Methods

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Animals: Male Sprague-Dawley rats ([H1a: (SD) CVF, n = 8 rats/group, 225-250 g at arrival or 6-8 weeks of age) were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). All animals were free of viral pathogens, parasites, mycoplasm, *Heliobacter* and cilia-associated respiratory bacillus. The rats were acclimated to the facilities for 1 week after arrival and housed in cages ventilated with HEPA (high efficiency particulate air)-filters under climate-controlled conditions and a 12-h light/12-h dark cycle (lights on 0600 h). Food (Teklad 7913, Invigo; Madison, WI) and tap water were provided *ad libitum*. The animal facilities are pathogen-free, environmentally controlled, and accredited by AAALAC International. All procedures were approved by the CDC Morgantown Animal Care and Use Committee and were in compliance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals.

Inhalation exposure system: An eight-kilowatt (kW) diesel generator (Onan QD 8000, part number 8HDKAK11451J, Cummins Inc., Columbus, IN) was used to produce diesel exhaust in real time during inhalation exposures. This diesel engine was Tier 2 EPA compliant. Tier 2 compliant engines were built between 2001 – 2005 and emit reduced levels of carbon monoxide, unburned hydrocarbons, nitrogen oxides and sulfur emission (What You Should Know About EPA Diesel Emissions Tiers - General Power Limited (genpowerusa.com)). A load bank was connected to the generator and set to 4 kW (50% generator load) for all inhalation exposures. Mobil 1 Delvac 1300 Super Motor Oil 15w40 was used as the engine oil. The fuel used in the

generator was ultra-low sulfur (15 ppm or less sulfur), No. 2 dyed winter blend from Jacobs petroleum products (Waynesburg PA).

A custom exposure system with software written in LabVIEW automatically controlled chamber air flows, particle concentration, and exposure duration. Several combustion gasses were monitored and recorded from inside the exposure chamber during each inhalation exposure run by using a multi-gas analyzer (Model PG-350Z, Horiba Instruments Inc., Irvine CA). These gases were SO2, NO, CO2 and O2, and were recorded every 2 seconds. The exhaust was further analyzed by GC/MS from samples collected over a 2- 6 hr collection periods (samples were collected into cannisters, and there were 1-2 cannisters used/exposure period at each dose of diesel). The measurement of these chemicals was conducted using a method published in the NIOSH Analytical Methods Manual (2018; 3900: Volatile organic compounds (VOC), C1 to C10 canister method (https://www.cdc.gov/niosh/nmam/default.html). A portion of the diesel exhaust was directed into the exposure chamber, and a computer-controlled ball valve determined the amount. Clean dry dilution air (80 L/min) from a mass flow controller was added to the diesel exhaust delivery line before it entered the top of the exposure chamber.

The aerosol mass concentration inside the exposure chamber was continuously monitored with a Data RAM (DR-40000 Thermo Electron Co.) and gravimetric determinations (37 mm cassettes with 0.45 µm pore-size Teflon filters (2 L/min. sample flow) were used to calibrate and verify the Data RAM readings during each exposure run. For this study the target diesel concentration was either 0.2 mg/m^3 or 1.0 mg^3 over 6-hr long exposures each day. Animals were exposed for 4 consecutive days. After each exposure, animals were returned to their home cage in the animal facility.

Particle size distribution inside the exposure chamber (count based) was collected (SMPS Model 3081, TSI Inc., Shoreview, MN). The count median electric mobility diameter was 120

nm. Mass-based aerodynamic particle size distribution was determined in the exposure chamber by using a Micro-Orifice Uniform Deposit Impactor (MOUDI, MSP Model 110, MSP Corporation, Shoreview, MN). The mass median aerodynamic diameter was found to be 170 nm. Table 1 provides the measured exposure chamber conditions for all exposures used in this study. Gravimetric filter data was used for the particle concentration data and combustion gasses were monitored in real time inside the exposure chamber.

Microvessel physiology: Animals were euthanized by i.p. injection of sodium pentobarbital solution (100-300 mg/kg) and exsanguination. Tails were dissected from rats after exsanguination and placed in cold Dulbecco's modified Eagle's medium with glucose (Invitrogen/Gibco; Carlsbad, CA). Microvessel studies were performed as previously described [1, 2]. Ventral tail arteries from the C18-20 region of the tail were dissected, mounted on glass pipettes in a microvessel chamber (Living System; Burlington, VT), and perfused with bi-carbonated HEPES buffer (130 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, 1.80 mM KH_2PO_4, 0.03 mM EDTA, plus 10 % glucose) warmed to 37 °C. Arteries were pressurized to 60 mm Hg and allowed to equilibrate for approximately 1 hr. After an hour acclimation period, the chamber buffer was replaced with fresh HEPES buffer and responsiveness of the arteries to phenylephrine (PE)-induced vasoconstriction and acetylcholine (ACh)-induced re-dilation was measured. All chemicals for microvessel exposures were purchased from Sigma (Indianapolis, IN) unless otherwise noted. To assess the effects of treatment on sensitivity to α_1-adrenoreceptor-mediated vasoconstriction, PE was added to the chamber so that changes in the concentration occurred in half-log increments (-9.0 to -5.5 M) and the internal diameter of the artery was recorded after the arteries stabilized (approximately 5 min between concentrations). After measuring the dose-dependent vasoconstriction that

occurred in response to PE, the chamber buffer, was removed and replaced with fresh, oxygenated HEPES buffer. After rinsing in oxygenated HEPES buffer for 15 min, arterial diameter returned to near baseline levels. Because ventral tail arteries usually display little basal tone, endothelial-mediated re-dilation was assessed after arteries were pre-constricted to approximately 50% of their baseline diameters with PE. In pilot work, we demonstrated that re-constricting arteries with PE did not affect subsequent responses to ACh or other vasomodulating factors. To assess the dilatory effects of ACh, the agonist was added cumulatively in half-log increments (-10.0 to -5.0 M) and changes in the internal diameter of the vessel were measured as described for PE.

Procedures for in vivo hemodynamic measurements

To obtain *in vivo* measurements of cardiac function and reactivity to adrenoreceptor agonists at 1, 7 and 27 d after DE exposure, rats were anesthetized with 3% isoflurane mixed with 1 L/min oxygen in an induction chamber and maintained at 1 – 2% isoflurane and 0.5 l/min of oxygen during surgery to instrument the animals [1, 2]. Cardiopulmonary responses [heart rate (HR); breathing rate and depth] and toe pinch spinal reflex were examined as intra-operative monitoring techniques. The concentration of isoflurane was adjusted to maintain the proper depth of anesthesia. A temperature-controlled heating pad was used to regulate normal body temperature, and temperature was monitored *via* an anal probe during the entire procedure. Before the surgery, surgical instruments and supplies were autoclaved, and the catheter was cold-sterilized using Cidex (Physician Sales and Services, Inc.; Jacksonville, FL) prepared according to the manufacturer's instructions and flushed with sterile saline solution. The rat was placed in dorsal recumbent position, and the incision sites were clipped and the aseptically prepared with povidone-iodine, followed by 70% alcohol. Mikro-Tip® ultra-miniature PV loop catheters (SPR-

901; Millar, Inc., Houston, TX) were inserted into the left ventricle through the carotid artery. The correct position of the catheter tip in the left ventricle was confirmed by the waveform of a pressure-volume loop visualized on a computer monitor. After stabilization for 20 min, signals were continuously recorded at a sampling rate of 1,000 samples/sec using a PV conductance system (MPVS-Ultra; Millar, Inc. Houston, TX) connected to the PowerLab4/30 data acquisition system (AD Instruments; Colorado Springs, CO), stored and displayed on a personal computer using the LabChart7 Software System (AD Instruments). Increasing doses of norepinephrine or dobutamine (Hospira, Inc.; Lake Forest, IL) were administrated through a catheter (polyurethane, 3 French size) that had been placed in the right jugular vein. Systolic (SBP), diastolic (DBP), mean arterial blood pressure (MAP) and left ventricular function were measured. Parallel conductance volume was calculated by the software and used for the correction of the cardiac mass volume, and each rat was euthanized by exsanguination under deep anesthesia at the conclusion of the experiment.

Measuring oxidative stress: Heart and kidney tissues were homogenized in 1 ml of 0.1 M phosphate buffered saline (PBS) with protease inhibitors [1]. The supernatant was removed and protein concentrations (absorbance ration of λ260/λ280 nM) were measured using the BCA protein assay (Fisher, Pittsburgh, PA). Concentrations of reactive oxygen species (ROS) were measured using 2′7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St. Louis, MO), or 2'7'- dichlorofluorescein (Sigma-Aldrich). When these dyes are added to tissue homogenates they fluoresce (i.e., higher levels of fluorescence indicate greater levels of ROS). To perform this assay, duplicates of the supernatant from each pellet (10 µl) were pipetted into a 96-well plate. Re-constituted dye was diluted so that the final concentration was 1 mM, and 50 µl was added per well. Plates were incubated in the dark for 45 min and then fluorescence was

measured at 490-540 nM using a Synergy H1 all in one microplate reader (Biotek; Winooski, VT). Background measures (wells with dye plus PBS) were subtracted from each sample, and fluorescence/μg tissue was calculated and analyzed as described below.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR): qRT-PCR was performed to determine if exposure to DE inhalation resulted in changes in transcript levels in the heart and kidney using the methods described in Hughes et al. [3]. Because the goal of these studies was to determine if exposure to DE inhalation affected the cardiovascular system, we examined transcripts in the heart and kidney (these tissues are involved in blood pressure regulation). We examined transcripts that were indicative of changes in the expression of inflammatory cytokines, apoptotic factors, and factors involved in vasomodulation, vascular remodeling and ionic status in the heart and kidney. The specific transcripts measured were chosen based on the results of previous studies examining the effects of inhaled particulate matter on the cardiovascular system and the kidneys [1, 2, 4, 5]. The transcripts measured in the heart included interleukin-1β (*Il-1β*), factors involved in regulating apoptosis (*Bax, Bad* and *Bcl2*), hypoxia induced factor 1α (*Hif1-α*), inducible and endothelial nitric oxide synthase (*iNos* and *eNos*, respectively) and tumor necrosis factor α (*Tnf*-α). Kidney transcripts examined included angiotensin converting enzyme (*Ace*), *Il1-β*, *Il-6*, tissue inhibitor of metallomatrix protein (*Timp)*, tumor necrosis factor (*Tnf)-α*, *nNos*, *iNos2, eNos3* and vascular endothelial growth factor (*Veg-f*). RNA was isolated from the tissue using RNAeasy lipid Miniprep kits (Qiagen; Valencia, CA), and first strand cDNA was synthesized from 1 μg of total RNA using a Reverse Transcription System (Invitrogen; Carlsbad, CA). Melt curves were run for each transcript using each tissue. Samples that did not show a single defined melt peak in the 80 ^{o}C range were not included in the

data set. To determine if the treatment resulted in changes in transcript levels, fold changes from the same day controls were calculated.

Superoxide dismutase concentrations: Because previous studies had demonstrated that the primary effects of inhalation to toxins are changes in tissue levels of oxidative stress, cytokines and concentrations of anti-oxidant enzymes, an ELISA was used to measure super-oxide dismutase-2 (SOD2, R&D Systems Inc.; Minneapolis, MN). Proteins concentrations in tissues were measured using the manufacturer's protocols.

Analyses: Microvessel data were analyzed using repeated measures analysis of variance (ANOVAs) to determine if there were treatment differences between the control and DE-treated groups. Significant pairwise interactions were probed with one-way ANOVAs or student's *t*-tests. Non-linear regression analyses using a log-linear transformation were performed to determine the effective dose 50 (ED50) for each artery. The ED50s were then analyzed using Student's *t*-tests (air vs. diesel at 1, 7 and 27 d). ROS, tissue protein concentrations and transcript levels were analyzed using a 2-way (treatment × time) ANOVAs. Post-hoc comparisons were made using Student's *t*-tests. Changes in cardiac physiology collected through telemetry, or *in vivo* PV-loop measurements were analyzed using 2 (treatment) × 3 (days of recovery) repeated measures ANOVAs. Subsequent pairwise comparisons were tested using Fishers LSD. All telemetry and PV loop data were analyzed using R [6]; other data were analyzed using Jmp (15.1.01). The values in the figures were expressed as the mean ± SEM. Differences with $p \leq 0.05$ were considered statistically significant.

References:

1. Krajnak, K., et al., *Biological effects of inhaled hydraulic fracturing sand dust. VI. Cardiovascular effects.* Toxicology and Applied Pharmacology, 2020. **406**: 115242.

2. Krajnak, K., et al., *Acute effects of COREXIT EC9500A on cardiovascular functions in rats.* J Toxicol Environ Health A, 2011. **74**(21): 1397-404.

3. Hughes, J.M., et al., *Increased oxidant activity mediates vascular dysfunction in vibration injury.* J Pharmacol Exp Ther, 2009. **328**(1): 223-30.

4. McKinney, W., et al., *Pulmonary and cardiovascular responses of rats to inhalation of a commercial antimicrobial spray containing titanium dioxide nanoparticles.* Inhal Toxicol, 2012. **24**(7): p. 447-57.

5. Roberts, J.R., et al., *Evaluation of Pulmonary and Systemic Toxicity of Oil Dispersant (COREXIT EC9500A((R))) Following Acute Repeated Inhalation Exposure.* Environ Health Insights, 2014. **8**(Suppl 1): p. 63-74.

6. Team, R.C. *R: A language and environment for statistical computing*. 2023.