Supplemental Materials

Expanded Materials and Methods

Animals and Inhalation Exposure Protocol. Ten-week-old male ApoE$^{-/-}$ 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$^{-/-}$ 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 *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^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 *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[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[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_2O 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 37ºC 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[4], as well as results in no adverse health effects and does not alter blood pressure in C57Bl6 mice[4]. 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/m^3PM 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 $O_2^{\bullet-}$, frozen aortic sections (6 μm thick) from each group were treated with dihydroethidium (DHE, Molecular Probes, Eugene, OR) using a previously published protocol[3], 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). $O_2^{\bullet-}$ production was analyzed using fluorescent densitometry and

quantified using Image J software (NIH); the percentage of $O_2^{\bullet-}$ 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. $O_2^{\bullet-}$ 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. ΔC_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 (OXItek, 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 4ºC 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/m^3 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/m^3 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/m^3; NOx: 4.7 ppm; NO_2: 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 -80ºC 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$^{-/-}$ mice.

We have previously reported that ApoE$^{-/-}$ mice subchronically exposed to GEE have significantly elevated vascular ROS[1]. 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$^{-/-}$ 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[1].

Acute GEE- exposure increases vascular MMP and TIMP mRNA expression in ApoE$^{-/-}$ 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$^{-/-}$ mice. Such findings suggest TIMP-2 expression is likely not mediated through an ET_A 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$^{-/-}$ 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_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 ($O_2^{\bullet-}$) 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 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, $gp91^{phox}$ 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 $p47^{phox}$ and $p67^{phox}$ 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

1. Lund AK, Knuckles TL, Obat Akata C, Shohet R, McDonald JD, Seagrave JC, Campen, MJ. Exposure to Gasoline Exhaust Results in Alterations of Pathways Involved in Atherosclerosis. *Toxicol Sci*. 2007;95:485-94.
2. McDonald JD, Barr EB, White RK, Kracko D, Chow JC, Zielinska B, Grosjean E. Generation and Characterization of Gasoline Engine Exhaust Inhalation Exposure Atmospheres. *Inhal Tox*. 2008 *In press.*
3. Troncoso Brindeiro CM, da Silva AQ, Allahdadi KJ, Youngblood V, Kanagy NL. Reactive Oxygen Species Contribute to Sleep Apnea-induced Hypertension in Rats. *Am J Physiol Heart Circ Physiol*. 2007;293:H2971-2976.
4. Ning L, Helwig BG, Feis RJ, Parimi S, Kenney MJ. Central Tempol alters basal sympathetic nerve discharge and attenuates sympathetic excitation to central ANG II. *Am J Physiol Heart Circ Physiol.* 2004;287:H2626-2633.
5. Muller PY, Janovjak H, Miserez HR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT–PCR. *BioTechniques* 2002;32:1372-1379.
6. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, Channon KM. Vascular superoxide production by NAD(P)H oxidase: association with endothelial dysfunction and clinical risk factors. *Circ Res*. 2000;86:E85-E90.
7. Sorescu D, Weiss D, Lassègue B, Clempus RE, Szöcs K, Sorescu GP, Valppu L, Quinn MT, Lambeth JD, Vega JD, Taylor R, Griendling KK. Superoxide production and exression of NOX family proteins in human atherosclerosis. *Circulation.* 2002;105:1429-1435.

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/m^3), 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/m^3), 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/m^3); 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$^{-/-}$ mice. Expression of aortic VEGF mRNA in ApoE$^{-/-}$ mice exposed for 6 h/d for 7 days to either filtered air (controls), or 60 μg/m^3 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$^{-/-}$ mice. Expression of aortic gp91phox (A), p47phox (B), and p67phox (C) mRNA in ApoE$^{-/-}$ mice exposed for 6 h/d for 1 or 7 days to either filtered air (controls), or 60 μg/m^3 GEE (n=6 each group). Data show mean normalized gene expression (to 18S) ± SEM, as determined by real time PCR. *$p \leq 0.050$ compared to controls.

Table I. Concentrations of Vehicular Exhaust Components.

	PM $\mu g/m^3$	**Mean PM MMAD** μm	**NO_2** ppm	**NO** ppm	**CO** ppm	**THC** ppm
Gasoline (mice)	61	0.150	2	16	80	12.7
Diesel (humans)	100	0.10 ± 0.02	0.4	3.5	9	0.9

PM, particulate matter; NO_2, 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.

MMP-9 FP:	5’ – TTGGTTTCTGCCCTAGTGAGAGA – 3’
MMP-9 RP:	5’ - AAAGATGAACGGGAACACACAGG – 3’
MMP-2 FP:	5’ – ACCAGGTGAAGGATGTGAAGCA – 3’
MMP-2 RP:	5’ – ACCAGGTGAAGGAGAAGGCTG – 3’
TIMP-1 FP:	5’ – CACTGATAGCTTCCAGTAAGGCC – 3’
TIMP-1 RP:	5’ – CTTATGACCAGGTCCGAGTTGC – 3’
TIMP-2 FP:	5’ – CTTCAAGCATCCAGGCTGAGC – 3’
TIMP-2 RP:	5’ – TCATCAGTTTGTGCAAAAGAGGGA – 3’
ET-1 FP:	5’ – AAGACCATCTGTGTGGCTTCTAC – 3’
ET-1 RP:	5’ – CAGCCTTTCTTGGAATGTTTGGAT – 3’
VEGF FP:	5’ – GGAGTACCCCGACGAGATAGAG -3’
VEGF RP:	5’ – CTCCAGGGCTTCATCGTTACAG – 3’
$gp91^{phox}$ FP:	5’– CACCCATTCACACTGACCTCTG – 3’
$gp91^{phox}$ RP:	5’ – CTTATCACAGCCACAAGCATTGAA – 3’
$p47^{phox}$ FP:	5’ – CTGCTGTTGAAGAGGACGAGATG – 3’
$p47^{phox}$ RP:	5’ – AGCCGGTGATATCCCCTTTCC – 3’
$p67^{phox}$ FP:	5’ – CTCGCCAGAACACACTAAACTGA – 3’
$p67^{phox}$ RP:	5’ - TCCTTCATGCTTTCTTCGGACAG - 3’
18S FP:	5’ – GCTTGCGTTGATTAAGTCCCTG – 3’
18S RP:	5 – GTTCGACCGTCTTCTCAGC – 3’

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.

Subject Gender		MMP-9 activity (ng/ml)		Total MMP-9 (ng/ml)		ET-1 (pg/mol)		NOx (μM)	
		FA	**DE**	**FA**	**DE**	**FA**	**DE**	**FA**	**DE**
Female	Pre	0.63±0.08	0.57±0.06	7.0±4.6	8.4±3.7	0.33±0.09	0.35±0.07	45.8±6.8	37.1±6.1
	Post	0.67±0.09	0.76±0.09*	10.2±5.6	18.6±8.3*	0.38±0.10	0.48±0.10	35.2±3.5	52.0±8.4*
	24 hrs Post	0.63±0.14	0.66±0.07	10.6±6.6	17.2±5.3	0.35±0.07	0.62±0.09*	38.0±8.0	41.5±5.0
Male	Pre	0.61±0.11	0.49±0.04	13.5±6.2	4.3±1.9	0.55±0.13	0.47±0.09	23.9±9.8	40.9±8.4
	Post	0.63±0.13	0.68±0.07*	12.3±4.9	12.5±1.7*	0.58±0.13	0.59±0.18	37.2±7.5	45.0±8.4
	24 hrs Post	0.55±0.07	0.65±0.13	12.3±6.8	10.0±1.5*	0.54±0.11	0.87±0.09*	26.2±5.6	26.3±6.9

MMP-9, matrix metalloproteinase-9; ET-1, endothelin-1; NOx, nitrogen oxides; FA, HEPA-filtered air controls; DE, diesel exhaust exposure 100 μg/m^3 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

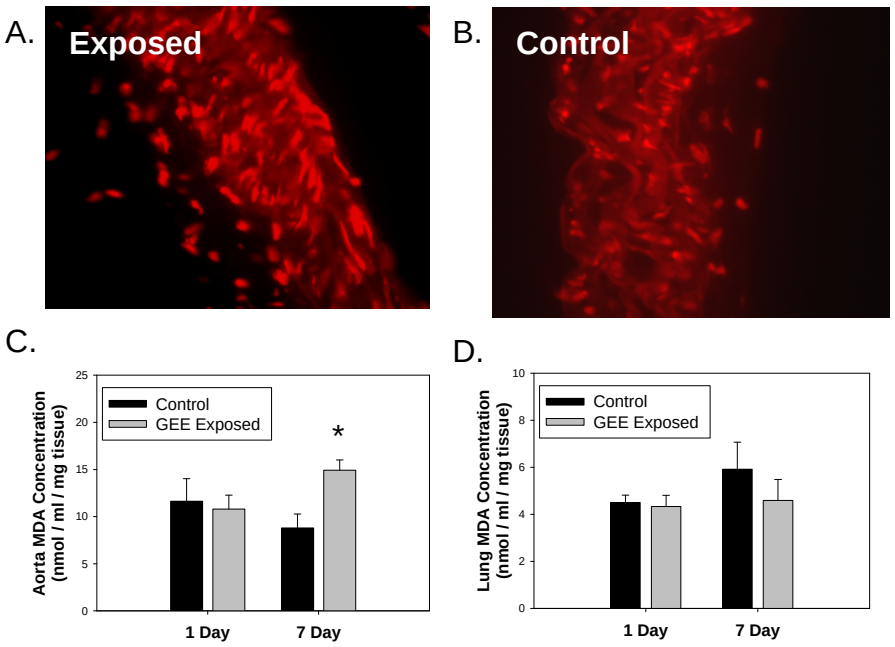

A.
Exposed
B.
Control
C.
Aorta MDA Concentration
(nmol / ml / mg tissue)
Control
GEE Exposed
*
1 Day
7 Day
D.
Lung MDA Concentration
(nmol / ml / mg tissue)
Control
GEE Exposed
1 Day
7 Day

Figure II

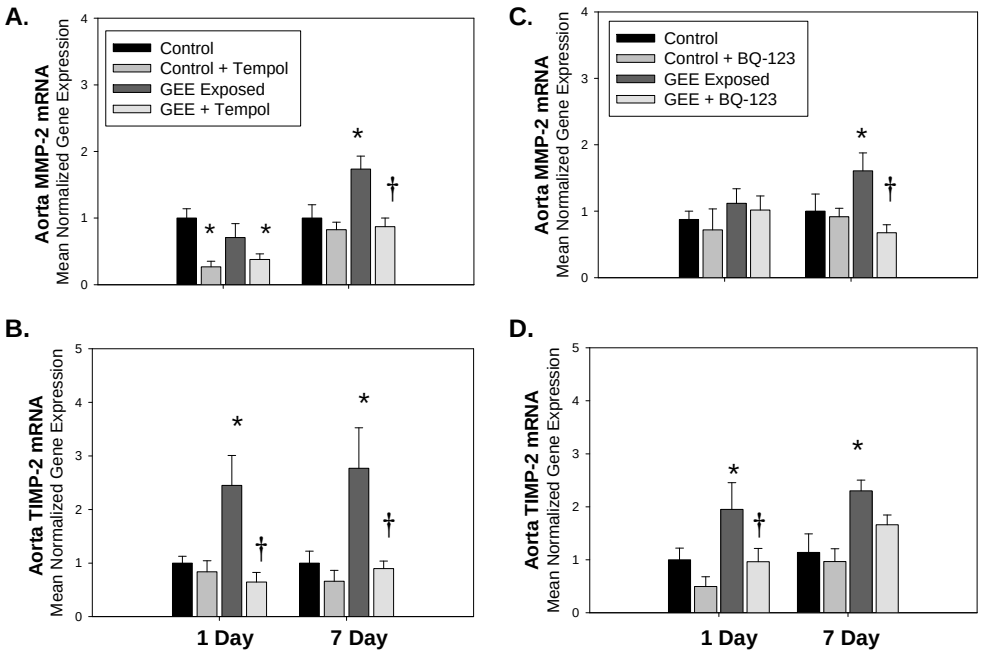

A.
Aorta MMP-2 mRNA
Mean Normalized Gene Expression
Control
Control + Tempol
GEE Exposed
GEE + Tempol
C.
Aorta MMP-2 mRNA
Mean Normalized Gene Expression
Control
Control + BQ-123
GEE Exposed
GEE + BQ-123
B.
Aorta TIMP-2 mRNA
Mean Normalized Gene Expression
1 Day
7 Day
D.
Aorta TIMP-2 mRNA
Mean Normalized Gene Expression
1 Day
7 Day

Figure III

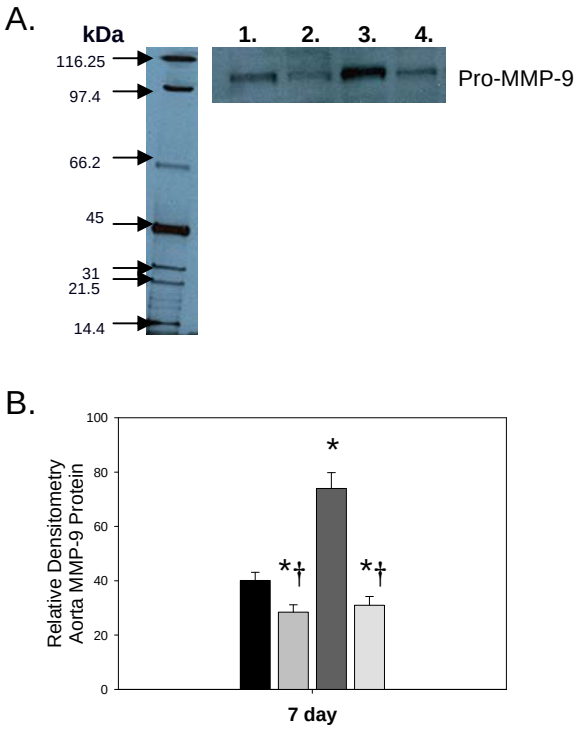

A.
kDa
1.
2.
3.
4.
116.25
97.4
66.2
45
31
21.5
14.4
Pro-MMP-9
B.
Relative Densitometry
Aorta MMP-9 Protein
100
80
60
40
20
0
*
*†
*†
7 day

Figure IV

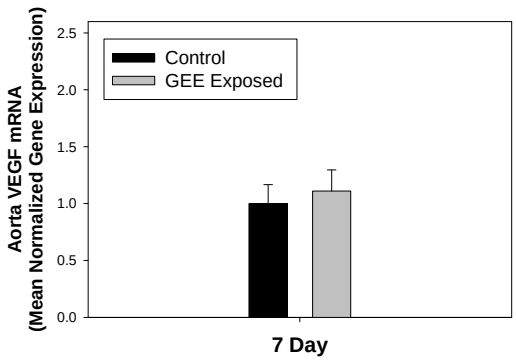
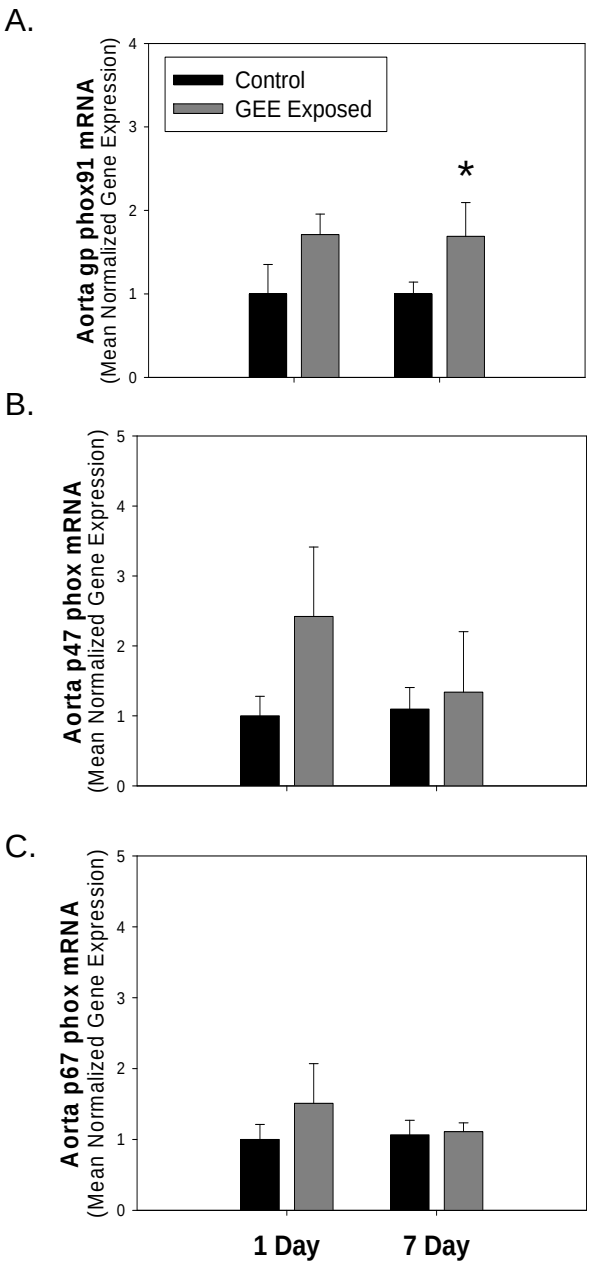

Aorta VEGF mRNA
(Mean Normalized Gene Expression)
2.5
2.0
1.5
1.0
0.5
0.0
Control
GEE Exposed
7 Day

Figure V


A.
Aorta gp phox91 mRNA
(Mean Normalized Gene Expression)
Control
GEE Exposed
*
B.
Aorta p47 phox mRNA
(Mean Normalized Gene Expression)
C.
Aorta p67 phox mRNA
(Mean Normalized Gene Expression)
1 Day
7 Day