Vehicular Emissions Induce Vascular MMP-9 Expression and Activity Associated With Endothelin-1–Mediated Pathways

Amie K. Lund, JoAnn Lucero, Selitá Lucas, Michael C. Madden, Jacob D. McDonald, Jean-Clare Seagrave, Travis L. Knuckles, Matthew J. Campen

Objective—Mechanisms of air pollution–induced exacerbation of cardiovascular disease are currently unknown, thus we examined the roles of vascular endothelin-1 (ET-1) and reactive oxygen species (ROS) in regulating mediators of vascular remodeling, namely matrix metalloproteinases (MMPs), after exposure to vehicle engine emissions.

Methods and Results—ApoE−/− mice were exposed by inhalation to filtered air or gasoline engine exhaust (GEE, 1:12 dilution) 6 hours per day for 1 or 7 days. Concurrently, mice were treated with either ET₀ receptor antagonist BQ-123 (100 ng/kg/d) via osmotic minipumps, Tempol (≈41 mg/kg/d, orally), or vehicle. GEE-exposure increased vascular MMP-2 and -9, endothelin-1 (ET-1), tissue inhibitor of metalloproteinases (TIMP)-2 mRNA and ROS levels. Aortic MMP protein and plasma MMP-9 were similarly upregulated. GEE-mediated increases in vascular ROS were attenuated by Tempol-treatment, as were MMP-2 and TIMP-2; whereas BQ-123 ameliorated GEE-induced vascular expression of MMP-9, MMP-2, ROS, and ET-1. In a parallel study, diesel exhaust exposure in volunteer human subjects induced significant increases in plasma ET-1 and MMP-9 expression and activity.

Conclusions—These findings demonstrate that acute exposure to vehicular source air pollutants results in upregulation of circulating and vascular factors associated with progression of atherosclerosis, mediated in part through activation of ET₀–ET₁ receptor pathways. (Arterioscler Thromb Vasc Biol. 2009;29:511-517.)

Key Words: atherosclerosis ■ endothelin-1 ■ matrix metalloproteinase ■ reactive oxygen species ■ air pollution

Atherosclerosis, a disease of the vasculature characterized by endothelial dysfunction and arterial plaque formation, has a multifactorial etiology that includes genetic, behavioral, and environmental influences. Numerous epidemiological studies indicate a positive correlation between exposure to common environmental air pollutants and increased risk of cardiovascular morbidity and mortality both chronically and acutely.1-3 Furthermore, a clear relationship between exposure to air pollution of vehicular origin and cardiovascular events has been established.2-4 Experimental findings have defined a role for components of environmental air pollution in the progression of atherosclerosis including: impaired vascular endothelial function,5 increase in plaque cell turnover and lipids in aortic lesions,6 and altered vasomotor tone and induced vascular inflammation.7 While these studies describe a relationship between exposure to environmental air pollutants and factors associated with atherosclerosis, the underlying mechanisms have not been fully elucidated.

A hallmark of atherosclerosis is inappropriate vascular remodeling, mediated by extracellular matrix (ECM) degradation. The matrix metalloproteinase (MMP) family of endopeptidases represents the primary mediators of vascular ECM degradation.8 Whereas MMP activity is essential for vascular homeostasis, dysregulation of MMPs also underlies pathobiological alterations in the vasculature,9 including progression of atherosclerosis and destabilization of advanced plaques.10 Recent reports have shown that increasingly diverse stimuli, including reactive oxygen species (ROS), can upregulate most MMPs in the vasculature.11,12

Other vascular factors, including endothelin-1 (ET-1), also mediate progression of atherosclerosis. ET-1, a constitutively secreted peptide, acts via the ET₁ and ET₂ receptors in the vasculature, where ET₁ predominantly mediates vasoconstriction and mitogenic pathways and ET₂ mediates vasodilation. ET-1 is significantly upregulated in atherosclerotic vessels13 and has been shown to increase MMP activity in cardiovascular pathologies.14,15

We have previously reported that subchronic inhalational exposure to the ubiquitous environmental air pollutant, gasoline engine emissions (GEE), results in increased expression of vascular factors associated with the progression of athero-
sclerosis, namely vascular ROS, ET-1, and MMPs in ApoE−/− mice.16 However, it has not been determined (1) what mediates expression of MMPs, or (2) whether these pathways are activated during acute exposures, in a manner that is consistent with epidemiological findings. Thus, we tested the hypothesis that acute exposure to GEE results in an ET-1–ETA–mediated increase in vascular ROS and MMP expression and activity, in atherosclerotic ApoE−/− mice.

Materials and Methods

An expanded Methods section is available in the online Data Supplement available at http://atvb.ahajournals.org.

Animals and Inhalation Exposure Protocol

Ten-week-old male ApoE−/− mice (Taconic, Oxnard, Calif) were placed on a high-fat diet (TD88137 Custom Research Diet, Harlan Teklad; 21.2% fat by weight, 1.5 g/kg cholesterol) beginning 30 days before exposure. ApoE−/− mice were inhalingally exposed to GEE (60 μg/m³ particulate matter whole exhaust) or filtered-air (controls) for 6 hours per day for a period of 1 or 7 days (n = 18 for each group/time point). The exposure concentration (supplemental Table I, available at http://atvb.ahajournals.org) was chosen to maximize the biological pathways relevant to exposures, without initiating lung injury or inflammation.16 All procedures were approved by the Lovelace Respiratory Research Institute’s Animal Care and Use Committee and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Gasoline emissions were generated as previously described.16 The engines were fueled with conventional unleaded, nonoxygencated, nonreforcluded gasol (ChevronPhillips Specialty Fuels Division), and emissions diluted approximately 1:12 with filtered air. Components of GEE were characterized and are summarized in supplemental Table I.

Tempol Dosing Groups

ApoE−/− mice assigned to filtered air or GEE, were randomly assigned to receive either 4-Hydroxy-TEMPO (Tempol, Sigma), 1 mmol/L (approximately 41 mg/kg/d, sterile-filtered) in their drinking water, or vehicle (ddH₂O), beginning 24 hours before and throughout exposure. Delivered dose was calculated from daily intake.

BQ-123 Dosing Groups

ApoE−/− mice exposed to either filtered air or GEE, were randomly assigned to receive either BQ-123 (100 ng/kg/d, Sigma) or vehicle (sterile saline) via osmotic minipumps (model #1007D Alzet). Animals were monitored daily for health status throughout the study.

Real-Time RT-PCR

Total RNA was isolated from the aorta (one-half, midsagittal cut from arch to common iliac bifurcation), n = 6 per group, using RNeasy Fibrous Tissue Mini Kit (Qiagen). cDNA was synthesized, and real-time PCR performed as previously described.17 Control reactions (no RT or RNA) were run to verify the absence of contaminated DNA and primer-dimerization. ΔCₚ was calculated as previously described.16 Results expressed as normalized gene expression as percentage of controls (18S).

TBARS Assay

Aortic thiobarbituric acid reactive substances (TBARS) levels were assessed using a TBARS assay kit (OXItk, ZeptoMetrix Corp Buffalo) measuring TBARS levels in whole uncentrifuged aorta homogenates per kit instructions, as previously described.16

Western Blot Analysis

Aortas were homogenized and protein isolated from cytosolic fractions. Aorta and plasma were concentrated on a Millipore column (YM-10, Fisher Scientific) and quantified using the Bradford assay (Biorad). 5 μg of protein was loaded (n = 3 to 5 per group) for SDS-PAGE electrophoresis under reducing conditions. Membranes were blocked overnight at 4°C in 5% blotto, incubated in either rabbit polyclonal antimouse MMP-9 (1:5000 dilution; Abcam), or anti-mouse TIMP-2 (1:1000, Abcam), and β-actin (1:2000, Abcam) for 1 hour at RT, and then antirabbit-HRP was used for 1 hour at RT. Bands were visualized with chemiluminescence and densitometry performed utilizing Image J software (NIH).

In Situ Zymography

Aorta sections (6 μm) were incubated with 45 μL of 10 μg/mL dye quenched (DQ)-gelatin (EnzChek, Molecular Probes, Invitrogen) and 1 μg/mL DAPI (Invitrogen) in 1% UltraPure LMP agarose (Invitrogen) cover-slipped, chilled for 5 minutes at 4°C, and then incubated for 6 hours in a dark humid chamber at 37°C. Some slides were preincubated with a gelatinase inhibitor (MMP -2, -9 inhibitor IV, Chemicon, Millipore).

Human Plasma MMP-9, ET-1, and NOx Assays

In conjunction with the Human Studies Division at the EPA, healthy subjects (n = 10; 18 to 40 years old, 4 male/6 female) were exposed to a target concentration of 100 μg/m³ diesel (DE) whole exhaust or HEPA and charcoal filtered “clean” air (controls) for 2 hours, on separate occasions. Subjects had 4 cycles of 15 minutes rest and 15 minutes exercise on a stationary bicycle at a target ventilation rate of 25 L oxygen/min/m² body surface area. DE was generated from a Cummins engine (5.9 L, 205 hp) operating at near idle conditions using a certified commercial #2 fuel purchased from ChevronPhillips. Components of DE were measured and are summarized in supplemental Table I. Blood was collected preexposure and 30 minutes and 24 hours postexposure, and plasma was stored at −80°C until analysis. MMP-9 ELISA (Biorad #RPN2614, Amersham), and MMP-9 activity ELISA (#RPN263A, Amersham), were performed on pre- and postexposure plasma samples (1:10 dilution in assay buffer) per manufacturer instructions. ET-1 levels were quantified by ELISA (#QET00B, R&D Systems) per manufacturer instructions. Plasma NOx levels were measured using a Nitrate/Nitrite colorimetric kit (#79001, Cayman Chemical), per manufacturer instructions. One sample was eliminated because of lack of preexposure sample. All procedures were approved by the Lovelace Respiratory Research Institutional Review Board under exemption #4 (protocol #07-001), and all subjects provided informed consent.

Statistical Analysis

Data expressed as mean±SEM. One-way analysis of variance (ANOVA) with a post-hoc Holm-Sidak test was used for analysis of multiple groups; human samples were analyzed with repeated measures ANOVA. A P<0.05 was considered statistically significant.

Results

Vascular MMP-2 and -9 Is Activated by Acute GEE-Exposure in ApoE−/− Mice

In situ zymography was used to quantify acute GEE-mediated effects in the aorta. Aorta gelatinase activity was increased at day 1 of exposure (Figure 1A), with further amplification at day 7 (Figure 1C), compared to controls (Figure 1B and Figure 1D, respectively). Localization of MMP-2/9 activity appears to be predominantly in the endothelial layer of the aorta at day 1 (Figure 1A, arrow), whereas by day 7 of exposure activity is found throughout the vasculature (Figure 1C, arrows). A MMP-2/9 inhibitor confirms fluorescence is
specific to gelatinase activity (Figure 1E). Densitometric quantification of relative fluorescence from all samples analyzed is shown in Figure 1F.

**Acute GEE- Exposure Augments Vascular MMP Protein Expression in ApoE\(^{-/-}\) Mice**

Because MMP expression is regulated through multiple mechanisms, we assessed aorta MMP-2, -9, and plasma MMP-9 protein levels from each study group by Western blot (Figure 2). In agreement with our transcriptional data, 7-day GEE-exposure resulted in a significant increase in aorta protein expression of MMP-9, MMP-2, and TIMP-2 (Figure 2A and 2B) and plasma MMP-9 (Figure 2C and 2D) in ApoE\(^{-/-}\) mice.

**Combustion Source Engine Emissions Increase Plasma ET-, MMP-9, and NOx in Human Exposures**

Plasma MMP-9 levels have been identified as a novel predictor of cardiovascular mortality in patients with CVD. Because plasma MMP-9 expression and activity was found significantly increased in GEE-exposed ApoE\(^{-/-}\) mice, we tested whether plasma MMP-9 may serve as a useful translational biomarker for human models of exposure, as well. Plasma samples collected from humans exposed to diesel engine exhaust (DE), for only 2 hours, showed a significant elevation in MMP-9 concentration (Figure 3A) and activity (Figure 3B). Intrasubject variation was apparent in baseline values of MMP-9, however the plasma MMP-9 concentration and activity was uniformly elevated post-DE exposure as compared to changes induced by filtered air exposure. Plasma ET-1 (Figure 3C) and NOx (Figure 3D) were also found to be significantly upregulated as a result of DE (supplemental Table II).

**BQ-123-Treatment Normalizes Vascular ROS, ET-1, and MMP-9, Whereas Tempol Treatment Attenuates Only Vascular ROS**

It has been previously reported that exposure to vehicular emissions induces vascular ROS, which was confirmed in
the present acute exposures (supplemental Figure IA and Figure IC), as well as increases vascular16 and circulating18 levels of ET-1. To elucidate the role of ROS versus ET-1 in mediating expression of vascular MMP-9, ApoE<sup>−/−</sup> mice were treated orally with either 1 mmol/L of Tempol (avg. 41 mg/kg/d) or vehicle, beginning 24 hours before exposure; in a separate study ApoE<sup>−/−</sup> mice were treated with either filtered air (controls), Tempol (10 mmol/L in drinking water), GEE (60 μg/m3), or Tempol+GEE (n=6 each). Expression of aortic TBARS levels (A), and ET-1 (B) and MMP-9 (C) mRNA in ApoE<sup>−/−</sup> mice exposed for 6 hours per day for 1 or 7 days to either filtered air (controls), tempol (10 mmol/L in drinking water), GEE (60 μg/m3), or Tempol+GEE (n=6 each). Expression of aortic TBARS levels (D), and ET-1 (E) and MMP-9 (F) mRNA in ApoE<sup>−/−</sup> mice exposed for 6 hours per day for 1 or 7 days to either filtered air (controls), BQ-123 (100 ng/kg/d, osmotic minipump), GEE (60 μg/m3), or BQ-123+GEE (n=6 each). "P<0.050 compared to controls, *P<0.050 compared to GEE-exposed.

Figure 4. Lipid peroxidation and transcriptional changes in aorta ET-1 and MMP-9, from ApoE<sup>−/−</sup> mice exposed to GEE and cotreated with either Tempol or BQ-123. Expression of aortic TBARS levels (A), and ET-1 (B) and MMP-9 (C) mRNA in ApoE<sup>−/−</sup> mice exposed for 6 hours per day for 1 or 7 days to either filtered air (controls), tempol (10 mmol/L in drinking water), GEE (60 μg/m3), or Tempol+GEE (n=6 each). Expression of aortic TBARS levels (D), and ET-1 (E) and MMP-9 (F) mRNA in ApoE<sup>−/−</sup> mice exposed for 6 hours per day for 1 or 7 days to either filtered air (controls), BQ-123 (100 ng/kg/d, osmotic minipump), GEE (60 μg/m3), or BQ-123+GEE (n=6 each). "P<0.050 compared to controls, *P<0.050 compared to GEE-exposed.

Discussion

The present study demonstrates that the ET<sub>A</sub> receptor pathway plays a central role in GEE-induced effects on the vasculature, including expression and activity of MMP-2 and -9, ET-1, and oxidative stress. In controlled human exposures to DE, we report a pattern of plasma MMP-9 expression and activity induction that is remarkably consistent with that observed in the GEE mouse model exposures. Although typically considered to be highly different exhausts in terms of chemical compositions, the biological findings of the present study suggest that the similar constituents in DE and GEE (eg, CO, NO, NO<sub>2</sub>, hydrocarbons) may be critical to vascular toxicity. It is important to note that the CO levels in these exposures did not result in apparent hypoxic conditions, as carboxyhemoglobin levels were not elevated in the human DE exposures (unpublished data, 2008 M.C.M.) and vascular endothelial growth factor (VEGF), which contains a hypoxia response element (HRE) in its promoter, was not altered in GEE exposures (supplemental Figure IV). Considering the relationship between MMP expression and vascular event outcomes,19,20 these results represent a very promising link to recent epidemiological findings that identify vehicular emissions as risk factors for acute cardiovascular events.4

Peters et al4 reported an increased risk of acute myocardial infarction (AMI) within 4 to 24 hours of traffic exposure. Other studies have also identified acute air pollution exposure, related to vehicular sources, as a significant risk factor for clinical events.21,22 It is plausible to suspect that activation of plaque-degrading enzymes, such as MMP-2 and -9, may play an important role in mediating air pollution-induced cardiovascular events such as AMI. This premise is further supported by recent animal studies that show condi-
ional overexpression of MMP-9 results in substantive enhancement of atherosclerotic plaque instability. The present study did not focus on plaque disruption per se, the biological dynamics of vascular MMP-2/9 regulation and activity, in response to exposure to vehicular emissions, provides a plausible link to epidemiological findings.

The results of the present study are temporally congruous with the epidemiological findings of acute induction of cardiovascular events associated with air pollution exposures, in that we demonstrate activation of vascular MMP-2 and -9 within 24 hours of exposure to GEE. Subsequently, transcription of MMP-2 and -9 is upregulated over the following week, leading to de novo synthesis of additional proteins and prolonged maintenance of vascular MMP response. The observed increase in activation of gelatinases may be attributable to a decreased interaction between these MMPs and their respective inhibitor (TIMP). The balance between MMPs and TIMPs is crucial in regulating vascular ECM remodeling; MMP-9 is preferentially inhibited by TIMP-1, whereas MMP-2 is preferentially inhibited by TIMP-2. Interestingly, we observe no change in expression of vascular TIMP-1 mRNA in ApoE−/− mice exposed to GEE; however, TIMP-2 mRNA is significantly elevated by day 1 of exposure to GEE (supplemental Figure II). The implications of these results are 2-fold: (1) the increased gelatinase activity observed in GEE-exposed ApoE−/− mice aortas are likely predominantly MMP-9 driven (because of the imbalance of expression of vascular MMP-9 to TIMP-1, and thus a decrease in MMP-9/TIMP-1 binding), and (2) TIMP-1 and TIMP-2 are regulated differently in the vasculature in response to both GEE-exposure and MMP activation.

Vascular ROS are significantly upregulated by GEE, as evidenced by increased aortic TBARs levels and DHE fluorescence (supplemental Figure IA and IC). Importantly, lung ROS levels are not increased as a result of GEE exposure (supplemental Figure ID), which is consistent with previous studies, thereby indicating that the observed vascular effects of GEE may be independent of pulmonary oxidative stress pathways. Vascular NAD(P)H oxidase activation appears to be one source of GEE-induced ROS (supplemental Figure V), although other sources (e.g., xanthine oxidase, uncoupled eNOS, mitochondria) cannot be ruled out. We show that Tempol-treatment ameliorates vascular oxidative stress, and attenuates acute increases in vascular MMP-2 and TIMP-2 in GEE-exposed ApoE−/− mice, although MMP-9 and ET-1 levels remain elevated above control. The observed partial antagonism of MMP-9, by Tempol, suggests regulation through additional signaling pathways, which based on our findings includes those mediated through the ETα receptor. Interestingly, we observe an increase in vascular TBARs in the BQ-123 study by day 1 of exposure (Figure 4D), not seen in the Tempol study (Figure 4A). Several factors may account for this observation including (but not limited to): variability in vascular pathology among animal groups (note the difference in day1 control groups between Figure 4A and 4D), surgical procedures required for osmotic minipumps in the BQ-123 study, differences in compensatory antioxidant pathway responses, as well as overall assay limitations.

A key feature of atherosclerosis is the imbalance of vasoactive factors produced by the endothelium, including ET-1, which may aggravate atherosclerosis through its ability to stimulate ROS via NADPH oxidases and MMPs. Although studies have confirmed the association between air pollution and elevated plasma ET-1 levels in humans, the mechanisms which mediate ET-1 expression, the respective contributions of environmental air pollutants, and the ultimate cardiovascular sequelae have not been elucidated. Our findings suggest that ET-1 may mediate expression of MMP-9 in this model, as BQ-123 treatment normalizes expression and activity of MMP-9. One plausible explanation for ET-1 regulation of MMP-9 may be through mitogen activated protein kinase (MAPK) signaling pathways, via the ETα
receptor, because MMP-9 is known to contain AP-1 sites in its gene promoter region.27 Nevertheless, we cannot discount the potential role of other factors whose expression is mediated or coregulated through ET-1/ET receptor pathways, which are also known to regulate expression of MMPs (eg, osteopontin, angiogenin II), nor can we exclude signaling pathways downstream of the ET \(_A\) receptor. It is possible that enhanced endothelial ET \(_B\) receptor activation after selective ET \(_A\) receptor blockade may lead to increased ET \(_B\)-mediated signaling; however, one might expect increased levels of oxidative stress attributable to increased nitric oxide and subsequent peroxynitrite generation, in this scenario. Further studies are required to definitively identify responsible signaling pathways.

Elevated plasma MMP-9 has been identified as a novel predictor of cardiovascular mortality.17 Our results show an abrupt increase in MMP-9, concomitant with elevated ET-1 and NOx, in plasma from humans exposed to DE, as well as plasma from ApoE \(^{-/-}\) mice exposed to GEE. The ubiquitous nature of both GEE and DE as components of air pollution has been well defined. While there are differences in the chemical fingerprint of each emission, their chemical species including CO, NOx, and volatile/semi volatile organic compounds are similar.16,28 Perhaps more relevant to our findings is that epidemiological reports of cardiovascular events associated with exposure to traffic-related air pollutants are assessments of overall traffic levels (not just one type of engine emission). Thus, it is reasonable to propose that the public health risk from vehicular emissions is attributable to the cumulative impact of these common chemical species present in the ambient air.

In conclusion, our findings show a significant increase in factors that are involved in mediating vascular remodeling and the progression of atherosclerosis, namely ET-1, MMP-2 and -9, and ROS, in response to acute exposure to the environmental air pollutant, GEE. Our data suggests that ET-1 signaling is regulating expression or activity of vascular MMP-9, as well as plays a role in ROS generation, in response to GEE exposure. Furthermore, the similarity in vascular response to engine emissions in both mice and humans lends credence to the translatability of this model, as well as identifies potential pathologically-relevant biomarkers that can assist in future mechanistic and risk assessment research. Considering the impact of cardiovascular disease on health care today, it is imperative to gain insight into pollution-induced alterations in pathways which mediate atherosclerotic plaque development and destabilization, leading to events such as AMI and stroke.

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**Disclosures**

None.

**References**


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Supplemental Materials

Expanded Materials and Methods

Animals and Inhalation Exposure Protocol. Ten-week-old male ApoE\textsuperscript{−/−} mice (Taconic, Oxnard, CA) were placed on a high fat diet (TD88137 Custom Research Diet, Harlan Teklad, Madison, WI; 21.2\% fat content by weight, 1.5g/kg cholesterol content) beginning 30 days prior to initiation of exposure protocol. ApoE\textsuperscript{−/−} mice were inhalationally, whole-body exposed to whole gasoline engine exhaust or filtered-air (controls) for 6 h/d for a period of 1 or 7 days. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-approved rodent housing facility (inhalational-exposure chambers) for the entirety of the study, which maintained constant temperature (20–24°C) and humidity (30–60\% relative humidity), and provided with high fat / high cholesterol mouse chow and water \textit{ad libitum} throughout the study period. During the study period, all animals were exposed concurrently to either filtered air (n=18 for each time point) or 60 µg/m\textsuperscript{3} particulate matter whole exhaust (n=18 for each time point). All procedures were approved by the Lovelace Respiratory Research Institute’s Animal Care and Use Committee and conform to the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Upon completion of the designated exposure period, animals were sacrificed within 18 hours after their last exposure.

Gasoline emissions were generated as previously described\textsuperscript{1}. The engines were fueled with conventional unleaded, non-oxygenated, non-reformulated gasoline blended to simulate a national average composition (ChevronPhillips Specialty Fuels Division, The Woodlands, TX), and exhaust was diluted approximately 1:12 with filtered air. On each day of exposure particle mass concentration was measured gravimetrically as previously described\textsuperscript{1}. As expected, the
particle number and size changed throughout the duty cycle. While the measurements were not traced as a function of point in the duty cycle, in general it was observed that larger particle size and higher concentration were associated with the higher workloads. The particle size ranged from 5.5–150 nm, with the majority of the particles between 5–20 nm. The mass median diameter was approximately 150 nm. The median diameter ranged from ~10 nm at the higher dilution levels to 15–20 nm at the lowest dilution\(^2\). Components of GEE were measured and are summarized in Table I.

**Tempol dosing groups.** ApoE\(^{-/-}\) mice assigned to either filtered air or 60 µg/m\(^3\) particulate matter whole exhaust, half of the animals from each group were randomly assigned to either 4-Hydroxy-TEMPO (Tempol, Sigma Aldrich, St. Louis, MO) or vehicle treatment groups. The treatment group received 1 mmol/L of Tempol (approximately 41 mg/kg/day, sterile-filtered) in their drinking water, beginning 24 hours before the 1 or 7 day exposure, while the control group received sterile-filtered ddH\(_2\)O water. Tempol doses were chosen based on previous experiments, both in our laboratory and others\(^3\) which show significant attenuation of vascular ROS levels, without any overt toxicological effects. Daily intake was assessed using low-drip water bottles, and this amount was used to calculate delivered dose. All mice were monitored daily to ensure health status.

**BQ-123 dosing groups.** ApoE\(^{-/-}\) mice assigned to either filtered air or 60 µg/m\(^3\) particulate matter whole exhaust, half of the animals from each group were randomly assigned to receive either BQ-123 (100 ng/kg/day, Sigma) or vehicle (sterile saline) via osmotic minipumps (model #1007D Alzet, Cupertino, CA) at a flow rate of 0.5 µl/day for 7 days. The pumps were primed by placing the filled pumps in 37°C sterile saline overnight prior to implantation. The BQ-123 dose was chosen based on previous experiments which show 100 ng/kg/day was successful at
attenuating ET-1-mediated cardiovascular ROS and NAD(P)H activity, as well as results in no adverse health effects and does not alter blood pressure in C57Bl6 mice. Mice were anesthetized (isoflurane by nose-cone, 5% induction, 2% maintenance), mini-pumps were implanted subcutaneously, incisions were closed, and mice were allowed to recover for 24 hrs before exposures. Mice were monitored throughout the study and no differences were noted in weight, eating, drinking, or activity compared to control animals.

**Plasma and Tissue Collection.** ApoE−/− mice were anesthetized with Euthasol (390 mg pentobarbital sodium, 50 mg phenytoin sodium/ ml; diluted 1:10 and administered at a dose 0.1 ml per 30 g mouse) and euthanized by exsanguination. The aorta tissue was dissected, weighed, split with a midsagittal cut, and frozen in liquid nitrogen. Tissue was stored at -80 ºC until assayed. N= 6 aortas from each exposure group for real time RT-PCR analysis and TBARs analysis; while control (n=3), and exposed (n=3) 60 µg/m³PM whole exhaust aortas were embedded in Tissue Tek® O.C.T. (VWR Scientific, West Chester, PA) and frozen on dry-ice for *in situ* zymography and dihydroethidium (superoxide) analysis.

**Superoxide analysis in aortas.** To assess levels and localization of vascular O₂•−, frozen aortic sections (6 µm thick) from each group were treated with dihydroethidium (DHE, Molecular Probes, Eugene, OR) using a previously published protocol, adapted for aorta tissue sections. Briefly, frozen aorta sections were incubated with 50 µl dihydroethidium dissolved in 0.1 mol/L phosphate buffered saline (PBS) containing 20% dimethyl sulfoxide to a final concentration of 10 µmol/L, cover-slipped, and incubated at 37 ºC for 30 min in a dark, humidified chamber. Tissue sections were viewed using excitation at 510–550 nm and emission at >580 nm to visualized ethidium fluorescence, and digital images were acquired (Everest Digitals Imaging Microscopy System). O₂•− production was analyzed using fluorescent densitometry and
quantified using Image J software (NIH); the percentage of $O_2^-$ production was quantified as the ratio of fluorescent area to total aortic tissue area. A minimum of 4-5 tissue sections (n=3 per group), with 3 sites from each section, were used for DHE quantification. $O_2^-$ signal specificity was confirmed by pre-incubating designated sections with Tempol (SOD mimetic, 0.3 mol/L) for 30 minutes at 37 ºC.

**Real time RT-PCR.** Total RNA was isolated from the aorta (one-half, midsagittal cut), n=6 per group, using RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA in a 60-µl final reaction volume containing 250 ng of sample RNA, 12.5 nM of 18S RT primer, 0.005 µg oligo dT, 0.0004 U RNAsin, 0.006 U M-MLV RT enzyme, 25 mM dNTP, 12 µl 5× RT buffer (pH 8.3), and sterile water to 60 µl volume. The mixture was heated at 42°C for 1 h and then cooled to 4°C. Real-time PCR was performed with gene-specific primers in the ABI 7900 (Applied Biosystems, Valencia, CA). Specific primers (500 nM concentration) used for each PCR reaction are listed in Table II. Control reactions without reverse transcriptase and those without RNA were run to verify the absence of contaminated DNA and primer-dimerization, respectively. PCR amplification was carried out in a 25-µl reaction volume containing 0.25 ng of cDNA, 500 nM each forward and reverse primers, 12.5 µl iQ SYBR green Supermix (BioRad, Hercules, CA), and 9.5 µl sterile water. The PCR reactions were initiated with denaturation at 95° for 60 s; followed by amplification with 40 cycles at 30s, 95°; annealing for 2 min at 54°; and an extension at 72° for 5 min. A melt curve was also obtained for each sample using the following parameters: 84 cycles starting at 54°C and increasing 0.5°C every 5 s. PCR products were run on an agarose gel, to confirm presence of single product (band) and bands were visualized using a Kodak Image Station (Perkin-Elmer, Boston, MA). Quantification of mRNA was evaluated using ABI software. Samples were run in triplicate and results for each run
were averaged. $\Delta C_T$ (change in threshold cycle) was calculated by subtracting the $C_T$ of the 18S control gene from the $C_T$ value of the gene of interest and mean normalized gene expression was calculated as previously described\(^5\). Results are expressed as normalized gene expression as percentage of 18S controls.

**TBARS Assay.** Aortic thiobarbituric acid reactive substances (TBARs) levels were assessed using a TBARS assay kit (OXItex, ZeptoMetrix Corp Buffalo, NY) measuring TBARS levels in whole, uncentrifuged aorta homogenates per kit instructions. Briefly, aortas (one-half, midsagittal cut) were resuspended by diluting 1:10 weight/volume in normal saline. Tissue was homogenized, and sonicated for 15 s at 40 V. Duplicate samples were read on a spectrophotometer (Perkin Elmer Lambda 35, Boston, MA), quantified using a malondialdehyde (MDA) standard curve, and are results expressed as MDA equivalents.

**Western blot analysis of MMP-9, TIMP-2 and MMP-2.** Pro-MMP-9, -2, and TIMP-2 protein levels were measured via Western blot. Aorta samples were homogenized and protein isolated from the cytosolic fraction; aorta and plasma samples were subsequently concentrated on a Millipore centrifugal separator (model YM-10, Fisher Scientific) and quantified using the Bradford assay (Biorad). 5 µg of protein was loaded into each lane (n = 3-5 for each group), and subsequently run through SDS-PAGE electrophoresis under reducing conditions. After membrane transfer, membranes were blocked overnight at 4°C in 5% blotto [5% weight/vol powdered milk: 100 ml 1X tris buffered saline (Biorad): 5% Tween vol/vol (Sigma Aldrich)]. Membranes were incubated in rabbit polyclonal anti-mouse MMP-9 (1:3000 dilution) (Abcam, Cambridge, MA), or rabbit polyclonal anti-mouse MMP-2 (1:3000, Abcam), or rabbit polyclonal anti-mouse TIMP-2 (1:1000, Abcam), and beta-actin primary antibody (1:2000, Abcam) for 1 hour at RT. Anti-rabbit antibody conjugated to HRP (1:2000 Abcam) was used for the
secondary antibody for 1 hour at RT. Bands were visualized with chemiluminescence and densitometry performed utilizing Image J software (NIH).

**In situ zymography.** MMP activity was analyzed on frozen serial aorta sections (6 µm thick), which were incubated with 45 µl of 10µg/ml dye quenched (DQ)-gelatin (EnzChek, Molecular Probes, Invitrogen, Carlsbad, CA) and 1 µg/ml DAPI (nuclei stain, Invitrogen) in 1% UltraPure™ low melting point agarose (Invitrogen) cover-slipped, chilled for 5 minutes at 4 ºC, and then incubated for 6 hours in a dark, humid chamber at 37ºC. Some slides were co-incubated with a specific gelatinase inhibitor (MMP -2, -9 inhibitor IV, Chemicon, Millipore, Temecula, CA). Slides were analyzed using fluorescent microscopy and densitometry was calculated using white/black images and quantified using Image J software (NIH, Bethesda, MD). Background fluorescence (fluorescence present in total image outside of the vessel) was subtracted from each section before statistical comparison between groups.

**Human plasma MMP-9 ELISA.** In conjunction with the Human Studies Division at the Environmental Protection Agency, healthy subjects (n=10; 18-40 years old, n=4 male and n=6 female) were exposed to a target concentration of 100 µg/m³ diesel (DE) particles or HEPA and charcoal filtered “clean” air (sham exposure) for 2 hours (controls), on a separate occasion. Subjects had 4 cycles of 15 min rest and 15 min exercise on a stationary bicycle at a target ventilation rate of 25 L oxygen/min/m³ body surface area. DE was generated from a Cummins engine operating at or near idle conditions using a certified commercial #2 fuel purchased from ChevronPhillips. Components of DE were measured as follows: PM: 106 +/- 9 µg/m³; NOx: 4.7 ppm; NO₂: 0.8 ppm; CO: 2.8 ppm; total hydrocarbons (HC-gas): 2.4 ppm; mass mean aerodynamic diameter (MMAD): 0.10 µm. The DE particle size was 0.10+/−0.02 µm and the
geometric standard deviation was 1.75+-0.11 (summarized in Table I). Further characterization of diesel chamber pollutant measurements for this study will be published in a future publication [personal communication, Sobus JR, Pleil JD, Madden MC, Funk WE, Hubbard HF, Rappaport SM. Identification of surrogate measures of diesel exhaust exposure in a chamber study with human subjects]. Blood was drawn prior to either filtered-air or DE exposure, 30 min after each type of exposure, 24 hrs post each type of exposure, and plasma stored at -80°C until analysis by ELISA. MMP-9 ELISA (Biotrak #RPN2614, Amersham, Piscataway, NJ), and MMP-9 activity ELISA (Biotrak #RPN263A, Amersham), were performed on pre- and post- exposure plasma samples (1:10 dilution in assay buffer), from both filtered air and DE exposures, per manufacture instructions. ET-1 levels were quantified by ELISA (QuantiGlo #QET00B, R&D Systems, Minneapolis, MN) per manufacturer instructions. Plasma NOx levels were measured by a Nitrate/Nitrite colorimetric kit (#79001, Cayman Chemical, Ann Arbor, MI). Briefly, plasma samples were processed through a pre-rinsed 10 kDa centrifuge ultrafilter (Millipore) to reduce background absorbance (due to hemoglobin) and improve color formation using the Griess reagents. Samples were run in triplicate for each ELISA, and concentrations determined from standard curve; whereas samples were run in duplicate in the colorimetric assay and calculated from standard curves for both nitrate and nitrite (µM), per manufacturer instructions. Sample #10 was eliminated from analysis due to lack of pre-exposure sample. Data are presented as the difference from pre-exposure to each post-exposure value (30 min-post and 24 h-post) for pro-MMP9, MMP9 activity, ET-1, and NOx (see Table III). To further identify whether individuals had a response to diesel exposure at either time point, as compared to air exposure, the difference between the pre-exposure value and the average of the two post-exposure values is presented.
All procedures were approved by the Lovelace Respiratory Research Institutional Review Board under exemption #4 (protocol #07-001) and all subjects provided informed consent.
RESULTS

**Acute exposure to GEE increases vascular ROS in ApoE<sup>−/−</sup> mice.**

We have previously reported that ApoE<sup>−/−</sup> mice subchronically exposed to GEE have significantly elevated vascular ROS<sup>1</sup>. To confirm effects of acute exposure to GEE, frozen aortas were analyzed using dihydroethidium staining. Ethidium fluorescence was nearly 2-fold higher in aortic nuclei from ApoE<sup>−/−</sup> mice exposed to GEE for 7 days (Figure IA), compared to filtered-air controls (Figure IB). Similar results were observed in vascular TBARs assay; where lipid peroxidation was observed to be approximately 1.5-fold higher by day 7 of GEE exposure, compared to controls (Figure IC). Interestingly, GEE-exposure does not appear to alter pulmonary oxidative stress levels (Figure ID), which is in agreement with our previous findings in subchronic GEE exposed lungs<sup>1</sup>.

**Acute GEE- exposure increases vascular MMP and TIMP mRNA expression in ApoE<sup>−/−</sup> mice.**

To elucidate whether acute GEE-exposure induced expression of vascular gelatinase MMP-2, and inhibitors TIMP-2, aorta mRNA levels were quantified. Exposure to GEE resulted in significant elevations in aorta MMP-2 (Figure IIA, IIC) and TIMP-2 (Figure IIB, D) mRNA by day 7 of exposure. Only TIMP-2 expression was significantly upregulated by day 1 of exposure (Figure IIB). Tempol-treatment ameliorated vascular expression of MMP-2 (Figure IIA), and TIMP-2 (Figure IIB). Similar reductions in MMP-2 were observed with BQ-123-treatment; however TIMP-2 remained elevated in 7 day BQ-123-treated - GEE-exposed ApoE<sup>−/−</sup> mice. Such findings suggest TIMP-2 expression is likely not mediated through an ET<sub>A</sub> signaling pathway. GEE-exposure had no effect on vascular TIMP-1 expression (data not shown).

**BQ-123-treatment attenuates vascular MMP-9 protein levels in GEE-exposed ApoE<sup>−/−</sup> mice.**
In an effort to determine the level at which ET-1 may be regulating MMP-9 expression, we examined the effects of BQ-123-treatment on aorta MMP-9 protein expression, using Western blot. GEE-exposure significantly increases expression of aorta MMP-9 protein, compared to filtered air controls (Figure IIIA: Lanes 1 and 3, respectively); whereas BQ-123 treatment reduced MMP-9 protein expression in the vasculature of both controls and GEE-exposed animals (Figure IIIA: Lanes 2 and 4, respectively). These findings suggest that ET-1-ET\(\text{A}\) mediated signaling pathways regulate expression of vascular pro-MMP-9, in response to GEE-exposure.

**GEE-exposure does not increase expression of hypoxia-induced genes in ApoE\(^{-/-}\) mice.**

To confirm that our GEE exposure is not inducing tissue hypoxia in the vasculature of our study animals, we analyzed aorta vascular endothelial growth factor (VEGF) expression. VEGF mRNA levels are not altered in the vasculature of our GEE-exposed mice, compared to controls (Figure IV), suggesting that exposure does not result in tissue hypoxemic, as expression of VEGF is induced by hypoxia through activation of the hypoxia response element (HRE) in its promoter. Additionally, the plasma which was used from human subject exposed to DE also did not show any changes in levels of carboxyhemoglobin, compared to their filtered air control exposures.

**Vascular NAD(P)H oxidase subunit expression is elevated in ApoE\(^{-/-}\) mice exposed to GEE.**

NAD(P)H oxidases have emerged as a major source of superoxide (O_2\(^{-}\)) generation in the vasculature\(^5\), which consist of a core heterodimers comprised of a phagocytic oxidase (p22phox) subunit and a glycoprotein (gp91phox) subunit (or homologs Nox1 – 5), and four regulatory subunits: p47phox, p67phox, p40phox, and rac1. Interestingly, NAD(P)H oxidase subunits are upregulated in the vasculature in pathophysiological states, such as atherosclerosis\(^6\). To determine a possible source of the ROS observed in the vasculature of GEE-exposed ApoE\(^{-/-}\) mice, we...
mice, we quantified the expression of aortic NAD(P)H oxidase subunit mRNA, using real time RT-PCR. GEE –exposure resulted in an elevation of the membrane bound NAD(P)H oxidase subunit, gp91phox mRNA by day 1 of exposure, with further increases observed in by day 7 (Figure VA). Similar trends in expression were observed in the expression of cytosolic NAD(P)H subunits p47phox and p67phox mRNA in the vasculature of GEE-exposed ApoE^{−/−} mice at day 1 of exposure, however the elevations observed were not statistically significant due to high variability (Figures VB and VC).
REFERENCES


FIGURE LEGENDS

Figure I. Vascular and pulmonary oxidative stress in 1 and 7 day acute GEE-exposed ApoE−/− mice. Representative photomicrograph of ethidium fluorescence in DHE-stained aortas, 7 day exposure (n=3 each group). A: Control, filtered-air; B: Exposed, 60 µg PM/m3. DHE enters the cell and is oxidized primarily by superoxide to yield fluorescent products, such as ethidium (red fluorescence). Aorta (C) and pulmonary (D) TBARS levels in response to GEE-exposure (60 µg PM/m3, n=6 per group). *p≤0.050 compared to controls.

Figure II. Aorta MMP-2 TIMP-2 mRNA in Tempol- or BQ-123-treated GEE-exposed ApoE−/− mice. Expression of aortic MMP-2 (A) and TIMP-2 (B) mRNA in ApoE−/− mice exposed for 6 h/d for 1 or 7 days to either filtered air (controls), Tempol (10mmol/L in drinking water), GEE (60 µg/m³), or Tempol + GEE (n=6 each) and MMP-2 (C) and TIMP-2 (D) mRNA in ApoE−/− mice exposed for 6 h/d for 1 or 7 days to either filtered air (controls), BQ-123 (100 ng/kg/day), GEE (60 µg/m³), or BQ-123 + GEE (n=6 each). *p≤0.050 compared to controls, †p≤ 0.050 compared to GEE-exposed.

Figure III. BQ-123 reduces vascular pro-MMP-9 levels in ApoE−/− mice exposed to GEE for 7 days. Aorta MMP-9 protein expression, as determined by Western blot, in ApoE−/− mice exposed for 6 h/d for 7 days to: (A) Lane 1: control (filtered air); Lane 2: control + BQ-123 (100 ng/kg/day); Lane 3: GEE (60 µg/m³); Lane 4: GEE + BQ-123. (B) Densitometric analysis of aorta MMP-9 (E). *p < 0.050 compared to controls; †p < 0.050 compared to GEE-exposed.
Figure IV. Transcriptional changes in aorta VEGF in GEE-exposed ApoE<sup>-/-</sup> mice.  
Expression of aortic VEGF mRNA in ApoE<sup>-/-</sup> mice exposed for 6 h/d for 7 days to either filtered air (controls), or 60 μg/m³ GEE (n=6 each group). Data show mean normalized gene expression (to 18S) ± SEM, as determined by real time PCR.

Figure V. Transcriptional changes in aorta gp91phox, p47phox, and p67phox in GEE-exposed ApoE<sup>-/-</sup> mice. Expression of aortic gp91phox (A), p47phox (B), and p67phox (C) mRNA in ApoE<sup>-/-</sup> mice exposed for 6 h/d for 1 or 7 days to either filtered air (controls), or 60 μg/m³ GEE (n=6 each group). Data show mean normalized gene expression (to 18S) ± SEM, as determined by real time PCR. *p≤0.050 compared to controls.
Table I. Concentrations of Vehicular Exhaust Components.

<table>
<thead>
<tr>
<th></th>
<th>PM  µg/m³</th>
<th>Mean PM MMAD µm</th>
<th>NO₂ ppm</th>
<th>NO ppm</th>
<th>CO ppm</th>
<th>THC ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gasoline (mice)</strong></td>
<td>61</td>
<td>0.150</td>
<td>2</td>
<td>16</td>
<td>80</td>
<td>12.7</td>
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<tr>
<td><strong>Diesel (humans)</strong></td>
<td>100</td>
<td>0.10 ± 0.02</td>
<td>0.4</td>
<td>3.5</td>
<td>9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

PM, particulate matter; NO₂, nitrogen dioxide; NO, nitric oxide; CO, carbon monoxide; THC, total hydrocarbons; ppm, parts per million; MMAD, mass median aerodynamic diameter.
Table II. Primer sequences used for real time RT-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9 FP</td>
<td>5’ – TTGGTTTCTGCCCTAGTGAGAGA – 3’</td>
<td></td>
</tr>
<tr>
<td>MMP-9 RP</td>
<td>5’ - AAAGATGAACGGGAACACACAGG – 3’</td>
<td></td>
</tr>
<tr>
<td>MMP-2 FP</td>
<td>5’ – ACCAGGTGAAGGATGTGAAGCA – 3’</td>
<td></td>
</tr>
<tr>
<td>MMP-2 RP</td>
<td>5’ – ACCAGGTGAAGGAGAAGGCTG – 3’</td>
<td></td>
</tr>
<tr>
<td>TIMP-1 FP</td>
<td>5’ – CACTGATAGCTTCCAGTAAGGCC – 3’</td>
<td></td>
</tr>
<tr>
<td>TIMP-1 RP</td>
<td>5’ – CTTATGACCAGGTCCGAGTTCG – 3’</td>
<td></td>
</tr>
<tr>
<td>TIMP-2 FP</td>
<td>5’ – CTTCAGCATCCAGGCTGAGC – 3’</td>
<td></td>
</tr>
<tr>
<td>TIMP-2 RP</td>
<td>5’ – TCATCAGTTTGTGCAAAAGAGGGA – 3’</td>
<td></td>
</tr>
<tr>
<td>ET-1 FP</td>
<td>5’ – AAGACCATCTGTGTGCTTCTAC – 3’</td>
<td></td>
</tr>
<tr>
<td>ET-1 RP</td>
<td>5’ – CAGCCTTTCTTGGAAATGTTGGAT – 3’</td>
<td></td>
</tr>
<tr>
<td>VEGF FP</td>
<td>5’ – GGAGTACCCCGACGAGATAGAG -3’</td>
<td></td>
</tr>
<tr>
<td>VEGF RP</td>
<td>5’ – CTCCAGGGCTTCATCGTTACG – 3’</td>
<td></td>
</tr>
<tr>
<td>gp91phox FP</td>
<td>5’– CACCCATTCACACTGACCTCTG – 3’</td>
<td></td>
</tr>
<tr>
<td>gp91phox RP</td>
<td>5’– CTTATCACAGCCACAAGCATTGAA – 3’</td>
<td></td>
</tr>
<tr>
<td>p47phox FP</td>
<td>5’ – CTGCTGTGTGAAGGAGGACGAGATG – 3’</td>
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</tr>
<tr>
<td>p47phox RP</td>
<td>5’ – AGCCGGTGATATCCCCTTTCC – 3’</td>
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</tr>
<tr>
<td>p67phox FP</td>
<td>5’ – CTGCTGTGTGAAGGAGGACGAGATG – 3’</td>
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<tr>
<td>p67phox RP</td>
<td>5’ – TCATCAGTTTGTGCAAAAGAGGGA – 3’</td>
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<tr>
<td>18S FP</td>
<td>5’ – GCTTGCCTTGTGATTAAGTCCTCAG – 3’</td>
<td></td>
</tr>
<tr>
<td>18S RP</td>
<td>5’ – GTTCGACCCTTCTTCAGC – 3’</td>
<td></td>
</tr>
</tbody>
</table>

FP, forward primer; RP, reverse primer; TIMP, tissue inhibitor of metalloproteinases; MMP, matrix metalloproteinase; ET-1, endothelin-1; VEGF, vascular endothelial growth factor
**Table III.** Plasma Active MMP-9, Total MMP-9, ET-1 and NOx Levels for Females vs. Males in Controlled Diesel Exposures.

<table>
<thead>
<tr>
<th>Subject</th>
<th>MMP-9 activity (ng/ml)</th>
<th>Total MMP-9 (ng/ml)</th>
<th>ET-1 (pg/mol)</th>
<th>NOx (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FA</td>
<td>DE</td>
<td>FA</td>
<td>DE</td>
</tr>
<tr>
<td>Female</td>
<td>Pre</td>
<td>0.63±0.08</td>
<td>0.57±0.06</td>
<td>7.0±4.6</td>
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<tr>
<td></td>
<td>Post 24 hrs</td>
<td>0.67±0.09</td>
<td>0.76±0.09*</td>
<td>10.2±5.6</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>0.63±0.14</td>
<td>0.66±0.07</td>
<td>10.6±6.6</td>
</tr>
<tr>
<td>Male</td>
<td>Pre</td>
<td>0.61±0.11</td>
<td>0.49±0.04</td>
<td>13.5±6.2</td>
</tr>
<tr>
<td></td>
<td>Post 24 hrs</td>
<td>0.63±0.13</td>
<td>0.68±0.07*</td>
<td>12.3±4.9</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>0.55±0.07</td>
<td>0.65±0.13</td>
<td>12.3±6.8</td>
</tr>
</tbody>
</table>

MMP-9, matrix metalloproteinase-9; ET-1, endothelin-1; NOx, nitrogen oxides; FA, HEPA-filtered air controls; DE, diesel exhaust exposure 100 µg/m² diesel for 2 hours; ng, nanograms; pg, picograms. Values shown ± standard error. *p<0.050 compared to pre measurements within that exposure group.
Figure I

A. Exposed  

B. Control  

C.  

D.  

Aorta MDA Concentration (nmol / ml / mg tissue)  

Control  

GEE Exposed  

Lung MDA Concentration (nmol / ml / mg tissue)  

Control  

GEE Exposed
Aorta TIMP-2 mRNA
Mean Normalized Gene Expression

Aorta MMP-2 mRNA
Mean Normalized Gene Expression

C.

D.

Figure II

Aorta TIMP-2 mRNA
Mean Normalized Gene Expression

Aorta MMP-2 mRNA
Mean Normalized Gene Expression

Control
Control + Tempol
GEE Exposed
GEE + Tempol

1 Day 7 Day

1 Day 7 Day
Figure III

A. kDa

1. 2. 3. 4. Pro-MMP-9

B. Relative Densitometry

Aorta MMP-9 Protein

*†
Figure IV

Aorta VEGF mRNA (Mean Normalized Gene Expression)

Control
GEE Exposed

7 Day
Figure V

A. Aorta gp phox mRNA (Mean Normalized Gene Expression)

B. Aorta p47 phox mRNA (Mean Normalized Gene Expression)

C. Aorta p67 phox mRNA (Mean Normalized Gene Expression)

* indicates significant difference.