Detailed Methods

Animals. All studies were conducted in facilities accredited by AAALAC International, were approved by the Institutional Animal Care and Use Committee (protocols 13-JF-R-014, 14-JF-R-011 and 16-JF-R-020 v. 3 and v. 4), and were in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats ([H1a: (SD) CVF, approximate body weight of 200 – 275 g at arrival], were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). All animals were free of viral pathogens, parasites, mycoplasm, *Heliobacter* and cilia-associated respiratory bacillus. Animals were acclimated for one week and housed in ventilated micro-isolator units supplied with HEPA-filtered laminar flow air (Lab Products OneCage; Seaford, DE), with Teklad Sanichip and Teklad Diamond Dry cellulose bedding (or Shepherd Specialty Paper's Alpha-Dri cellulose; Shepherd Specialty Papers; Watertown, TN) instead of Diamond Dry). They were provided tap water and autoclaved Teklad Global 18% protein rodent diet (Harlan Teklad; Madison, WI) *ad libitum*. Rats were housed in pairs under controlled light cycle (12 h light/12 h dark) and temperature (22 – 25 °C) conditions.

Exposure system. Crude oil that was a surrogate oil from the Macondo well was used to generate vapors for exposure. The exposure system used to generate COV was described in McKinney *et al.* (2021). In Experiment 1, rats were exposed to a single (acute), 6-h exposure to COV (300 ppm) or filtered air in a whole-body inhalation system and euthanized 1 or 28 d following the exposure. In Experiment 2, rats were exposed to filtered air or 300 ppm COV 6 h/d × 4 d/wk × 4 wk (subchronic) and euthanized 1, 28 or 90 d following the exposure.

Procedures for microvessel, transcription and protein measurements

After each 6 h exposure, the animals were returned to the colony room. In Experiment 1, rats were administered an overdose of sodium pentobarbital euthanasia solution (i.p.; 100 – 300 mg/kg; Fatal Plus;

Vortech Pharmaceuticals, Dearborn, MI) and euthanized by exsanguination 1 or 28 d following the last exposure. The ventral tail nerve, heart and kidney were collected for analyses. In Experiment 2, rats were euthanized as described above and tissues were collected 1, 28 or 90 d after the end of exposure.

Microvessel physiology

Tails were dissected from rats after exsanguination and placed in cold Dulbecco's Modified Eagle's Medium with glucose (Invitrogen/Gibco; Carlsbad, CA). Ventral tail arteries from the C18-20 region of the tail were dissected shortly after euthanasia, mounted on glass pipettes in a microvessel chamber (Living System; Burlington, VT), and perfused with bi-carbonated HEPES buffer warmed to 37 °C and oxygenated. Arteries were pressurized to 60 mm Hg and allowed to equilibrate for approximately 1 h. Then, the chamber buffer was changed and vascular responsiveness to phenylephrine (PE)-induced vasoconstriction and acetylcholine (ACh)-induced re-dilation were measured. All chemicals were purchased from Sigma-Aldrich (Indianapolis, IN) unless otherwise noted. To assess the effects of treatment on sensitivity to α_1-adrenoreceptor-mediated vasoconstriction, PE was applied to the organ chamber in half-log increments (-9.0 to -5.0 M) and the internal diameter of the artery was measured using software that detected the internal and external edge of the artery (Living Systems) after vessels stabilized (approximately 5 min between application of doses). After assessing vasoconstriction, the chamber buffer containing PE was removed and replaced with fresh HEPES buffer. After rinsing, the arterial diameter was returned close to baseline levels. Because ventral tail arteries in adult animals 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 a pilot study, we demonstrated re-constricting arteries with PE did not affect subsequent responses to ACh. To assess the effects of ACh on vascular re-dilation, ACh was added in half-log increments (-9.5 to -5.0) using the same procedures that were used to apply PE.

Nitrate/nitrite (N_{ox}), hydrogen peroxide (H_2O_2), and reactive oxygen species (ROS) assays

N_{ox} and H_2O_2 were measured in heart and kidney tissues using the nitrate/nitrite kit (Caymen Biologics; Ann Arbor, MI) and the Fluoro H_2O_2 kit (Cell Technologies, Inc.; Hayward, CA), respectively. Assays were performed using the manufacturer's protocols. To account for potential differences in the amount of the tissue from which N_{ox} and H_2O_2 were isolated, a BCA protein assay was performed (Pierce Chemicals, Dallas, TX) and the concentrations of the ROS were expressed as nanomoles/µg protein. These assays were used to measure N_{ox} and H_2O_2 concentrations in kidney tissue from Experiment 1 and heart tissue from both experiments. Because of an equipment failure, the isolation of N_{ox} and H_2O_2 could not be completed in kidney tissue collected during Experiment 2. Thus, instead, the tissue pellet was reconstituted in 1 ml PBS with protease inhibitors (ULTRA protease inhibitor tablets (without EDTA; Roche, Indianapolis, IN)) and total ROS were measured using 2´7´-dichlorofluorescien diacetate (DCFH-DA; Sigma-Aldrich). 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 1:20 in phosphate buffered saline (PBS final concentration 1 M, pH 7.4) 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 COV resulted in changes in transcript levels in the heart and kidney using the methods described in (Krajnak et al. 2006). Because the goal of these studies was to determine if COV inhalation affected the cardiovascular system, we examined transcripts in the heart and kidney that are involved in regulation of blood pressure. We specifically examined transcripts that were indicative of changes in the expression of inflammatory cytokines, apoptotic

factors, and factors involved in vasomodulation, vascular remodeling, and concentration of ions and ion receptors in the heart and kidney. 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*), *il-1β*, *il-6*, *timp*, *tnf-α*, *inos*, *nnos*, and vascular endothelial growth factor (*vegf*). RNA was isolated from the tissue using RNAeasy lipid Miniprep kits (cat # 74804; 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 a change in transcript levels, fold changes from the same day controls were calculated. This was done by calculating the average response for the control group and then subtracting the individual CT values for each sample from the average of the controls.

Protein analysis via *plates*

In Experiment 1, heart and kidney tissue were homogenized in 0.1 M PBS with 0.2% Triton-X 100 and complete ULTRA protease inhibitor tablets (without EDTA; Roche, Indianapolis, IN), centrifuged at approximately 180 *g*, and the supernatant was removed and frozen at -80 °C until used for the assays. The pellet also was decanted and frozen at -80 °C until total protein concentrations could be measured using the BCA protein assay (Pierce, Rockford, IL USA). Rat kidney and vascular multiplex protein kits were purchased from EMD Millipore (Billerica, MA) and assays were run according to the manufacturer's instructions with the following exception: homogenates from kidney tissue were run using a 1:3 dilution instead of a 1:2 dilution because of the high protein concentrations. Heart samples were processed as described in the original protocol. Bioluminescence of the antibody labeled for each protein bound to the beads was measured using the Luminex 200 (Luminex Corp.; Austin, TX) and

concentrations were calculated using the standards provided by the supplier and X-ponent Software (Luminex X-ponent, 3.1; Austin, TX). The proteins measured in the arteries were: caveolin-1 (CAV1), connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1), interleukin-6 (IL-6), tumor necrosis factor (TNF)-α, the chemokine ligand 1-related proteins; Gro/KC/CINC-1, and tissue plasma activator inhibitor-1 (tPA-1). The kidney protein panel assessed the following proteins: calbindin (CAL), clusterin (CLUST), glutathione-S-transferase (GST)-α, interferon-gamma inducible protein (IP)-10, kidney injury molecule (KIM-1), osteopontin (OPN), tissue-inhibitor of metalleoproteinase-1 (TIMP1), and vascular endothelial growth fact (VEGF).

Protein measurements made using the Simple Protein western system

In Experiment 2, proteins were measured in heart tissues using the Simple Protein system (Protein Simple, Biotechne; San Jose, CA). Western blots were performed based on changes in protein levels and PCR results from Experiment 1. This system performs a Western assay using small volume samples ($\leq$ 4 µl). The proteins analyzed were chosen based upon changes seen using the multiplex plates described above (Experiment 1). Proteins were isolated from heart and kidney tissue using the methods described for the protein plates. Protein concentrations in 2 µl aliquots from each sample were measured using the 260/280 reading from a using Nanodrop One microvolume UV-Vis Spectrophotometer (Thermo-Fischer; Carlsbad, CA). Each sample (10 µg total protein) was prepared with a denaturing solution that also contains a molecular weight ladder, denatured at 95 °C for 5 min, cooled on ice, and loaded into a micro-well plate (4 µl/well). Blocking solution, primary antibody (rabbit TNF-α, and NOS2, each used at a final dilution of 1:1000; Santa Cruz Biotech; Santa Cruz, CA), secondary antibody and luminol reagent (Protein Simple kit) were added to each well using the volume recommended in the manufacturer's instructions. The plate was placed into the Simple Western machine (Protein Simple) which transfers samples, antibodies, buffers and luminal reagent from the plate into a

microgel. Once the proteins were separated, labeled and identified by chemiluminescence, the band densities were measured using the Compass Program (Protein Simple). The analysis program produced a histogram for each sample and measured peak height and width for each labelled band. To ensure all samples ran appropriately and that there was proper separation, the location of each marker in the ladder was compared across samples. If the representative markers in a sample did not run at the same rate as the molecular weight ladder or those of the other samples, the data were not used.

Procedures for in vivo *hemodynamic measurements*

Another group of rats (*n* = 8/group) were exposed to air or COV so that *in vivo* measurements of cardiac function could be measured. At 1 and 7 d after the COV exposure, rats (n = 8/grp) were anesthetized with 3% isoflurane and 1 l per min of oxygen in an induction chamber and maintained at 1-2% isoflurane and 0.5 l per min of oxygen during the surgery. Cardiopulmonary responses (heart rate, breathing rate and depth of anesthesia) and toe pinch and spinal reflexes 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 maintain 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 by Cidex (Physician Sales & 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. Millar's Mikro-Tip® ultra-inerature 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 Instruments; Houston TX) connected

to the PowerLab 4/30 data acquisition system (AD Instruments; Colorado Springs, CO), stored, and displayed on a personal computer by the LabChart7 Software System (AD Instruments). Increasing doses of the agonists, norepinephrine (Sigma-Aldrich) or dobutamine (Hospira, Inc.; Lake Forest, IL), were administrated through a catheter (polyurethane, 3 French size) pre-placed in the right jugular vein. Changes in various measures of cardiac function systolic and diastolic blood pressure were measured following administration of increasing doses of each agonist agent. At the end of the experiment, 10 µl of 30% saline were injected intravenously, 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.

References

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