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Biological effects of crude oil vapor. IV. Cardiovascular effects

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Abstract

Workers in the oil and gas extraction industry are at risk of inhaling volatile organic compounds. Epidemiological studies suggest oil vapor inhalation may affect cardiovascular health. Thus, in this hazard identification study we investigated the effects of inhalation of crude oil vapor (COV) on cardiovascular function. Male rats were exposed to air or COV (300 ppm) for 6 h (acute), or 6 h/day × 4 d/wk. × 4 wk. (sub-chronic). The effects of COV inhalation were assessed 1, 28, and 90 d post-exposure. Acute exposure to COV resulted in reductions in mean arterial and diastolic blood pressures 1 and 28 d after exposure, changes in nitrate-nitrite and H_2O_2 levels, and in the expression of transcripts and proteins that regulate inflammation, vascular remodeling, and the synthesis of nitric oxide (NO) in the heart and kidneys. The sub-chronic exposure resulted in a

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Walter McKinney was involved in designing the exposure system, quantifying and characterizing the exposure, monitoring the exposure, editing the manuscript.
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Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of brand name does not constitute product endorsement.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest in relation to this publication.

reduced sensitivity to α_1-adrenoreceptor-mediated vasoconstriction *in vitro* 28 d post-exposure, and a reduction in oxidative stress in the heart. Sub-chronic COV exposure led to alterations in the expression of NO synthases and anti-oxidant enzymes, which regulate inflammation and oxidative stress in the heart and kidneys. There seems to be a balance between changes in the expression of transcripts associated with the generation of reactive oxygen species (ROS) and antioxidant enzymes. The ability of antioxidant enzymes to reduce or inhibit the effects of ROS may allow the cardiovascular system to adapt to acute COV exposures. However, sub-chronic exposures may result in longer-lasting negative health consequences on the cardiovascular system.

Keywords

Cardiovascular; Peripheral vascular; Renal; Adrenoreceptor modulation; Inflammation; Oxidative stress

1. Introduction

This is the third paper in a series of seven tandem papers which focus on an investigation of the effects of inhaled crude oil vapor (COV) on the pulmonary, cardiovascular, immune and nervous systems, brain and kidneys (Fedan et al. 2022; Sager et al. 2022; Sriram et al. 2022; Weatherly et al. 2022) in an animal inhalation model (McKinney et al. 2022). The purpose of the overall investigation is introduced in Fedan, 2022, and the results obtained from these investigations have been summarized (Investigative Team, 2022).

According to the North American Industry Classification System, workers employed in the oil and gas extraction industry are working in one of three areas 1) extraction, 2) drilling, or 3) processing and refinement (Bureau of Labor Statistics (BLS) 2014). These workers are exposed to a number of physical and chemical hazards that can result in musculoskeletal disorders, traumatic injuries due to contact with equipment, hearing loss, and respiratory, cardiovascular, digestive, reproductive and nervous system disorders (Blackley et al. 2014; Bureau of Labor Statistics (BLS) 2014; Esswein et al. 2014; Laffon et al. 2016; Nance et al. 2016; Stenehjem et al. 2015; D'Andrea and Reddy 2014; Cipolla et al. 2016; Mazet et al. 2001). Many of these health problems have also been found in people living in communities that are in close proximity to areas where gas and oil extraction and refinement occurs because of ingestion or inhalation of fumes generated during crude oil extraction and refining processes (Chivu et al. 2007; Anttila et al. 2015; Cipolla et al. 2016).

A number of studies have demonstrated that inhalation of volatile organic compounds (VOCs) released into the air during the drilling process can have severe health effects on workers, and, if high concentrations of these vapors are released rapidly, exposure may result in death (Esswein et al. 2014; Harrison et al. 2016). Fortunately, most of the VOCs dissipate shortly after the oil has been extracted, reducing levels of exposure (Eide 1990; Anttila et al. 2015; Harrison et al. 2016). However, there also are a number of VOCs such as benzene, xylene and toluene that do not disperse as rapidly and can be inhaled by workers or others living in close proximity to areas where oil production is occurring (Cipolla et al. 2016). Epidemiology studies performed during and after oil spills (Laffon et al. 2016; Nance et al. 2016) have demonstrated that the exposure of the skin and inhalation of fumes

is correlated with the development of a number of different negative health consequences. In humans, inhalation of VOCs is associated with an increased risk of developing inflammation and fibrosis of the lungs, neurological dysfunction, reproductive dysfunction, cardiovascular dysfunction and lung, liver, and kidney cancer (Anttila et al. 2015; Stenehjem et al. 2015). In laboratory studies performed in animals, pulmonary exposures that result in inflammation have been associated with an increased risk of developing respiratory and cardiovascular problems (Mauderly et al. 2014). The cardiovascular system may be more sensitive than the pulmonary system to the negative health consequences that occur in response to the inhalation of particles; inhalation exposures that do not induce significant changes in pulmonary function or inflammation can result in changes in physiological and biological markers indicative of peripheral and cardiovascular dysfunction (Nurkiewicz et al. 2011).

The goal of these studies was to determine how inhalation of crude oil vapor (COV) affects cardiovascular function by examining its effects on responsiveness of peripheral arteries to agonists that induce vasoconstriction and dilation, changes in gene and protein expression of factors that are markers of cardiovascular disease, and ROS levels in the heart and kidney. The oil used for this study was a surrogate of the crude oil that leaked into the Gulf of Mexico after the Deepwater Horizon oil spill (McKinney et al. 2022). We tested the hypothesis that changes in cardiovascular function after inhalation of crude oil fumes are manifested as changes in the responsiveness of the heart and peripheral vascular system to vasoconstricting and vasodilating agonists and changes factors that regulate inflammation, oxidative stress and vascular remodeling. Based on previous studies, we also predicted that inhalation of COV would affect the expression of biomarkers of kidney function that are associated with blood pressure regulation (Mediouni et al. 2011; Ketan et al. 2015).

2. Methods

2.1. 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.

2.2. Exposure system

Crude oil was obtained from BP Exploration and Production, Inc. This oil was a surrogate to the oil from the Macondo oil well that was released during the Deepwater Horizon spill in 2010. The exposure system used to generate COV, the various components of the oil and concentrations of the volatile organic compounds in the vapors are described in McKinney et al. (2022).

In Experiment 1, rats were exposed to a single, 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. and euthanized 1, 28 or 90 d following the exposure. Because workers performing different jobs during crude oil production may be exposed to a wide range of concentrations of oil vapors, an exposure concentration that fell in the mid-range of what many workers would be exposed to (300 ppm) was chosen for the current study (Esswein et al. 2014).

2.3. Procedures for micro-vessel, 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 artery, 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.

2.4. Micro-vessel 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 were dissected from the region of the C18–20 vertebrate in the tail (which is in the distal 3rd of the tail) shortly after euthanasia, mounted on glass pipettes in a micro-vessel chamber (Living System; Burlington, VT), and perfused with bi−carbonated HEPES buffer warmed to 37 °C and oxygenated. Arteries were pressurized to 60 mmHg 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 measuring changes in vascular diameter in response to PE, the chamber buffer containing PE was removed and replaced with fresh HEPES buffer. After rinsing, the arterial diameter 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 measure 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.

2.5. 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 N_{ox} and H_2O_2 were expressed as nanomoles/μg protein. Because of an equipment failure, the isolation of N_{ox} and H_2O_2 could not be completed in tissue collected during Experiment 2. Thus, instead, the tissue pellets from each tissue were 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. Reconstituted DCFH-DA 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 DCFH-DA plus PBS) were subtracted from each sample, and fluorescence/μg tissue was calculated and analyzed as described below.

2.6. 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). 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 (*Il1β*), factors involved in regulating apoptosis (*Bax, Bad* and *Bcl2*), hypoxia induced factor 1α (*Hif1α*) inducible and endothelial nitric oxide synthase (*iNos* and *eNos*, respectively) and tumor necrosis factor α (*Tnf*-α). Kidney transcripts examined included *catalase, Il1β*, *Il6*, *Timp*, *Tnfα*, *iNos*, *nNos*, *eNos, Sod2* 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 °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.

2.7. Protein analysis via plates

In both Experiments 1 and 2, 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 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 manufacturer's protocol. Bioluminescent antibodies for each protein on the plate were bound to beads, and after performing the assay the luminescence 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 heart 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).

2.8. 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 collected. At 1 and 7 d after the COV exposure, rats (n = 8/grp) were anesthetized with 3% isoflurane and one 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 1000 samples/s 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.

2.9. Statistical analyses

To determine if COV exposure affected responsiveness to PE-induced vasoconstriction or ACh-induced re-dilation, an effective dose (ED_{50}) was calculated by curve fitting a concentration-response curve for each animal. The ED_{50} in Experiment 1 was analyzed using a 2 (treatment) × 2 (days of recovery) and in Experiment 2 using a 2 (treatment) × 3 (days of recovery) ANOVA.

ANOVA. These analyses were performed using Prism GraphPad 5.0 (La Jolla, CA). Additional analyses of micro-vessel data were also performed using 2 (treatment) × 2 or 3 (days of recovery) × 8 (doses) repeated-measures ANOVAs. Protein concentrations were analyzed using ANOVAs. In addition, changes in the concentrations of individual proteins were associated with changes in other proteins, using multivariate analyses. Bi-variate linear analyses were then performed to determine if treatment-induced changes in protein-protein associated variations were significant. ANOVAs and *t*-tests used to perform comparisons between individual groups were done using JMP (SAS Institute; Cary, NC; 11.1.1). Changes in PV-loop measurements were analyzed using 2 (treatment) × 2 (days of recovery) ANOVA or repeated measures ANOVAs (for studies performed with dobutamine). Subsequent pairwise comparisons were tested using Fishers LSD tests. All PV loop data were analyzed using SAS software (Version 9.3). Differences were considered statistically significant at the level of $p < 0.05$. All results values are expressed as the mean ± SEM unless otherwise noted.

3. Results

3.1. Micro-vessels

3.1.1. Experiment 1: Acute exposures—Responses to PE-induced vasoconstriction are presented in Fig. 1A and B and to ACh-mediated re-dilation are presented in Fig. 1C and D. A single, acute exposure (6 h at 300 ppm) to COV did not result in a change in PE-induced vasoconstriction or ACh-induced re-dilation at either 1 or 28 d following the exposure.

3.1.2. Experiment 2: Sub-chronic exposures—Responses to PE-induced vasoconstriction are presented in Fig. 2A – C. On post-exposure days 1 and 90 (A and C), there were no significant effects of COV exposure on PE-induced vasoconstriction. However, 28 d following exposure, arteries from animals exposed to COV were less

sensitive to PE-induced constriction than those from air-exposed animals (B). Reactivity to ACh was not different between the air-controls and COV-exposed animals at any time point.

3.2. Cardiac performance in vivo

3.2.1. Experiment 1: Acute exposures—Acute exposure to COV resulted in a reduction in the ventricular end-systolic pressure (Ves; an estimate of pre-load) one day after exposure. Baseline Ves, was significantly reduced in COV-exposed animals (Fig. 3A). However, dobutamine-induced recovery from vasoconstriction not affected by 1 d following COV exposure (Fig. 3B). Ventricular end-diastolic pressure (Ved) also was reduced at baseline in COV-exposed rats (Fig. 3C) and, dobutamine-induced recovery from vasoconstriction was also reduced in COV-exposed animals (Fig. 3D). One day of crude oil vapor inhalation also resulted in a reduction in diastolic blood pressure (DPB; Fig. 4A) and mean arterial pressure (MAP; Fig. 4B). There were no significant effects 28 d after acute exposure to COV (data not shown). *In vivo* cardiac performance measures could not be collected after the sub-chronic exposure (Experiment 2) because of a change in personnel.

3.3. N_{ox}, H_2O_2 or reactive oxygen species (ROS) in heart and kidneys

3.3.1. Experiment 1: Acute exposures—N_{ox} concentrations in the heart were significantly increased 28 d after exposure to COV (Fig. 5A). However, in the kidney, N_{ox} concentrations were not significantly affected by COV exposure (Fig. 5C). H_2O_2 concentrations were significantly higher in heart tissue collected from COV-exposed than in tissue collected from air- control animals 1 d following exposure (Fig. 5B). In the kidney, exposure to COV resulted in a significant reduction in H_2O_2 concentrations 28 d following the exposure (Fig. 5D).

3.3.2. Experiment 2: Sub-chronic exposures—Because of equipment failure, we were unable to measure N_{ox} and H_2O_2 in kidney collected from animals exposed to COV sub-chronically using ELISA kits. However, we were able to measure total ROS using the fluorescent dye, DCFH-DA. Twenty-eight days following the exposure, ROS concentrations in the heart were reduced in both air and COV-treated animals in comparison to air-control animals on day 1. In addition, ROS concentrations were also lower in COV animals than in air-control animals on day 28 (Fig. 6A). Although there appeared to be changes in ROS measures in the heart 1 and 90 d after exposure (Fig. 6A), and in the kidney 28 and 90 d after exposure, these differences were not significant (Fig. 6B).

3.4. qRT-PCR

3.4.1. Experiment 1: Acute exposures—Transcript levels in heart tissue from air- and COV-treated are presented Table 1. In the heart, there were significant reductions in transcript expression for interleukin-6 (*Il6)* and tumor necrosis factor (*Tnfα*) and an increase in hypoxia-induced factor-1a (*Hif1a)* 1 d after exposure to COV. By 28 days following the exposure, only *Tnfα* expression was different, with *Tnfα* being reduced in COV-exposed animals.

Results from the kidney PCR experiments are presented in Table 2. One and 28 d following inhalation of COV, there were significant reductions in interleukin 1β (*Il1β*), *Tnfα*, inducible nitric synthase (*iNos)*, endothelial *NOS* (*eNos)*, and vascular endothelial-mediated growth factor (*Vegf*) expression.

3.4.2. Experiment 2: Sub-chronic exposures—Transcript levels in the heart 1, 28 and 90 d following air or COV exposure are presented in Table 3. One day following sub-chronic exposure to COV inhalation there were no significant changes in gene expression in heart tissue. However, 28 d following exposure, there was a reduction in *eNos* and *iNos*, and the reduction in *eNos* was maintained 90 d after exposure.

Transcript levels in the kidney after a sub-chronic exposure to COV are in Table 4. In the kidney *Il1β* expression was reduced 1 d after exposure to COV; however, it returned to pre-exposure levels on days 28 and 90 post-exposure. *Il6* was increased 1 d following exposure, but also returned to air control levels 28 and 90 d following exposure. Levels of the anti-oxidant enzyme, *catalase,* were increased in COV-exposed as compared to air-control animals 28 d after exposure. Ninety d following exposure, *Timp, Tnfα* and superoxide dismutase 2 (*Sod2)* expression increased in tissue from COV-exposed animals.

3.5. Protein concentrations in heart and kidneys: Acute exposure

Protein concentrations in the heart measured using the multiplex assay are presented in Table 5. One day following COV exposure, there was a significant increase in *Il6* in the heart. There was no significant treatment related-changes 28 d following exposure to COV.

The multiplex arrays used in these studies measure proteins are markers vascular disease and dysfunction, it is possible that although proteins may not show significant changes in response to an exposure, the concentrations of proteins may vary together, as part of a cellular or signaling pathway, and it may be variations in these groups of proteins that are important and serve as biomarkers of dysfunction (Krajnak et al., 2010). To determine how protein concentrations varied in relationship to other proteins being measured, we performed multivariate analyses at each time point. The analyses revealed that there were a number of significant correlations in changes between specific proteins. One day after exposure to COV, there were correlations between GRO and MCP1, in heart tissue from COV-exposed animals and between GRO and IL6, CAV-1, TIMP, and CTGF in air-control tissue (Fig. 7). In tissue from air exposed animals there were correlations between IL6 and MCP-1 and CTGF. In COV-exposed animals, there were correlations between IL6 and CAV1 and TIMP. Twenty-eight days after exposure to air or COV, there were correlations between TIMP and GRO, or IL6.

Protein concentrations in the kidney are presented in Table 6. There were no significant exposure-related differences in protein concentrations. However, when multivariate analyses were performed, there were significant correlations between VEGF and IP10, or clusterin, or calbindin in kidney tissue 1 d following COV exposure (Fig. 8). In the air-exposed rats, there were correlations between KIM-1 and IP-10, between IP-10 and clusterin and between clusterin and calbindin.

Twenty-eight days after air and COV exposure, clusterin and calbindin correlated. In air-exposed animals, there were significant correlations in between VEGF and IP10 or clusterin or calbindin. These factors may be induced by inflammation and/or changes in blood flow to the kidney (Panduru et al. 2015; Roberts et al. 2013). There were also correlations between IP10 and clusterin or calbindin in air-exposed rats. The correlations between these groups of proteins are most likely indicative of changes due to normal kidney function.

3.6. Protein concentrations in heart and kidneys: Sub-chronic exposure

Proteins measurement using multi-plex assays were performed on tissue collected 1 and 28 days after exposure. Exposure to COV did not result in significant changes in the concentrations of any proteins measured in heart (Table 7) or kidney (Table 8) tissues.

4. Discussion

Inhalation of crude oil fumes by workers, or people living in communities near oil and gas mining sites and refineries, can result in a number of negative health effects including cardiovascular and renal disease (Laffon et al. 2016). Because previous studies have suggested that the pulmonary inflammation associated with inhalation of workplace toxicants can contribute to the development of cardiovascular problems, in this hazard identification study we assessed the effects of the inhalation of COV on the peripheral and cardiovascular system, and on the kidneys (Nurkiewicz et al. 2011; Roberts et al. 2013; Krajnak et al. 2011). The results of this study demonstrated that acute inhalation of COV had transient effects on measures of blood pressure, and responsiveness to vasoconstrictors and vasodilators, and affected measures of oxidative stress and expression of pro-inflammatory markers as well as other factors associated with cardiovascular disease (Bicu et al. 2010; Panduru et al. 2015; Aragon et al. 2016).

Exposure to COV had a transient effect on PE-induced vasoconstriction but did not affect ACh-induced re-dilation in either experiment. Other studies examining the effects of inhaled toxicants on tail artery responsiveness to vasoconstricting, and vasodilating factors have produced varying results; inhalation of some toxicants resulted in changes in PE-induced vasoconstriction or ACh-mediated vasodilation (Krajnak et al. 2011), while other toxicants had no effect on tail artery responsiveness to these vasomodulating agents (McKinney et al. 2012).

In vivo measurements of cardiac responsiveness to oil vapor inhalation also resulted in transient effects on various measures of cardiac output. Baseline measures of end-systolic and end-diastolic volumes were reduced, along with diastolic and mean arterial blood pressures. However, changes in responsiveness to dobutamine- or NE-induced vasoconstriction were observed and, are consistent with the results seen in the micro-vessel studies. There were also transient reductions in sensitivity to PE-induced vasoconstriction 28 d following COV exposure in the sub-chronic experiment. However, there were no changes 1 or 90 d following the exposure. This is in contrast to epidemiological and experimental studies that have found that inhalation of crude oil vapor increases the risk for developing cardiovascular disease (Mauderly et al. 2014; Laffon et al. 2016). It is likely that, longer exposures, or exposures to higher concentrations of COV, would have resulted

in more permanent changes in cardiac and peripheral vascular functions. For example, studies examining the systemic effects of exposure to crude oil and polycyclic aromatic hydrocarbons (PAHs) in fish and aquatic mammals have shown that exposure to PAHs can have cardiotoxic effects on the developing cardiovascular system of fish (Incardon and Scholz 2016) and that over time, PAHs can accumulate in various organs of aquatic species (Meador et al. 1995). In addition, epidemiological studies examining cardiovascular disease in people that worked as part of the Deepwater Horizon spill clean-up had an increased risk of developing cardiovascular disease, and that this risk was greatest in workers with the highest exposure (Denic-Roberts et al. 2022). People living near the spill also displayed an increased risk of developing cardiovascular disease (Stelitz et al. 2019).

Although the functional changes in the peripheral vascular system were only transient after COV inhalation, there was an increase in N_{ox}, and in H_2O_2 concentrations in the heart 28 days after completion of the acute exposure. This increase in oxidative stress may have been due to a transient hypoxic event after COV inhalation; 1 day following exposure to COV there was an increase in *hif-1* transcript levels. There may also have been changes in other proteins or enzymes involved in cell signaling that may have contributed to the transient changes seen in cardiac and peripheral vascular function after an acute exposure. For example, one of the mechanisms regulating NO signaling involves the cell membrane protein CAV-1 (Godo et al. 2016). Depending on the assembly of the signaling proteins, which can be mediated by caveolins, NO can signal the production of ROS, and these factors can have negative consequences for cells. If increase in oxidative stress or inflammation are maintained over extended periods of time, there is an increased risk of a number of different disorders, including cardiovascular disease (Stapleton et al. 2012; Miller et al. 2017; Zelko et al. 2016).

In experiment 2, COV inhalation was associated with a reduction in the expression of *eNos*, *iNos* and ROS production in the heart. NOS enzymes regulate the production of NO, and the fluorescent dye used to determine levels of ROS measured a number of NO-associated by-products and H_2O_2 (Stapleton et al. 2012). Thus, it is likely that the reduction in *nos* transcription resulted in a reduction in NOS enzyme concentrations and a reduction in the production of ROS. By day 90 following the COV exposure, *eNos* expression was increased, and ROS levels in air- and COV-exposed rats were similar. Thus, in these experiments, the effects of COV inhalation on the cardiovascular system were transient. Epidemiological studies have shown that oil vapor inhalation is a risk factor for developing a number of diseases and disorders, including cardiovascular disease (Mauderly et al. 2014; Harrison et al. 2016; Anttila et al. 2015; Stenehjem et al. 2015; Aguilera et al. 2010; Zock et al. 2009). It is possible that the present experiments did not detect any prolonged effects on measures of cardiovascular health because the duration of the COV exposure was not sufficiently long.

In the kidney, *Il6* and *Timp1* transcript expressions were increased 1 d after exposure (experiment 2). These changes in gene expression were not associated with a change in ROS levels in the kidney. There were no effects of COV exposure on ROS or gene expression in the kidney 28 d after exposure. However, catalase gene expression was increased in COV-exposed rats, suggesting that there may have been some oxidative activity at this time point. Ninety d after exposure, the expression of *Timp1* and *Tnf-α* were increased. The increase

in *Tnf-α* suggests that there was some inflammation in the kidneys of COV-exposed rats. However, no other pro-inflammatory genes were increased. There was also an increase in the anti-oxidant enzyme, *Sod2*. This increase may have been associated with the increase in *Tnf-α*, which could induce an increase in oxidative stress. An increase in *Timp1* expression could result in an inhibition of matrix-metalloproteinases and inhibit the breakdown of cell matrix proteins. There are studies showing an increase in kidney disease in workers exposed to COV inhalation (Anttila et al. 2015; Aguilera et al. 2010; Zock et al. 2009). In this study we measured renal factors that could have had an effect on blood pressure. However, the changes induced by COV inhalation may affect other aspects of kidney function, resulting in disease. Additional studies investigating the effects of longer exposures, and histological changes in the kidney, would help elucidate the mechanisms by which the COV inhalation affects kidney function and disease.

The protein arrays did not reveal many changes in protein concentrations that occurred as a result of COV-exposure. However, looking at how the various proteins on each array varied in correlation with one another provides some insight as to some of the cellular pathways that may be affected by and responding to COV exposure. For example, after acute exposure, the positive correlations between changes IL6 and MCP1, TIMP and CTGF in heart tissue are all indicative of inflammation and these factors may also play a role in vascular remodeling and regeneration (Krajnak et al. 2010; Aragon et al. 2016). CAV1 is a protein involved in mediating cell to cell and intracellular signaling (Li et al. 2005). This protein has been shown to play a role in regulating cell signaling pathways that mediate responses to factors that result in vascular dysfunction, and, thus, it is not surprising that inhalation of COV resulted in positive correlations between changes in CAV1 and other proteins that respond to vascular insults. Changes in VEGF and IP10, or clusterin, or calbindin in kidney tissue 1 d following COV exposure are indicative of changes in kidney function and remodeling. Changes KIM-1 in kidneys are usually a marker of kidney injury or dysfunction (Panduru et al. 2015). However, it is also expressed during development and may be involved in cell turnover in the kidney. Therefore, in the air-exposed animals it may be indicative of changes that occurred as a result of normal development.

With chronic exposure to COV, the changes in protein concentrations that varied in correlation with each other in the heart 1 d following exposure, such as VEGF and clusterin, calbindin and IP10 and IL6 and CTGF, suggest that pathways involved in remodeling may have been affected by the sub-chronic exposure to COV (Krajnak et al. 2010; Aragon et al. 2016). However, 28 d following the sub-chronic exposure to COV, different pairs of proteins showed changes, and the changes in Gro and MCP-1, CTGF and CAV1, along with changes in TIMP1 and CTGF are consistent with other findings in this study showing that there were increases in oxidative stress, and that cellular mechanisms that respond to oxidative stress were activated. One day following the sub-chronic exposure, changes in correlations between proteins in COV exposed tissues, such as correlation in changes between VEGF and clusterin, and calbindin and IP10, suggest that there may have been vascular remodeling the in the kidney as well as in the heart (Li et al. 2005; Zelko et al. 2016). The correlation between KIM-1 and IP10 1d after the exposure suggest there may have been some changes in kidney function shortly after the sub-chronic exposure. However, 28 d following the sub-chronic exposure, the majority of the proteins that were correlated were in the air

exposed animals, and these correlations may have been indicative of normal kidney function. Although additional studies need to be done, the correlations between changes in these proteins provide information regarding cellular pathways that may be affected by crude oil vapor inhalation, and the changes in these pathways could serve as early biomarkers of dysfunction.

In conclusion, both acute and sub-chronic exposures to crude oil vapor inhalation resulted in transient effects on vascular function, blood pressure, gene expression, ROS production, and inflammation in the heart and kidney. Numerous studies have shown that workers exposed to crude oil develop significant health problems. However, the composition of crude oil depends on where it is extracted (Farombi et al. 2010; American Petroleum Institute 2008; Di Toro et al. 2007). Therefore, the fact that only transient effects of COV were seen on the cardiovascular system in the current experiments may be due to the specific oil used in this experiment or the length of the exposure. Regardless, the findings of these studies identify potential biomarkers that may be used to predict if a worker exposed to an exposure to oil vapor or another inhaled toxicant is at risk for developing cardiovascular or renal problems.

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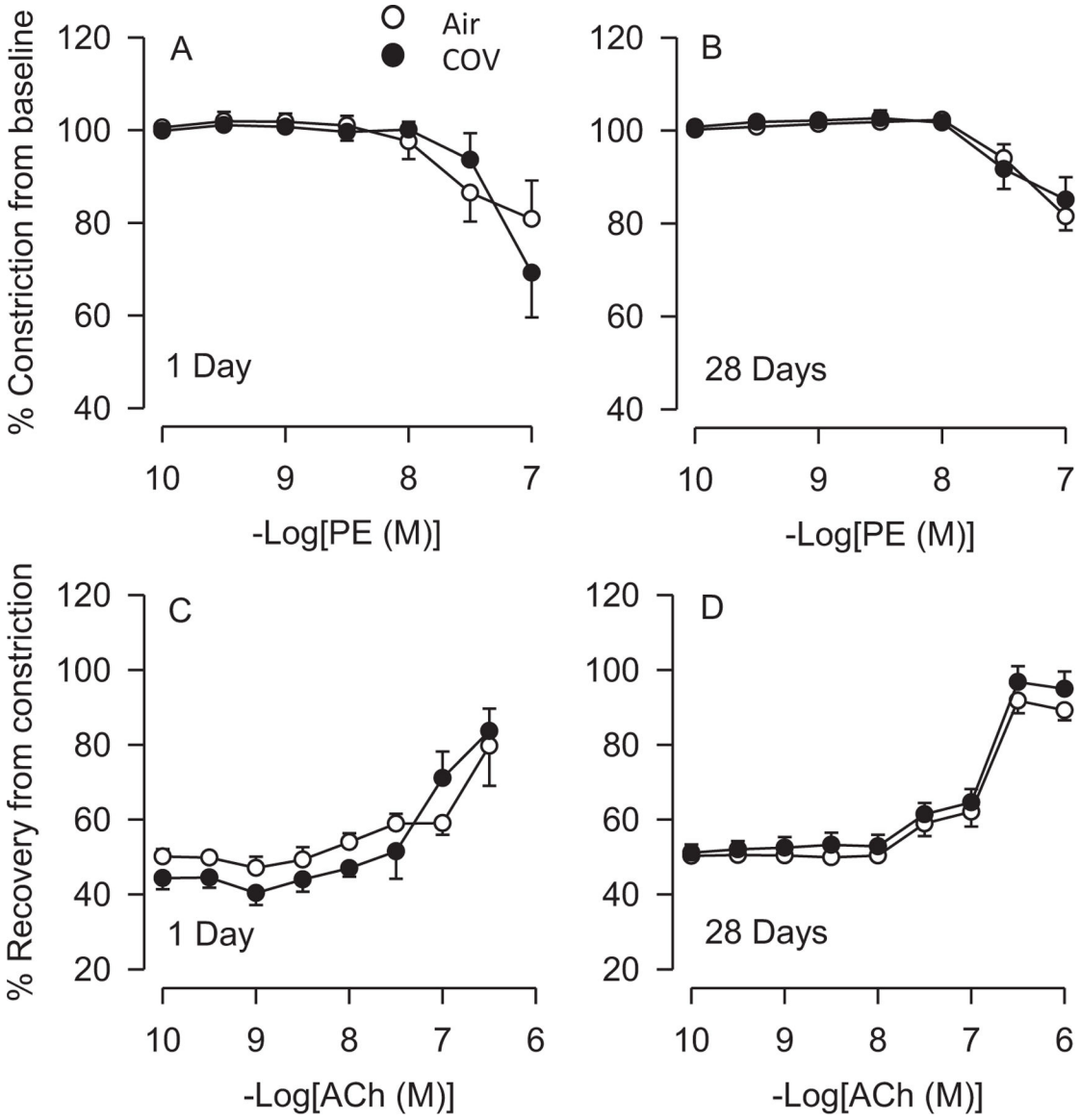

Fig. 1.

Fig. A and B show the dose-dependent vasoconstriction in the tail artery of rats in response to phenylephrine (PE) 1 d (A) and 28 d (B), and acetylcholine (ACh) 1 d (C) and 28 d (D) after an acute exposure to air or COV (n = 8 animals/group). No significant changes in vascular responses to either PE-induced vasoconstriction or ACh-induced re-dilation in response to treatment or days of recovery were observed.

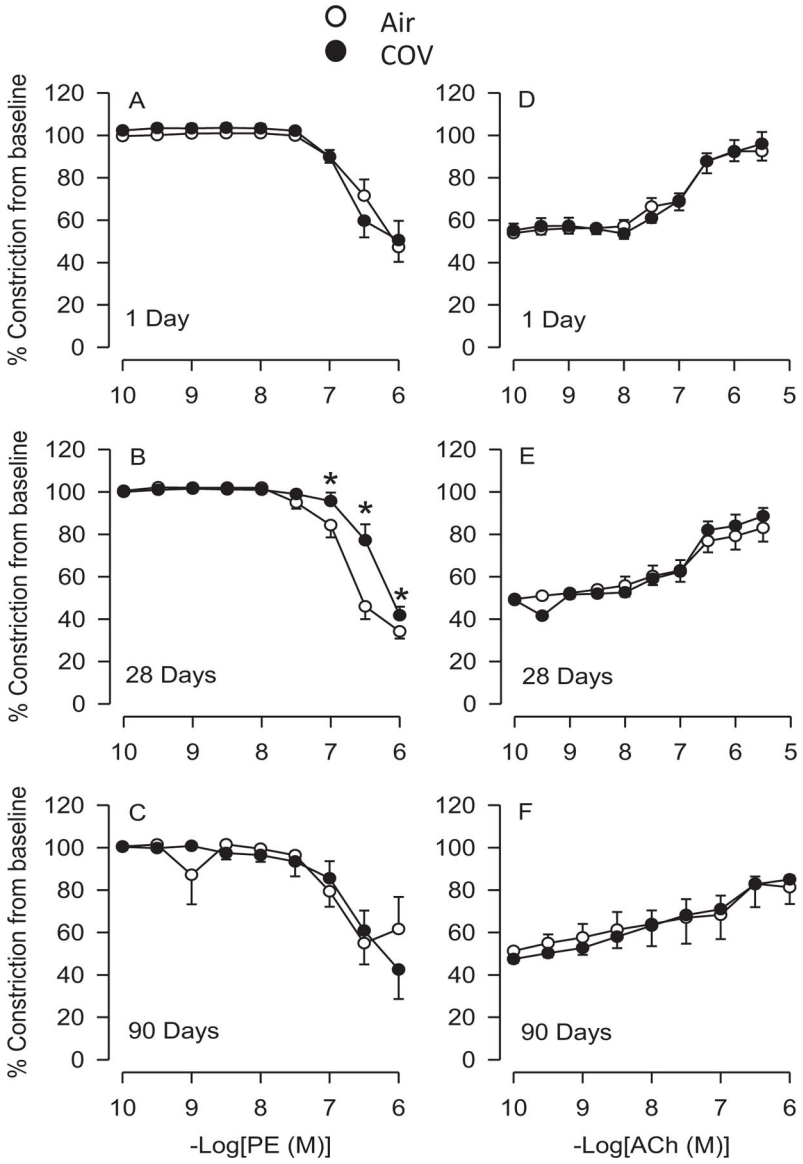

Fig. 2.

Fig 2. A-C show the dose-dependent vasoconstriction in response to PE-induced vasoconstriction 1 d (A), 28 d (B) and 90 d (C) after a sub-chronic exposure to COV in the ventral tail arteries of rats. $n = 5–8$ animals/group. Twenty-eight days following exposure, arteries from COV-exposed rats were less sensitive to PE-induced vasoconstriction than those from air-exposed animals ($*p < 0.05$). Fig. D-F show responses to ACh-induced re-dilation in the same arteries. There were no effects of air or COV on ACh-induced re-dilation.

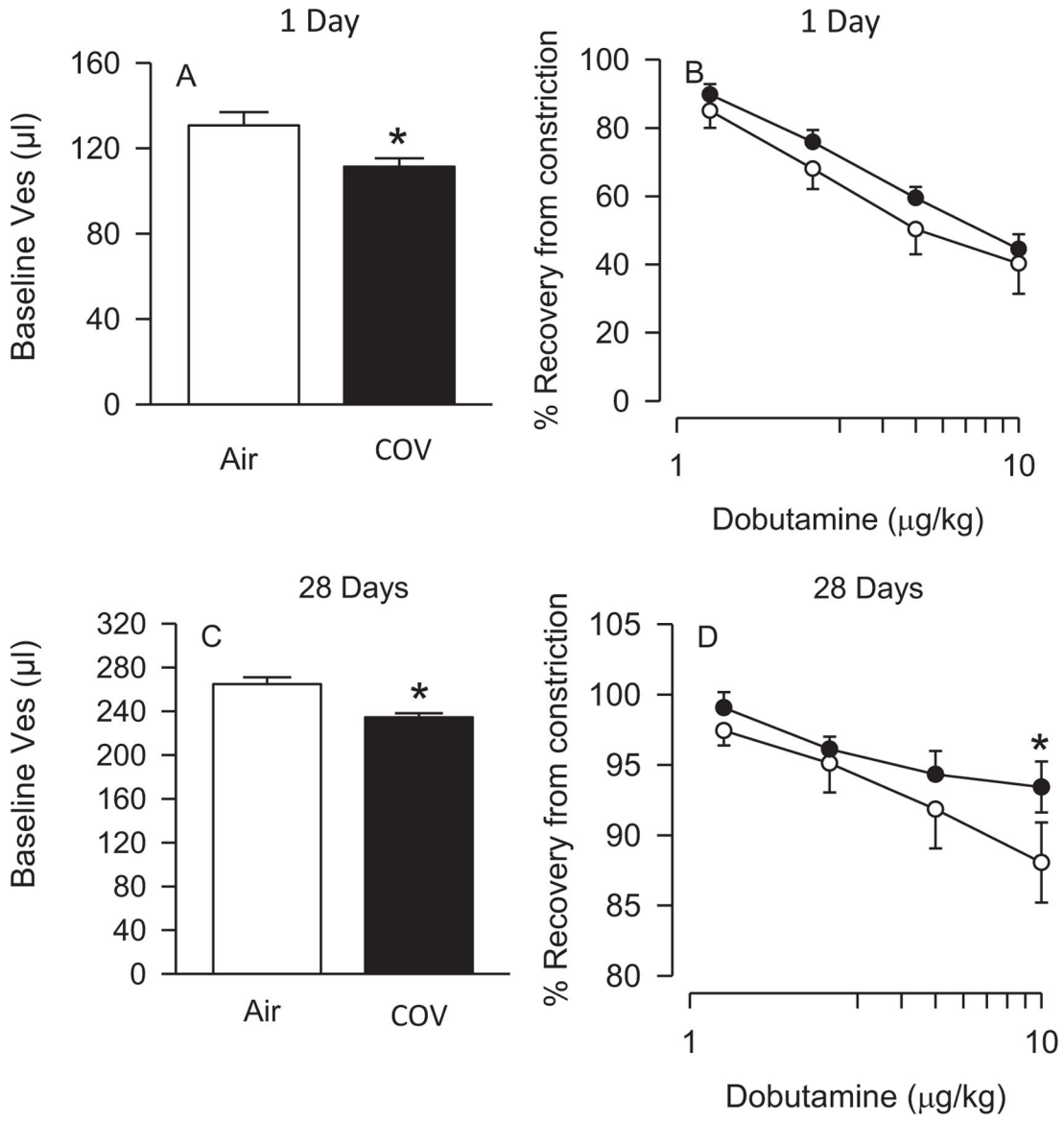

Fig. 3.

The effects of COV inhalation on ventricular systolic-end pressure (Ves) and diastolic-end pressure (Ved). One d following an acute exposure, baseline Ves was reduced, and 28 d following exposure Ved was reduced in COV-exposed rats (A and C, respectively). Dobutamine-induced recovery from vasoconstriction was not affected 1 d following COV exposure (Fig. 3B). However, 28 d following COV exposure, dobutamine-induced recovery from vasoconstriction was reduced as compared to controls (Fig. 3D).

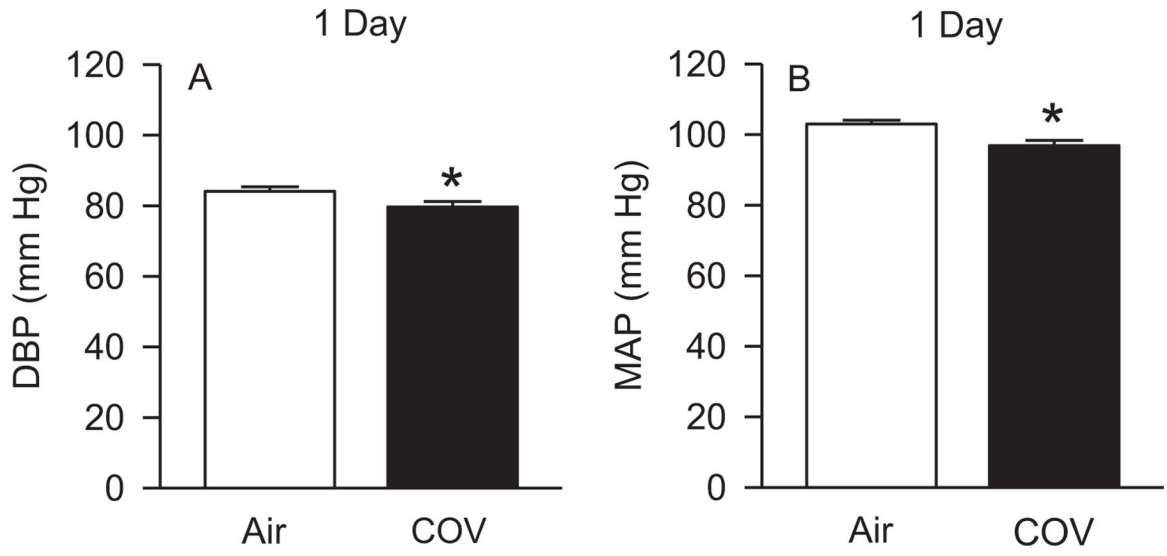

Fig. 4.

Diastolic blood pressure (DBP) and mean arterial blood pressure (MAP) measured by the PV-loop method after an acute exposure to air or COV. One d following exposure, both DBP (A) and MAP (B) were significantly reduced as compared to air controls. *$p < 0.05$. $n = 8$ animals/group.

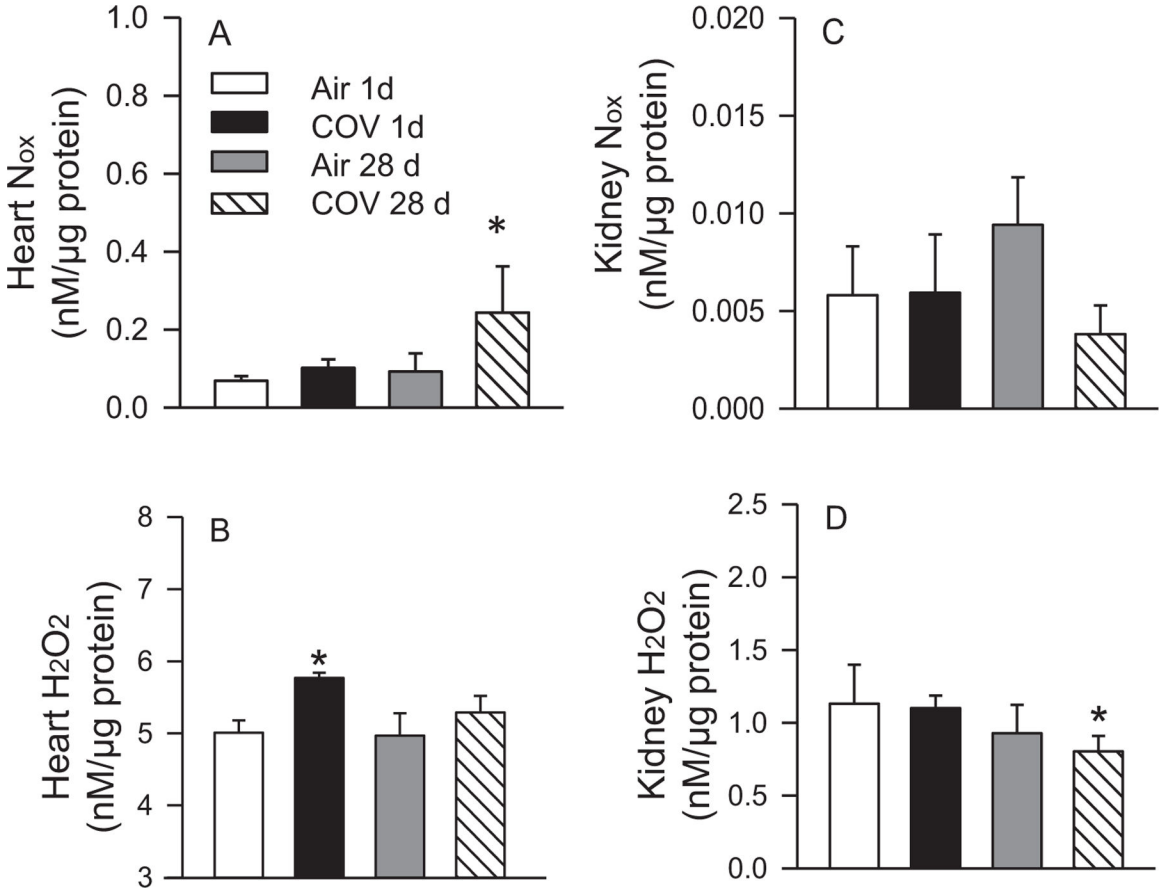

Fig. 5.

N_{ox} concentrations in the heart (A, B) and kidney (C, D) 1 and 28 d after an acute exposure to air or COV. In the heart, N_{ox} concentrations were significantly increased 28 d after exposure to COV (A). However, there were not significant differences in N_{ox} expression in the kidney (B) after exposure to COV. However, H_2O_2 concentrations were significantly increased 1 d after COV exposure in the heart and reduced 27 d following exposure in the kidney. *$p < 0.05$, as compared to time-matched controls. $n = 8$ animals/group.

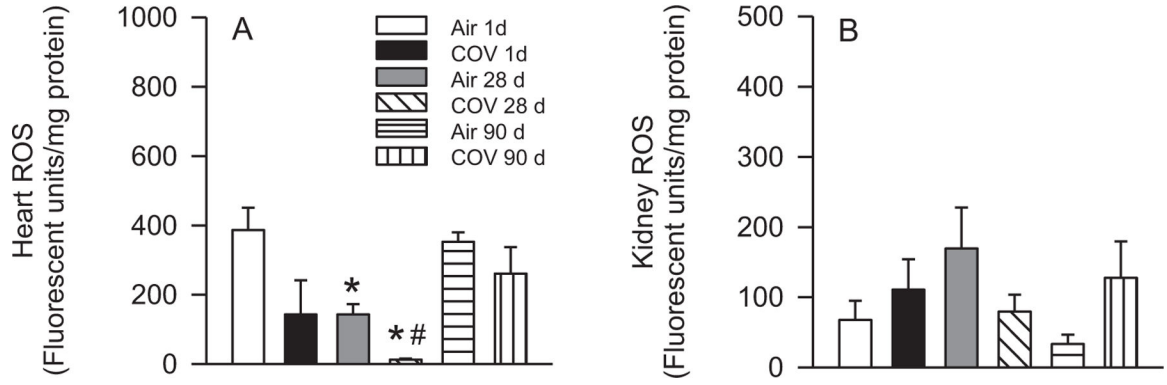

Fig. 6.

Reactive oxygen species (ROS) concentrations in the heart (A) and kidney (B) of rats 1, 28 or 90 d following a sub-chronic exposure to air or COV. In the heart, ROS concentrations in air and COV exposed animals were not significantly different 1 and 90 d after exposure to COV. However, 28 d after exposure, air control ROS levels were less than levels on 1 d (* $p < 0.05$). ROS concentration in the 28 d after COV exposure were lower than air controls (# $p < 0.05$) and lower than ROS concentrations in COV animals after 1 and 90 d of exposure. In the kidney (B), ROS concentrations were variable, but there were no significant COV exposure-related changes in ROS on any day of the study. $n = 8$ animals/group.

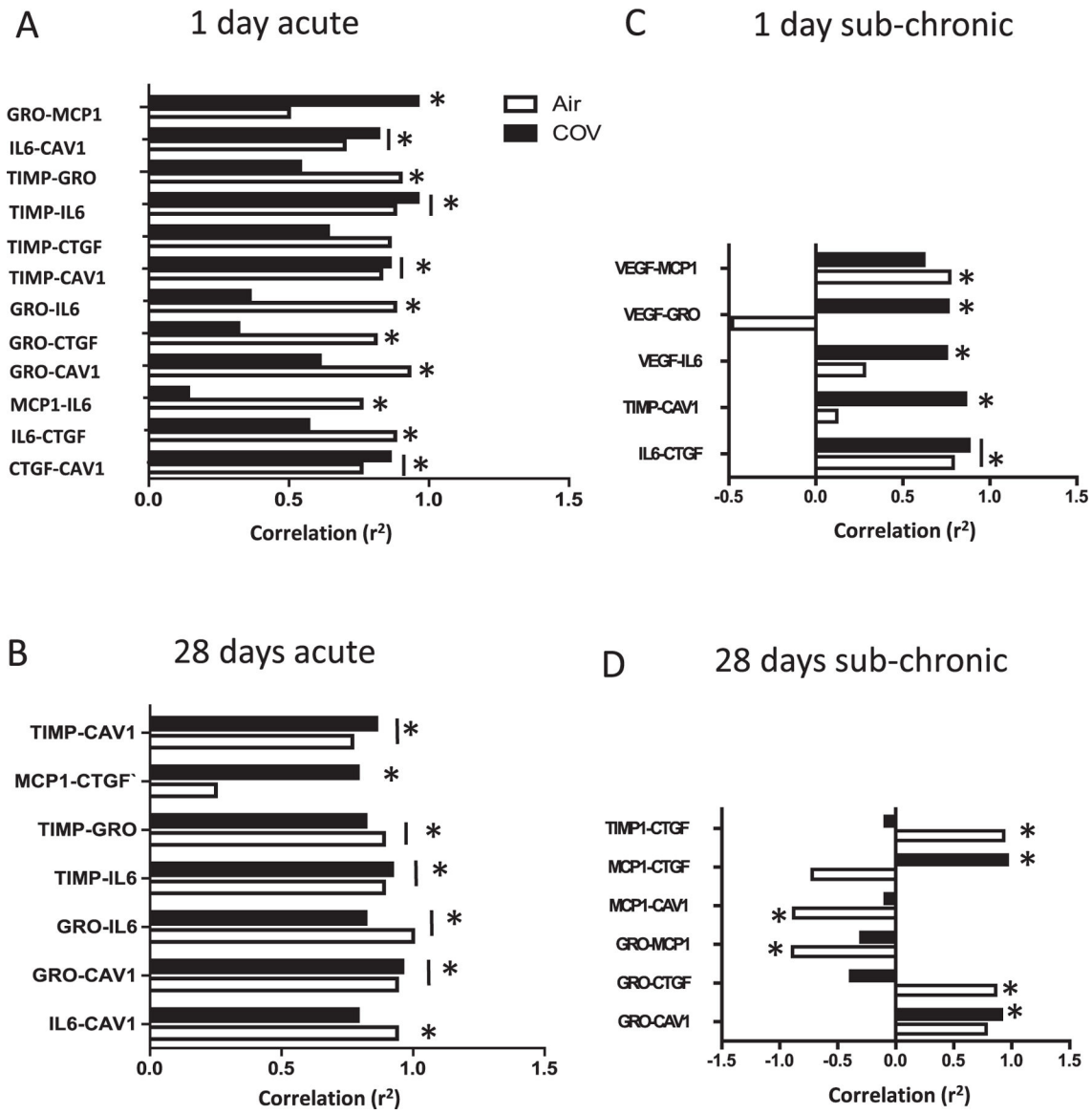

Fig. 7.

Correlations between changes in different proteins in a protein panel for factors implicated in the development of cardiovascular disease after acute exposure to COV using multivariate regression analysis. Protein pairs are listed on the left, and bars with asterisks indicate that there was a significant correlation between changes in those proteins ($p < 0.05$). If there is a bar in the graphs, it indicates that there were significant correlations in the changes in that pair of proteins in both the air- and COV-treated animals. Correlations proteins measured in tissue collected 1 d (A) and 28 d (B) following acute COV exposure, and 1d (C) and 28 d (D) following sub-chronic COV exposure are presented. $n = 6–8$ animals/group.

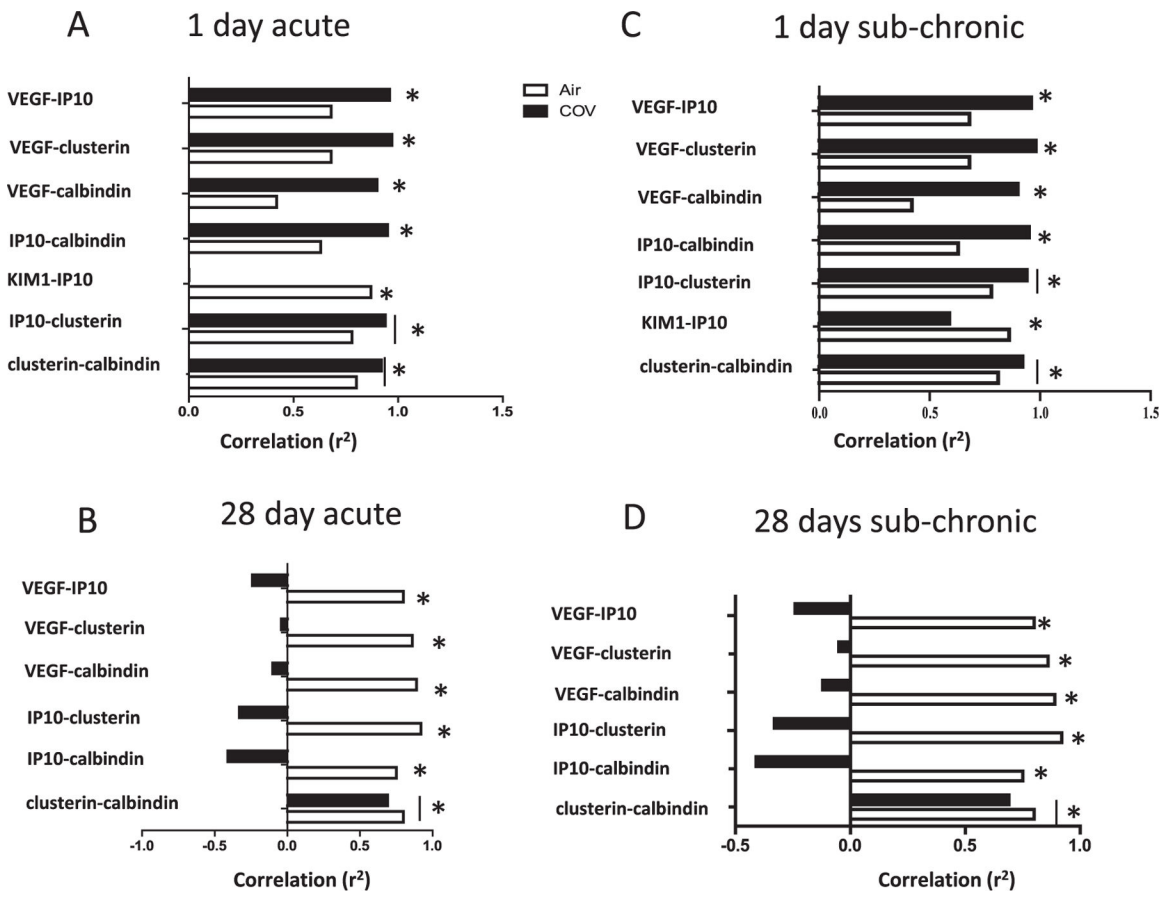

Fig. 8.

Correlations between changes in different proteins on a protein panel, measuring changes in proteins indicative of kidney injury after acute exposure to COV. Protein pairs are listed on the left, and bars with an asterisk indicate that there was a significant correlation between changes in those proteins ($p < 0.05$). If there is an asterisk over a bar in the graph, it indicates that there were significant correlations in the protein changes in both the air- and COV-treated animals. Correlations between proteins measured in tissue collected 1d (A) and 28 d (B) following acute COV exposure, and 1d (C) and 28 d (D) following sub-chronic COV exposure are presented. $n = 6–8$ animals/group.

Table 1

Fold changes (± SEM) in transcript levels in the heart 1 or 28 d following an acute inhalation exposure to air or COV.

	Air day 1	COV day 1	Air day 28	COV day 28
eNos	1.09 (0.15)	1.28 (0.20)	0.79 (0.08)	0.88 (0.12)
iNos	1.05 (0.09)	1.14 (0.13)	1.02 (0.07)	1.08 (0.10)
Il6	1.31 (0.48)	**0.52* (0.06)**	1.36 (0.45)	1.31 (0.47)
Hif1a	0.93 (0.05)	**1.06* (0.09)**	1.16 (0.09)	1.13 (0.10)
Tnfα	1.47 (0.55)	**0.87* (0.07)**	1.85 (0.18)	**0.40* (0.12)**
Il1β	0.79 (0.15)	0.78 (0.09)	1.36 (0.49)	1.78 (0.31)
Bad	0.95 (0.06)	1.02 (0.08)	1.05 (0.12)	1.01 (0.10)
Bax	1.02 (0.09)	1.22 (0.25)	1.09 (0.17)	1.14 (0.19)
Bcl2	1.00 (0.52)	0.86 (0.07)	1.02 (0.07)	0.90 (0.04)

Bold text with * designates different than air controls; $p < 0.05$.

Table 2

Fold changes in transcript levels (± SEM) in the kidney 1 or 28 days following a single exposure to air or COV

	Air day 1	COV day 1	Air day 28	COV day 28
Catalase	1.06 (0.07)	1.01 (0.10)	1.10 (0.08)	1.00 (0.11)
Sod2	1.02 (0.09)	0.71 (0.13)	1.13 (0.08)	0.97 (0.24)
Il1β	1.32 (0.38)	**0.60* (0.10)**	1.70 (0.48)	**0.37* (0.09)**
Il6	1.17 (0.22)	0.92 (0.22)	1.42 (0.39)	0.72 (0.52)
Timp	1.11 (0.19)	1.25 (0.15)	1.08 0.16	1.39 (0.24)
Tnfα	1.47 (0.54)	**0.49* (0.08**)	1.85 (0.71)	**0.37* (0.10)**
nNos	1.04 (0.11)	0.83 (0.06)	1.10 (0.19)	0.93 (0.13)
iNos	1.22 (0.27)	**0.52* (0.10)**	1.58 (0.57)	**0.40* (0.06)**
eNos	1.07 (0.14)	**0.68* (0.09)**	1.20 (0.27)	**0.67* (0.07)**
Vegf	1.25 (0.30)	**0.58* (0.09)**	1.57 (0.50)	**0.46* (0.09)**

Bold text with * designates different than air controls; $p < 0.05$.

Table 3

Fold changes in transcript levels (± Sem) in the heart 1, 28 and 90 d following a single exposure to air or COV.

	Air day 1	COV day 1	Air day 28	COV day 28	Air day 90	COV day 90
eNos	1.04 (0.39)	0.93 (0.16)	0.86 (0.12)	**0.36* (0.05)**	0.78 (0.16)	**1.38* (0.26)**
iNos	1.02 (0.27)	1.04 (0.28)	1.04 (0.10)	**0.74* (0.07)**	1.03 (0.09)	1.08 (0.11)
Il6	1.19 (0.11)	1.15 (0.35)	0.99 (0.14)	1.14 (0.28)	1.06 (0.14)	0.97 (0.24)
Timp	1.02 (0.01)	1.13 (0.21)	1.20 (0.21)	1.08 (0.22)	1.07 (0.15)	1.12 (0.18)
Hif1a	1.11 (0.20)	1.13 (0.21)	1.08 (0.13)	0.79 (0.09)	1.02 (0.06)	1.19 (0.12)
Tnfα	1.62 (0.23)	0.81 (0.39)	1.59 (0.20)	1.56 (0.36)	1.24 (0.16)	0.71 (0.19)
Il1β	1.33 (0.19)	1.31 (0.13)	0.95 (0.33)	0.72 (0.42)	1.29 (0.14)	1.29 (0.15)
Bad	1.05 (0.14)	1.02 (0.18)	1.10 (0.13)	0.87 (0.09)	1.01 (0.05)	1.07 (0.12)
Bax	1.11 (0.20)	1.11 (0.11)	0.98 (0.12)	0.93 (0.11)	1.02 (0.08)	1.24 (0.14)
Bax	1.03 (0.11)	0.97 (0.21)	1.27 (0.22)	0.87 (0.10)	1.02 (0.09)	1.29 (0.19)

Bold text with * designates different than air controls; $p < 0.05$.

Table 4

Fold changes in transcript levels in the kidney 1, 28 and 90 days following a single exposure to air or oil vapors.

	Air day 1	COV day 1	Air day 28	COV day 28	Air day 90	COV day 90
Catalase	1.16 (0.77)	0.81 (0.24)	1.42 (0.51)	**3.66*^ (1.10)**	0.82 (0.22)	1.62 (0.60)
Il1-β	1.51 (0.20)	**1.09* (0.05)**	1.41 (0.21)	1.26 (0.07)	1.18 (0.10)	0.31 (0.12)
Il6	1.55 (0.21)	**3.35* (1.42)**	1.75 (0.96)	1.34 (0.24)	1.58 (0.37)	1.23 (0.53)
Timp	1.22 (0.12)	2.32 (0.81)	1.34 (0.14)	1.49 (0.42)	1.29 (0.13)	**2.04* (0.93)**
Tnf-α	1.53 (0.27)	0.60 (0.52)	1.49 (0.20)	1.93 (0.67)	1.40 (0.13)	**8.01* (1.58)**
nNos	1.14 (0.09)	0.81 (0.41)	1.40 (0.90)	1.46 (1.07)	1.73 (0.51)	0.70 (0.27)
iNos	1.63 (0.23)	0.56 (0.19)	1.44 (0.81)	1.26 (0.53)	1.57 (0.13)	1.37 (0.33)
eNos	1.19 0.08	0.23 (0.16)	1.99 (0.70)	1.28 (0.12)	1.36 (0.12)	0.48 (0.32)
Sod2	1.93 (0.19)	1.31 (0.34)	1.48 (0.64)	1.78 (0.21)	1.33 (0.32)	**3.48^ (0.88)**
Vegf	1.13 (0.06)	0.24 (0.12)	0.76 (0.46)	1.22 (0.10)	1.34 (0.11)	0.45 (0.24)

Bold text and * designates different than same day air controls; $p < 0.05$. Bold text and ^ indicates different than other days for that treatment; $p < 0.05$ controls.

Table 5

Protein concentrations (pg/μg total protein) in the heart 1 and 28 d after an acute exposure to COV.

	Air day 1	COV day 1	Air day 28	COV day 28
CAV1	41.47 (8.39)	57.95 (13.96)	56.32 (16.09)	36.09 (5.09
CTGF	6.23 (1.92)	11.39 (2.76)	9.11 (3.20)	9.51 (1.43)
IL6	3.21 (0.59)	**5.63* (0.82)**	4.43 (0.76)	5.69 (1.07)
MCP1	0.22 (0.45)	0.31 (0.09)	0.28 (0.07)	0.19 (0.02)
GRO/KC/CINC1	1.27 (0.22)	1.75 (0.28)	1.66 (0.53)	1.52 (0.20)
TIMP	5.01 (0.82)	7.05 (1.38)	7.44 (1.82)	5.81 (0.59)

Data are presented as the mean (sem). Bold with * designates significantly different than same day air control ($p < 0.05$). Proteins were measured using a multiplex assay for markers of vascular disease.

Table 6

Protein concentrations (pg/μg total protein) in the kidney 1 and 28 d after an

	Air day 1	COV day 1	Air day 28	COV day 28
calbindin	19.78 (4.26)	32.59 (11.50)	23.69 (4.00)	26.57 (3.32)
clusterin	103.24 (45.72)	406.91 (236.82)	275.56 (81.86)	477.78 (119.90)
IP10	0.03 (0.01)	0.07 (0.03)	0.06 (0.02)	0.07 (0.01)
KIM-1	0.02 (0.003)	0.03 (0.01)	0.04 (0.02)	0.07 (0.02)
TIMP	0.08 (0.14)	1.07 (0.34)	1.45 (0.33)	1.69 (0.37)
VEGF	0.04 (0.02)	0.12 (0.05)	0.09 (0.03)	0.08 (0.02)

Data are presented as the mean (sem). Proteins were measured using a multiplex assay for kidney disease.

Table 7

Protein concentrations (pg/μg total protein) in the heart 1 and 28 d after a sub-chronic exposure to air or COV.

	Air day 1	COV day 1	Air day 28	COV day 28
CAV1	44.52 (3.47)	41.78 (5.23)	37.74 (6.84)	28.94 (2.60)
CTGF	7.54 (1.96)	8.30 (1.99)	6.52 (2.42)	8.41 (1.92)
IL6	3.60 (0.39)	4.33 (0.49)	4.31 (0.45)	4.46 (0.48)
MCP1	0.22 (0.03)	0.23 (0.03)	0.22 (0.05)	0.16 (0.02)
GRO/KC/CINC1	1.39 (0.10)	1.31 (0.11)	1.03 (0.14)	1.22 (0.09)
TIMP	5.36 (0.28)	5.28 (0.33)	5.28 (0.64)	4.73 (0.06)

Data are presented as the mean (sem). Proteins were measured using a multiplex assay for vascular disease.

Table 8

Protein concentrations (pg/μg total protein) in the kidney 1 and 28 d after a sub-chronic exposure to air or COV.

	Air day 1	COV day 1	Air day 28	COV day 28
calbindin	24.17 (4.41)	34.81 (11.23)	20.90 (3.47)	29.91 (7.32)
clusterin	155.92 (57.65)	465.05 (229.59)	228.79 (84.72)	431.29 (129.08)
IP10	0.05 (0.02)	0.08 (0.03)	0.05 (0.01)	0.01 (0.05)
KIM-1	0.02 (0.004)	0.02 (0.01)	0.09 (0.05)	0.10 (0.04)
TIMP	1.74 (0.30)	0.90 (0.38)	1.26 (0.25)	1.73 (0.29)
VEGF	0.06 (0.02)	0.12 (0.05)	0.08 (0.03)	0.07 (0.02)

Data presented are the mean (sem). Proteins were measured using a multiplex plate for kidney disease.