effects in animals, we wanted to use a similar range of concentrations, although one of our earlier studies with DE provided only marginal effects on selected biological endpoints in healthy and hypertensive rats using a concentration of 500 $\mu g/m^3$ (Gottipolu et al., 2009).

Largely negative toxicity findings with many biological endpoints from exposure to all exhausts somewhat limited our ability to precisely compare the effects of B0, B20 and B100 on different biological processes and organ systems, however, changes in lung injury and inflammation markers as well as changes in aorta biomarkers at gene expression level allowed us important insights into how B0 and B100 are likely to target different organ systems. Of the many routine BALF endpoints assessed, we found that levels of GGT activity were most affected, specifically by B0, indicating that this is perhaps the most sensitive marker of lung epithelial cell integrity. GGT is involved in transport of amino acids across the cell membrane for glutathione synthesis. Lung type II and Clara cells express GGT and it has been shown that cleavage of the extracellular domain of this membrane protein accounts for the presence of its activity in extracellular lining fluid (Jean et al., 2003). Mice lacking GGT are highly sensitive to oxygen-induced oxidative damage (Jean et al., 2003). Small but significant increases in BALF GGT activity in general, could thus be accounted for by transcriptional activation of the gene in lung secretory epithelial cells and/or increased shedding of the catalytic unit from cell membrane. The biological significance of GGT release in the BALF is not clear, but this increase can serves as a sensitive indicator of lung cell injury following inhalation of pollutants. The finding that B0 caused greater effect on BALF GGT activity than B100 suggests that B0 likely induces greater cellular changes in the lung when compared to B100. This is further supported by trends of increased neutrophilic inflammation in rats exposed to B0 and not to B100.

Very acute aorta effects (immediately after a single 4-h exposure) seen with B100 (B100>B0) are of interest (Supplemental Table S10). Although only four markers were significant, the trend of mRNA increases with most examined markers of vasoconstriction, thrombosis and atherogenesis in the aortas of WKY rats exposed to B100, suggests that this biofuel exhaust could potentially have greater acute systemic vascular impact than B0. We have previously shown that B0 (diesel exhaust) exposure at a much higher (~4 times) concentration and after a longer duration of exposure, increased the expression of these markers in WKY and SH rat aortas (Kodavanti et al., 2013). Thus, we believe that although rats do not develop atherosclerotic lesions, some of these changes in aortas could reflect impairment in vascular contractility and thrombogenic potential of inhaled vehicular pollutants. Our data show that B100 caused immediate acute aorta effects which were

markedly reduced in WKY rats undergoing 4-week exposure. It is likely that higher solubility of B100 exhaust in airway lining fluid might facilitate systemic translocation of components resulting in a rapid vascular response while sparing the lung. Since 4-week B100 exposure effects on mRNA expression for ET-1 were noted in aortas of SH rats, chronic outcomes will need to be examined further.

It has been shown that the chemical profile of B20 exhaust could be markedly different from B0, even though B20 contains 80% B0 (Brito et al., 2010). Examining the data set for each fuel type and exposure scenarios, no unique changes or patterns emerged in biological endpoints with B20 exposure in our study. Since we did not have robust biological responses in pulmonary, systemic and cardiovascular endpoints, it is likely that any potential toxicity differences that might exist between B0, B20 and B100 are not distinguishable, even at a 500 $\mu g/m^3$ concentration. A higher concentration of biofuel exhaust might reveal toxicity differences between B20 and B0 or B100.

We have recently reported that pulmonary injury and inflammation resulting from DE (B0) exposures at high concentration of ~2000 $\mu g/m^3$ could be accounted for by gas phase components (Kodavanti et al., 2013). While NO_x concentrations did not differ markedly between B100 and B0 in this study, there were significant differences in the level of CO (Table 1; Mutlu et al., 2015). In B0, the chamber concentration of CO reached clinically relevant concentration in this study and therefore it is possible that gas phase components were as important as particle phase components in driving the pulmonary effects of B0 in this study. Since we did not expose rats to CO alone at the concentrations encountered with B0, we cannot affirm its contribution in pulmonary injury/inflammatory response. Differences in the levels of PAHs and EC could also contribute to the observed toxicity differences between B0 and B20.

Spontaneously hypertensive (SH) rats have been extensively used as a model of human hypertension. We did not determine the degree of hypertension in SH rats in this study, but blood pressure begins to increase as early as 8 weeks of age and progresses over time. We have used WKY rats as a healthy rat model and SH as a model of cardiovascular disease in many of our air pollution studies to understand susceptibility differences and to examine how pulmonary injury might lead to systemic and cardiovascular impairments. Dependent on the biological endpoint the responses of hypertensive rats have been found to be different from that of healthy WKY (Gottipolu et al., 2009; Kodavanti et al., 2000, 2013; Shannahan et al., 2012). In a recent DE study, we noted that, while WKY rats show a more robust acute pulmonary inflammation response, SH rats in general seemed to be more susceptible to 4 week vascular effects. Some of these vascular effects could be reversed by pharmacologically normalizing hypertension (Kodavanti et al., 2013). Our present data supports greater sensitivity of WKY rats to pulmonary effects of B0, even at lower exposure concentrations than were used in our previous study. Since only a few changes were observed in rats exposed to different exhausts, the strain differences in susceptibility to a given biofuel exhaust could not be ascertained for all markers in this data set. However, the lung injury marker GGT was detected more abundantly in B0-exposed WKY when compared to SH rats at the 4-week time point. ET-1 expression was increased only in the

aortas of SH rats exposed to B100, not in WKY rats at this time point, suggesting that some strain differences might become more pronounced upon longer exposure.

In conclusion, environmentally relevant concentrations of B0, B20 and B100 exhausts produced only modest to mild pulmonary and vascular effects. We show here that B100, in general, produced slightly less pulmonary injury and inflammation than B0 in both healthy WKY and cardiovascular compromised SH rats. In contrast, B100 produced greater acute vascular effects than B0 in WKY rats. Since these vascular effects were not exacerbated after 1 month exposure in either WKY or in SH rats the significance of these effects will need to be further examined at higher concentrations and chronic exposures.

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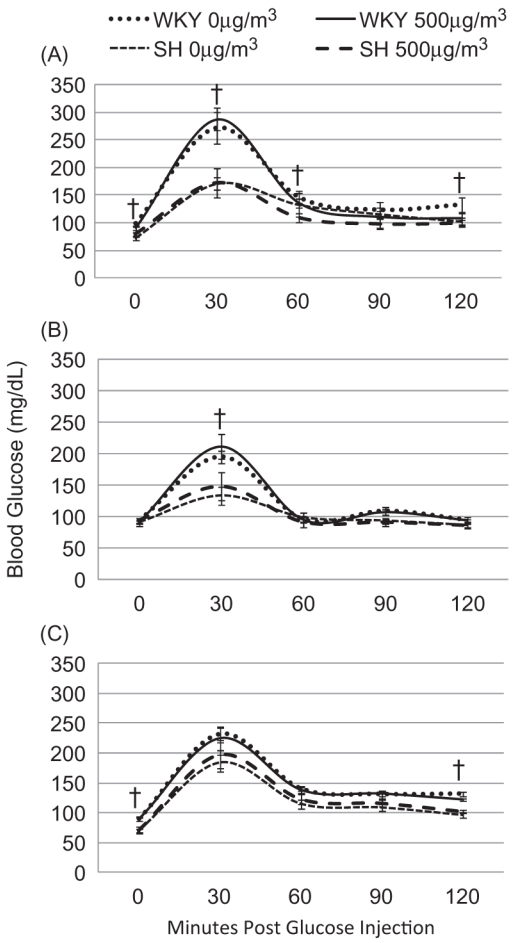

Figure 1.

Glucose tolerance test (GTT) comparison of WKYand SH rats during weekly exposures to air or B0 (100% DE). Only air control and high concentration groups are shown for clarity. Time points shown are prior to start of exposure (A), immediately after 2 days (B) and immediately after 4 weeks of exposure to B0 (C). Each value is the mean blood glucose measurement ± SE of 6–8 rats. Mean blood glucose values of air exposed SH rats at each time point are compared for significant strain differences relative to air exposed WKY group (†$p<0.05$). No B0 exposure related differences are noted. 0 µg/m^3 indicates air exposure. No GTT were done in B100 and B20 studies.

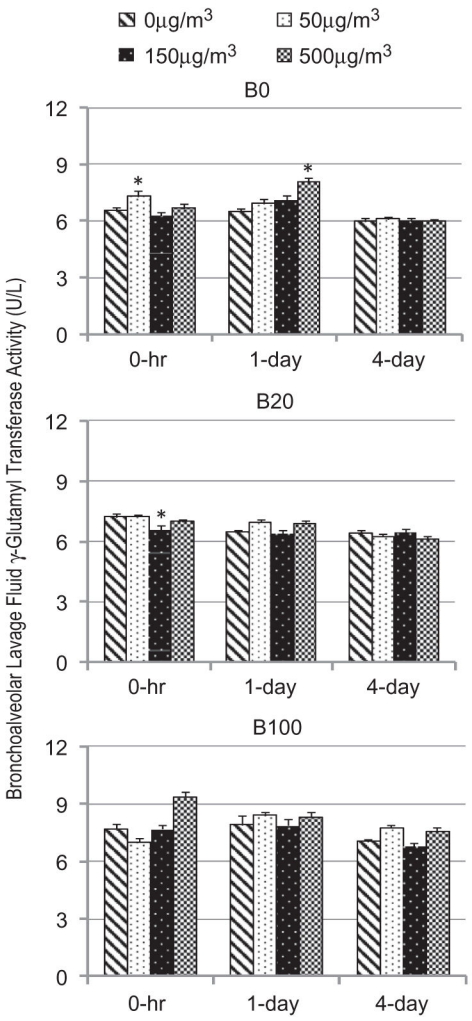

Figure 2.

Bronchoalveolar lavage fluid levels of γ-glutamyl transferase (GGT) activity in WKY rats at different time points after a single 4-h exposure to different biofuel exhausts. Each value is the mean measurement ± SE of 6–8 rats. Mean values of each time point are compared for significant exposure effects relative to air exposed group (*p<0.05). 0 μg/m^3 indicates air exposure.

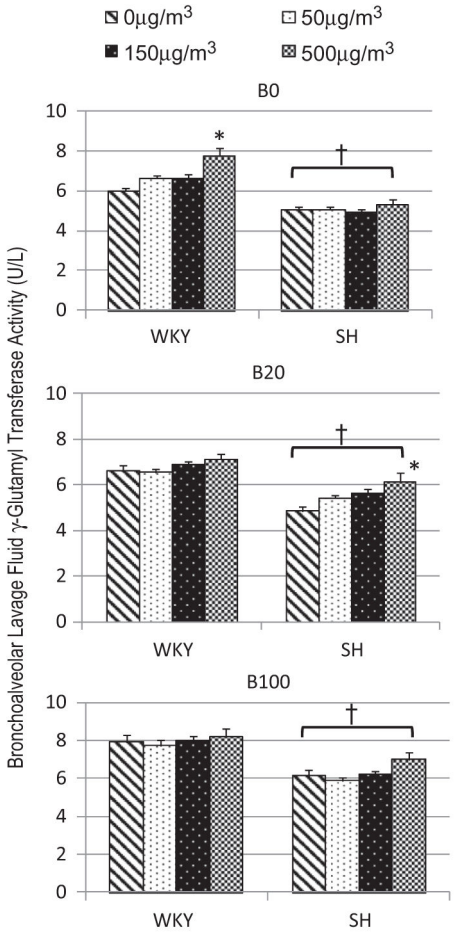

Figure 3.

Bronchoalveolar lavage fluid levels of γ-glutamyl transferase (GT) activity in WKY and SH rats after 2-day exposure to different biofuel exhausts. Each value is the mean measurement ± SE of 6–8 rats. Mean values of each time point are compared for significant exposure effects relative to air exposed group (*p<0.05). 0 μg/m^3 indicates air exposure. †Indicates a significant strain difference (p<0.05).

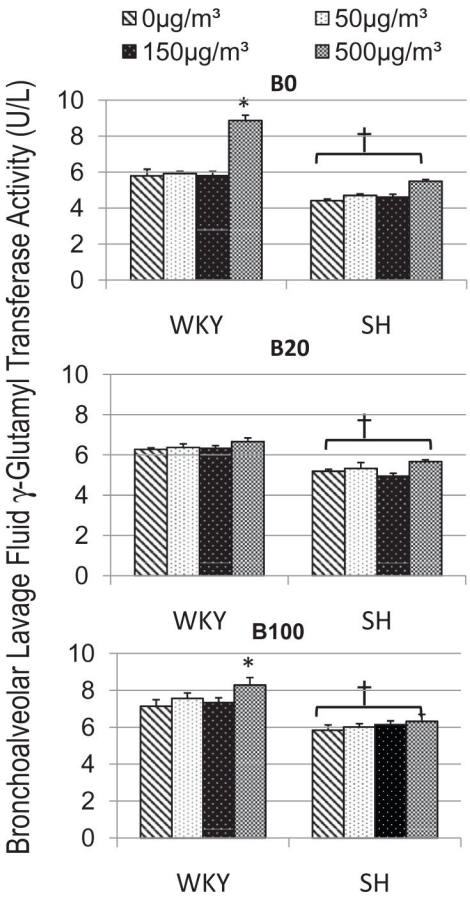

Figure 4.

Bronchoalveolar lavage fluid levels of γ-glutamyl transferase (GT) activity in WKY and SH rats after 4-week exposure to different biofuel exhausts. Each value is the mean measurement ± SE of 6–8 rats. Mean values of each time point are compared for significant exposure effects relative to air exposed group (*p<0.05). 0 μg/m^3 indicates air exposure. †Indicates a significant strain difference (p<0.05).

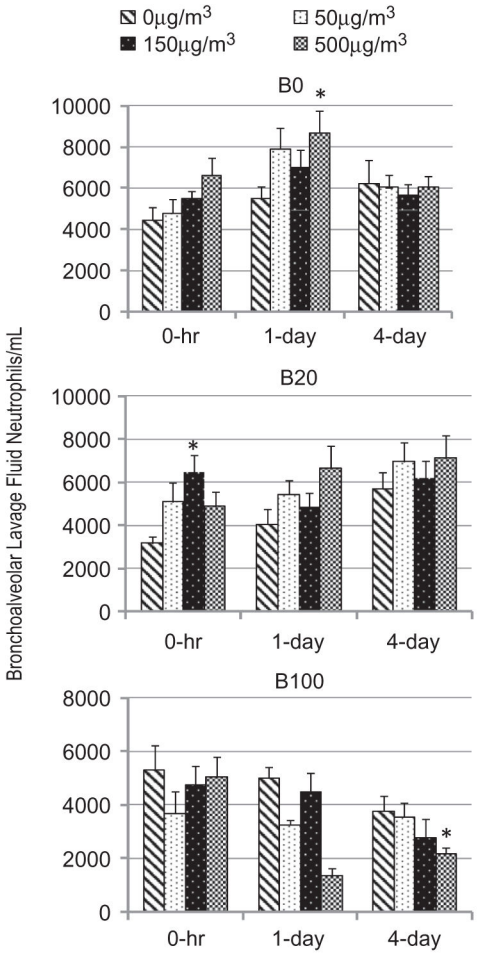

Figure 5.

Bronchoalveolar lavage fluid levels of neutrophils in WKY rats at 0, 24 or 120 h after a single 4-h exposure to different biofuel exhausts. Each value is the mean measurement ± SE of 6–8 rats. Mean values of each time point are compared for significant exposure effects relative to air exposed group (*p<0.05). 0 μg/m^3 indicates air exposure.

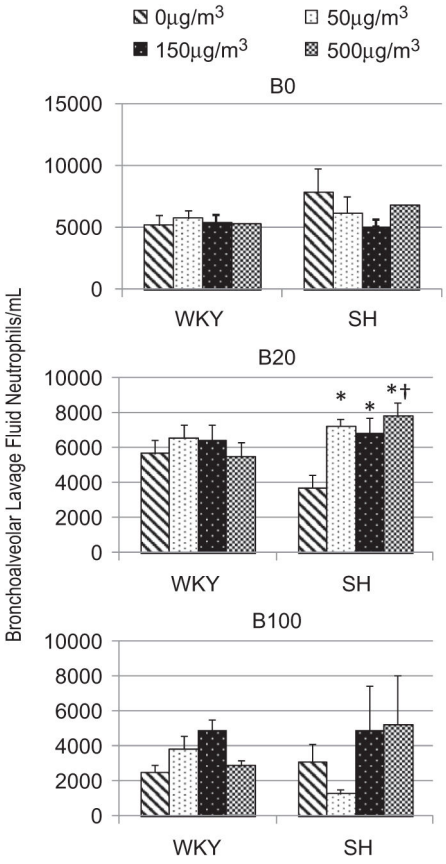

Figure 6.

Bronchoalveolar lavage fluid levels of neutrophils in WKY rats after 2 day exposure to different biofuel exhausts. Each value is the mean measurement ± SE of 6–8 rats. Mean values of each time point are compared for significant exposure effects relative to air exposed group (*p<0.05). 0 μg/m^3 indicates air exposure. †Indicates a significant strain difference (p<0.05).

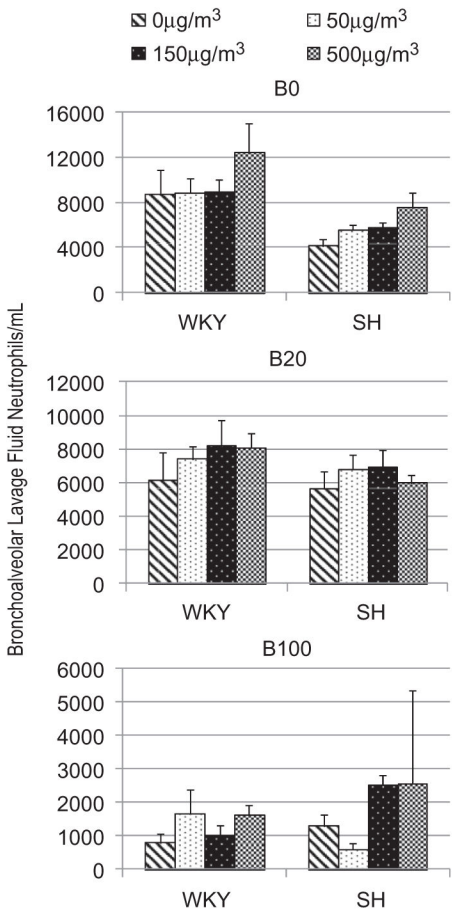

Figure 7.

Bronchoalveolar lavage fluid levels of neutrophils in WKY rats after 4-week exposure to different biofuel exhausts. Each value is the mean measurement ± SE of 6–8 rats. No exposure or strain-related differences reached significance. 0 μg/m^3 indicates air exposure.

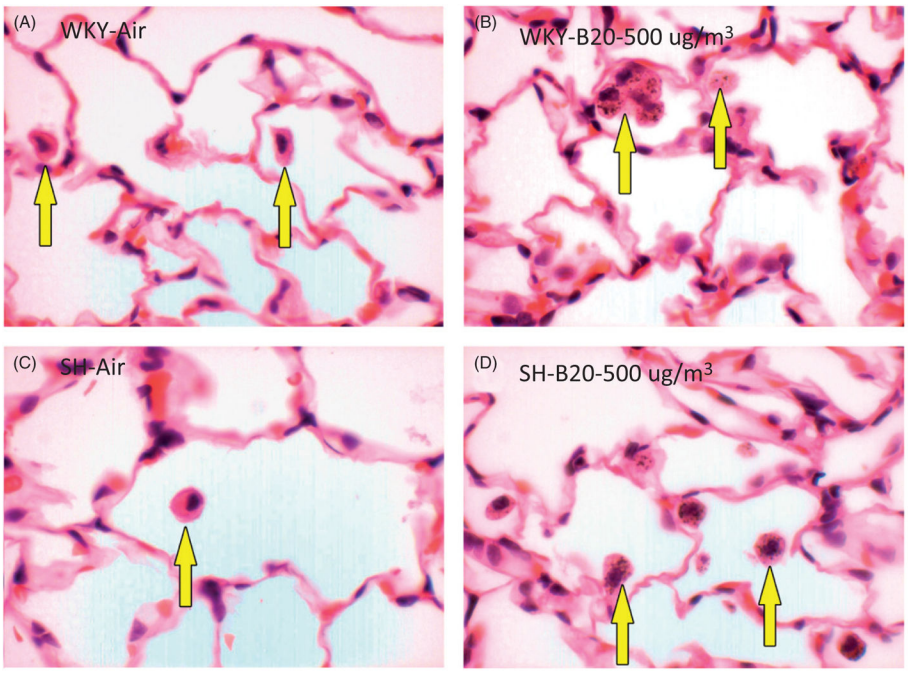

Figure 8.

Light microscopic images of hematoxylin and eosin stained lung tissue sections from 1-month Air (B and D) and B20 (A and C)-exposed WKY and SHR rats, respectively. Note (green arrows) the dark pigmented granules within the cytoplasm of alveolar macrophages seen in the B20 exposed rats, in contrast to the normally present non-pigmented alveolar macrophages in the air exposed rats. Note that the pathology pictures from B100 and B0 are not shown since no unique differences between air and exhaust exposed rats were present other than the presence of pigmented macrophages.

Table 1

Chamber operating conditions during 2-day and 4-week exposure of rats to B100, B20 and B0 biofuel blends.

A				
Chamber parameters	**Filtered air**	**B100; 50 μg/m^3**	**B100; 150 μg/m^3**	**B100; 500 μg/m^3**
PM mass, TEOM, μg/m^3	13 ± 0.4	51.1 ± 9.5	147.5 ± 19.4	476 ± 56.4
O_2, %	21.0 ± 0.01	20.0 ± 0.1	20.9 ± 0.1	20.6 ± 0.2
CO, ppm	<1	3.1 ± 3.1	6.4 ± 6.5	11.7 ± 5.8
SO_2, ppm	<1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
NO, ppm	<1	2.4 ± 1.8	4.8 ± 2.7	10.7 ± 5.1
NO_2, ppm	<1	<1	<1	1.0 ± 0.2
NO_x, ppm	<1	2.6 ± 1.9	5.3 ± 2.8	11.5 ± 5.4
Temperature, °F	74.7 ± 1.2	75.8 ± 1.5	75.6 ± 1.7	74.4 ± 0.7
RH, %	46.8 ± 6.0	43.4 ± 5.1	45.9 ± 3.3	53.2 ± 4.1
B				
	Filtered air	B20; 50 μg/m^3	B20; 150 μg/m^3	B20; 500 μg/m^3
PM mass, TEOM, μg/m^3	2.2 ± 4.1	53.0 ± 3.6	153.5 ± 5.8	513.6 ± 16.2
O_2, %	21.0 ± 0.2	20.8 ± 0.2	20.7 ± 0.2	20.4 ± 0.2
CO, ppm	<1	3.3 ± 1.0	7.0 ± 1.1	15.4 ± 2.2
SO_2, ppm	<1	<1	<1	<1
NO, ppm	<1	2.7 ± 1.1	6.3 ± 1.3	13.1 ± 1.6
NO_2, ppm	<1	<1	<1	1.0 ± 0.3
NO_x, ppm	<1	2.9 ± 1.1	6.9 ± 1.5	14.0 ± 1.8
Temperature, °F	73.2 ± 1.3	73.3 ± 1.4	73.7 ± 1.2	73.1 ± 0.8
RH, %	45.5 ± 3.8	44.5 ± 5.4	46.3 ± 3.9	53.7 ± 3.1
C				
	Filtered air	B0; 50 μg/m^3	B0; 150 μg/m^3	B0; 500 μg/m^3
PM mass, TEOM, μg/m^3	1.35 ± 0.1	50.3 ± 3.2	146.7 ± 5.6	484.4 ± 17.7
O_2, %	21.0 ± 0.3	20.9 ± 0.2	20.8 ± 0.2	20.4 ± 0.2
CO, ppm	0.2 ± 0.2	5.9 ± 2.0	10.8 ± 3.0	29.1 ± 7.0
SO_2, ppm	0.4 ± 0.2	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2
NO, ppm	0.17 ± 0.05	2.63 ± 0.58	5.12 ± 0.93	12.84 ± 1.88
NO_2, ppm	0.11 ± 0.06	0.46 ± 0.17	0.79 ± 0.25	1.44 ± 0.38
NO_x, ppm	0.28 ± 0.10	3.09 ± 0.65	5.92 ± 0.94	14.28 ± 1.88
Temperature, °F	74.0 ± 1.4	72.6 ± 1.9	74.3 ± 1.6	74.8 ± 0.7
RH, %	41.1 ± 8.0	42.3 ± 10.7	43.2 ± 7.1	51.2 ± 4.3

Each value in the table represents the average ± SE of the mean daily values for each exposure day. Values indicated by<1 shows below instrument detection limit. Note that different detection limits were set for measuring CO and NO_X during each study and therefore values in Panel C are measured below 1 ppm. B100 = 100% soy methyl esters; B20 = 20% soy methyl esters plus 80% petroleum diesel; B0 = 100% petroleum diesel.

Table 2

Exposure protocols for each biodiesel exhaust type.

Exhaust type	Rat strain	Experiment	Exposure groups	Duration of exposure	Time to necropsy after start of the final exposure
B100; B20; or B0	WKY	Acute-time course	0.0, 50, 150, and 500 μg/m^3	4h × 1 day	4-h, 24-h (1 day) and 96-h (4 days)
	WKY and SH	Acute-2day	0.0, 50, 150, and 500 μg/m^3	4h × 2 days	24-h
	WKY and SH	4-week	0.0, 50, 150, and 500 μg/m^3	4 h/day × 5 days/week × 4 weeks	24-h

B100 = 100% Soy methyl ester; B20 = 80% soy methyl ester plus 20% petroleum diesel; B0 = 0% soy methyl ester plus 100% petroleum diesel.

Table 3

Biological end points assessed in experiments examining the toxicities of biodiesel and petroleum diesel exhausts.

Organ system	Tissue sampled	Pathological process	Endpoints assessed
Lung	Bronchoalveolar lavaged fluid (right lung)	Injury	Total protein
			Albumin
			N-acetylglucosaminidase activity
			g-Glutamyl transferase activity
			Lactate dehydrogenase activity
		Inflammation	Total cells
			Alveolar macrophages
			Neutrophils
Systemic	Left Lung Blood	Morphological effect Hematological	Histopathology
			Red blood cells
			Hemoglobin
			Hematocrit
			Mean corpuscular hemoglobin concentration
			Platelets
			Total white blood cells
			Lymphocytes
	Citrated plasma	Platelet Aggregation/Thrombosis	Primary aggregation
			Primary slope
			Area under the curve
			Disaggregation
			Maximum aggregation
Vasculature	Aorta (mRNA for limited samples)	Inflammation and oxidative stress (mRNA)	Heme oxygenase-1 (HO-1)
			Tumor necrosis factor alpha (TNF-α)
			Matrix metalloproteases-14 (MMP-14)
			Matrix metalloproteases-2 (MMP-2)
			High mobility group box-1 (HMGB-1)
		Vasoconstriction (mRNA)	Endothelin-1 (ET-1)
			Endothelial nitric oxide synthase (eNOS)
			Inducible nitric oxide synthase (iNOS)
		Thrombosis (mRNA)	Plasminogen activator inhibitor-1 (PAI-1
			Tissue type plasminogen activator inhibitor (tPA)
			Tissue Factor (TF)
		Atherogenesis mRNA	Lectin-like receptor for oxidatively modified low density lipoprotein 1 (LOX-1)
			Receptor for advanced glycation endproduct (RAGE)
Cardiac	Left ventricle (mRNA for limited samples)	Cardiac contractility	Endothelin (ET-1)
			Forhood box-1 (FOXO-1)

Organ system	Tissue sampled	Pathological process	Endpoints assessed
			Myelin 7 (MYL-7)
			Endothelin receptor A (ETR-A)
			Tumor necrosis factor alpha (TNF-α)
			Plasminogen activator inhibitor -1 (PAI-1)

A limited number of aortic ring samples were examined *ex vivo* for vasocontractile effects of biofuels; however, no significant effects on contractile response were noted. The phenylephrine induced vasoconstriction and acetylcholine induced vasodilations were different for WKY and SH rats regardless of exposure condition. These data are not provided in the manuscript.