The Oxidized Low Density Lipoprotein Receptor Mediates Vascular Effects of Inhaled Vehicle Emissions

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ONLINE DATA SUPPLEMENTS

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 7 days. Mice were housed in standard shoebox cages within an Association for Assessment and Accreditation of Laboratory Animal Care International-approved rodent housing facility (2m^3 exposure chambers) for the entirety of the study, which maintained constant temperature (20–24°C) and humidity (30–60% relative humidity). Mice had access to chow and water *ad libitum* throughout the study period, except during daily exposures when chow was removed. During the study period, all animals were exposed concurrently to either filtered air (n=16 for each treatment group: 8 vehicle, 8 treatment) or a mixture of 50 µg PM/m^3 gasoline engine emissions + 250 µg PM/m^3 diesel engine emissions (n=16 for each treatment group: 8 vehicle, 8 treatment). 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 14-16 hours after their last exposure, tissue were collected and either snap frozen in liquid nitrogen or embedded in OCT medium for cryosectioning and stored at -80°C.

ME were created by combining exhaust from a 1996 GM gasoline engine and a Yanmar diesel generator system, as previously reported (E1, E2). Gasoline emissions were generated using a 1996 model 4.3L General Motors V-6 engine equipped with stock exhaust systems

(including muffler and catalyst). 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). The diesel generator (Yanmar) was operated on a steady state load condition by drawing voltage to a bank of flood lamps, and fueled with a diesel blend characterized by 300 ppm sulfur content. Exhausts from both systems were combined after their primary dilutions into a $2m^3$ mixing chamber. The residence time in the mixing chamber was approximately 4 minutes. The exhaust from the mixing chamber was then diverted into a plenum that permitted diversion to exposure chambers. The proportion of gasoline engine exhaust was balanced by measuring carbon monoxide (CO) concentration, where >90 % of the CO originated. Diesel exhaust was similarly balanced based on particle mass concentration, because the majority of the particle mass came from that source. On each day of exposure particle mass concentration was measured gravimetrically (differential filter weight) using Teflon membrane (47-mm TEFLO, Pall-Gelman, East Hills, NY) filters. Particle size distribution was measured with a fast mobility particle sizer (TSI, St. Paul, MN), which conducts particle size scans from ~5–500 nm in 1 s time resolution. General characteristics of mixed emission exposures (mouse) and diesel emission exposures (humans) in are shown in Table 1.

For the filtered atmosphere containing only mixed vehicular gases (MEG), PM was removed from the MVE by an in-line HEPA filter that proved 99 + % effective. Sulfate aerosol atmospheres were generated with an evaporation-condensation approach. Dilute sulfuric acid was nebulized through a diffusion dryer, heated to 150ºC, and condensed by a counter-current heat exchanger at 4ºC. This yielded aerosol particle sizes with bimodal distributions at 0.1 and 1

micron, which is similar to what is observed in ambient air for sulfate. Sulfate is partially neutralized by ammonia that comes from the animals (E3).

LOX-1 Ab Treatment Group. ApoE$^{-/-}$ mice assigned to filtered air or ME, were randomly assigned to receive either LOX-1 Ab (R &D, anti-mouse LOX-1/SR-E1 antibody: 16 μg/ml, 0.1 ml/mouse, n=16) or vehicle (n=16 mouse IgG control, 16 μg/ml, 0.1 ml/mouse, n=16) i.p. beginning 24 hours prior to exposures and then every other day throughout the 7 day exposure. Doses were chosen based on previous studies which showed this dose effectively knocked down vascular LOX-1 expression (E4). Animals were monitored daily for health status throughout the study.

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=8 aortas (arch to bifurcation) from each exposure group were split midsagittaly and used for either RNA (real time RT-PCR analysis) or protein (TBARs and Western blot) assays. Hearts with ascending aorta attached were immediately embedded in Tissue Tek® O.C.T. (VWR Scientific, West Chester, PA) and frozen on dry-ice for MOMA-2, LOX-1 and *in situ* zymography analysis.

Real time RT-PCR. Real time PCR was done as previously described (E5). Total RNA was isolated from the aorta (one-half, midsagittal cut), n=8 per group, using RNeasy Fibrous Tissue

Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA in a 20 µl final reaction volume, per manufacturer instructions (Biorad, iScript Select cDNA synthesis kit). 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 reported in Table E1. 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 GAPDH 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 GAPDH controls.

Immunohistochemistry. Aorta sections (6 µm) were prepared for either LOX-1 or macrophage – monocyte (MOMA) staining as previously described (E6). Briefly, aortic sections were incubated with 10% normal goat serum for 30 min at room temperature, washed in PBS, and

incubated with a primary rat monoclonal antibody against mouse macrophages (1:400 dilution, 150 µl per section; MOMA-2; BioSource International, Camarillo, CA), diluted in rinse wash buffer [1 part 5% blocking solution (0.5 ml Normal Rabbit Serum in 10 ml 3% w/v Bovine Serum Albumin) and 4 parts Phosphate Buffered Saline (PBS)] for 1 hr at room temperature. Slides were then rinsed 3 times with PBS. The slides were then incubated in secondary antibody - biotin-horseradish peroxidase-conjugated goat anti-rat IgG in rinse wash buffer (1:2000 dilution, 150 µl per section; Vector Laboratories, Biovalley, Marne la Vallée, France) in the dark for 1 hr at room temperature. Immunostainings were visualized after incubation with a peroxidase detection system (Vectastain ABC kit, Vector Laboratories) using Vector Red as a substrate (incubation was in the dark, approximately 5-10 minutes, or until red color developed), and hemotoxylin-counterstain. Slides were then coverslipped with Glycerol Gelatin Mount (Sigma) on a slide warmer at 40°C. For LOX-1 staining, slides were submerged in cold acetone for 10 min, rinsed and blocked with 5% goat serum in 3% BSA (Sigma-Aldrich, St. Louis, MO) PBS in the dark for 2 h. Sections were incubated for 1 h (dark, humidified chamber) with rabbit polyclonal anti-LOX-1 antibody (1:1000; Abcam, Cambridge, MA) at room temperature and goat anti-rabbit secondary antibody – Biotin (1:100; Abcam) for 1 h at room temperature. Sections were visualized with a VECTASTAIN Universal ABC-AP kit (Vector Labs, Burlingame, CA) following manufacturer instructions, counterstained with hematoxylin, and coverslipped with Glycerol Gelatin Mount (Sigma) on a slide warmer at 40°C. Slides were imaged by light microscopy at 10x, digitally recorded, and analyzed by image densitometry using Image J software (NIH, Bethesda, MD). For MOMA-2 slides, total valve and total lesional areas were the sum of all individual areas measured throughout the valve (6 sections/animal, 4-5 animals/group). Specific MOMA-2 stained areas were divided by the total lesion area for

calculation of the "percentage stained area". For LOX-1 slides, total positive staining was calculated per unit area on each section. A minimum of 3 locations on each section (2 sections per slide), 3 slides and n=3-5 per group were processed/analyzed

Double Immunofluorescence. Aorta sections (6 μm) were prepared for either LOX-1 and vonWillebrand factor (vWF) or LOX-1 and MOMA-2 double immunofluorescence. Aortic sections were incubated with 10% normal goat serum for 30 min at room temperature, washed in PBS, and incubated with 150 μl per section of mixed anti-rabbit LOX-1 (1:1000 dilution, Abcam) and anti-goat vWF (1:1000 dilution, Abcam) or 150 μl per section of mixed anti-rabbit LOX-1 (1:1000 dilution, Abcam) and anti-rat monoclonal antibody against mouse macrophages (1:1000 dilution,; MOMA-2; BioSource International, Camarillo, CA), diluted in rinse wash buffer [1 part 5% blocking solution (0.5 ml Normal Rabbit Serum in 10 ml 3% w/v Bovine Serum Albumin) and 4 parts Phosphate Buffered Saline (PBS)] for 1 hr at room temperature. Slides were then rinsed 3 times with PBS. The slides were then incubated in 150 μl per section of a mixture of secondary antibodies Alexa Fluor 488 (anti-rabbit) and Alexa Flour 594 (anti-goat or anti-rat) (1:2000 dilution,; Vector Laboratories, Biovalley, Marne la Vallée, France) in the dark for 1 hr at room temperature. Slides were then rinsed 3 times in PBS, and coverslipped with Acqueous Gel Mount (Sigma). Slides were imaged by fluorescent microscopy at 10x, 40x, and 63x using the appropriate excitation/emission filters, digitally recorded, and analyzed by image densitometry using Image J software (NIH). A minimum of 3 locations on each section (2 sections per slide), 3 slides and n=3-5 per group were processed/analyzed.

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 manufacturer's instructions, as previously described (5). Briefly, aortas (one-half, midsagittal cut) were resuspended by diluting 1:10 weight/volume in normal saline. Tissues were 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 results expressed as MDA equivalents.

oxLDL Assay. Total cholesterol and oxLDL were quantified as previously described (E5). Briefly, 150 µl of plasma was transferred to a microcentrifuge tube containing 2µl of 1mM BHT, centrifuged at 13.2xg for 10 min at 4°C. 100 µl of the infranatant was transferred to a clean tube (LDL), 100 µl of LDL precipitating reagent (Pointe Scientific) was added, mixed by inversion, and centrifuged for 5 min at 13.2xg, 4°C. The remaining pellet was resuspended (1 mM EDTA and 0.01 mM BHT) and processed through both a TBARs assay (200 µl), per manufacturer instructions, and cholesterol assay, using cholesterol detection reagent (2 µl sample + 40 µl reagent, incubated for 5 min at 37°C and read at an OD of 520 nm). Values reported as TBARS/µg of cholesterol.

Western blot analysis. Plasma sLOX protein levels were measured via Western blot. Plasma samples were 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 LOX-1 (1:3000 dilution), 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 using ECL Plus (GE Healthcare, Amersham Biosciences, Piscataway, NJ) and imaged on the FLA-5100 (Fujifilm, USA) digital image scanner; densitometry was 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 min at 4ºC, and then incubated for 6 h 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; performed on 6 sections per sample, 3 regions per section, 6 samples per group). Background fluorescence (fluorescence present in total image outside of the vessel) was subtracted from each section before statistical comparison between groups.

Dihydroethidium Staining. Dihydroethidium (DHE) staining of aortas from all exposure treatment groups were processed on 6 μm thick frozen sections. Hearts were collected at euthanization, the aortas were removed just proximal to the aortic arch and the top one-third of

the heart was immediately embedded in OCT (Tissue Tek), including filling the remaining ascending aorta with OCT, and frozen on dry ice. The aorta was sectioned on a cryostat and immediately processed through DHE staining as previously described (E5). Briefly, slides were washed in PBS for 30 s, and rinsed three times in ddH2O. Slides were allowed to air dry, and then each section (2 per slide) was treated with 50 µl of 10 µM DHE. Slides were cover-slipped and then incubated at 37°C for 45 min. Ethidium staining was visualized by fluorescent microscopy at 63x, digitally recorded, and analyzed by image densitometry (color images converted to white/black) using Image J software. Superoxide signal specificity was confirmed by incubating selected sections with polyethylene glycol-conjugated superoxide dismutase (PEG-SOD, 50 U/ml) for 30 min at 37°C.

Human exposures and plasma sLOX analysis. In conjunction with the Human Studies Division at the Environmental Protection Agency, healthy subjects (n=8, after excluding 2 with incomplete sample collections; 18-40 years old, n=3 male and n=5 female; Table E2) were exposed to a target concentration of 100 µg PM/m^3 whole diesel emissions (DE) or HEPA- & charcoal-filtered "clean" air (sham exposure) for 2 hours (controls), on separate occasions. 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: (Table 1) 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. Further characterization of diesel chamber pollutant measurements for this study are available in Sobus JR, et al., 2008 (E7). Banked samples were

analyzed from exposures of healthy subjects (n=10; 18-40 years old) exposed to 100 μg/m^3 diesel (DE) whole exhaust or HEPA and charcoal filtered "clean" air for 2 hrs, on separate occasions, as previously described (7). Blood was drawn prior to both filtered-air and DE exposures, 30 minutes after each exposure and 24 hrs after each exposure; plasma was stored at -80ºC until analysis by ELISA. Samples were coded and randomized to ensure that the assay was conducted under blinded conditions; data was then decoded and analyzed by another contributor (MJC). A sLOX ELISA (DuoSet #1798 Human LOX-1/SR-E1 ELISA, R&D Systems, Minneapolis, MN) was performed on pre- and post- exposure plasma samples (1:10 dilution in assay buffer), from both filtered air and DE exposures, per manufacture instructions. Samples were run in triplicate for the ELISA. Data are presented as the difference from pre-exposure to each post-exposure value (2 h-post and 24 h-post) for plasma sLOX concentrations (Table E2) and as raw absolute data (Table E3). Lipid panel analyses for total cholesterol (C), HDL-C, and triglycerides were performed at LabCorp (Burlington, NC) within 24 hr using standardized automated methods; LDL-C and VLDL-C are reported as calculated values. 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 2-way repeated measures ANOVA. The relationship between specific serum lipids and the magnitude of the sLOX response was analyzed by linear regression. Statistical

analyses were conducted in GraphPad Prism v5.02. A $p < 0.05$ was considered statistically significant.

RESULTS

Acute exposure to ME increases vascular reactive oxygen species levels in ApoE$^{-/-}$ mice.

We have previously reported that ApoE$^{-/-}$ mice exposed to acute gasoline engine emissions exhibit elevated vascular reactive oxygen species (ROS) (E5). To confirm effects of acute exposure to ME, frozen aortas were analyzed using dihydroethidium staining. Ethidium fluorescence was nearly 2-fold higher in aortic nuclei from ApoE$^{-/-}$ mice exposed to ME for 7 days (Fig E1, panel C), compared to filtered-air controls (Fig E1, panel A). Anti-LOX-1 neutralizing antibody treatment resulted in a significant decrease in DHE fluorescence in both FA (Fig E1, panel B) and ME (Fig E1, panel D), indicating that ME-mediated increases in ROS are mediated, at least in part, through LOX-1 receptor signaling.

ME exposure increases LOX-1 expression in aortic valve region of ApoE$^{-/-}$ mice, which is attenuated through LOX-1 neutralizing antibody treatment.

To confirm reduction in LOX-1 protein in the aorta of ApoE$^{-/-}$ mice treated with anti-LOX-1 neutralizing antibody, aorta sections were analyzed for LOX-1 expression through immunohistochemistry. Compared to filtered air controls (both IgG and anti-LOX-1 treated groups; Fig E2 panels A, C), inhalational exposure to ME for 7 days resulted in a significant increase in LOX-1 protein in the aorta of ApoE$^{-/-}$ mice (Fig E2, panel B). Concurrent treatment with anti-LOX-1 resulted in attenuation of LOX-1 expression in the vasculature in ME-exposed ApoE$^{-/-}$ mice (Fig E2, panel D).

Acute ME- exposure increases vascular TIMP mRNA expression in ApoE$^{-/-}$ mice.

To elucidate whether acute ME-exposure induced expression of vascular tissue inhibitors of matrix metalloproteinases (TIMPs) -1 and -2, aorta mRNA levels were quantified. Interestingly, anti-LOX-1 neutralizing antibody-treatment resulted in an increase in TIMP-2 mRNA expression, which may be a protective mechanism to increase TIMP:MMP inhibition in the vasculature. ME-exposure resulted in an even further increase in TIMP-1, but not TIMP-2, vascular mRNA expression above the values observed for anti-LOX-1-treated animals. Such findings suggests there may be a LOX-1 – mediated feedback mechanism to increase TIMP expression and maintain normal levels of TIMP:MMP binding (inhibition), possibly due to the increased MMP expression resulting from exposure. However, as TIMPs have biological roles independent of inhibiting MMPs, it is plausible that the mechanisms responsible for the increase in TIMP expression with anti-LOX-1 antibody vs. ME-exposure are likely result through different mechanisms. Further research will need to be done to confirm MMP and TIMP localization in this model, as well as determine if ME-exposure alters TIMP-MMP binding.

References.

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Table E1. Primer sequences used for real time RT-PCR analysis.

MMP-9 FP:	5’ – TTGGTTTCTGCCCTAGTGAGAGA – 3’
MMP-9 RP:	5’ - AAAGATGAACGGGAACACACAGG – 3’
ET-1 FP:	5’ – AAGACCATCTGTGTGGCTTCTAC – 3’
ET-1 RP:	5’ – CAGCCTTTCTTGGAATGTTTGGAT – 3’
LOX-1 FP:	5’ – CACAAGACTGGCTCTGGCATA – 3’
LOX-1 RP:	5’ – GCAGGTCTGCCGGTTTTTT – 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’
GAPDH FP:	5’ – CATGGCCTTCCGTGTTCCTA – 3’
GAPDH RP:	5 – GCGGCACGTCAGATCCA – 3’

FP, forward primer; RP, reverse primer; MMP, matrix metalloproteinase; ET-1, endothelin-1; LOX-1, lectin-like oxLDL receptor; TIMP-1, tissue inhibitor of matrix metalloproteinase-1; TIMP-2, tissue inhibitor of matrix metalloproteinase-2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table E2. Normalized Plasma sLOX Concentration Response to Filtered Air or Diesel Emission-Exposure.

Subject Number	Subject Gender	BMI kg/m^2	Plasma sLOX pg/ml			
			FA Pre	FA Post	DE Pre	DE Post
1	M	28.22	no sample	excluded	excluded	excluded
2	M	23.59	100	63.9 ± 3.9	100	140.9 ± 32.1
3	M	26.17	100	28.3 ± 2.4	100	121.0 ±1.41
4	F	24.60	100	85.2 ± 20.9	100	231.7± 34.5
5	M	20.99	100	82.6 ± 11.5	100	102.9 ±12.5
6	F	25.11	100	38.0 ± 6.5	100	198.1 ± 122.4
7	F	21.12	100	68.9 ± 1.16	100	98.2 ± 4.2
8	F	25.51	100	79.5 ± 27.5	100	105.8 ± 9.3
10	F	23.39	excluded	excluded	no sample	excluded
11	F	23.81	100	80.0 ± 1.5	100	101.2 ± 8.4

FA, HEPA-filtered air controls; DE, diesel exhaust exposure 100 μg/m^3 diesel for 2 hours. Pre, blood sample taken before exposure to FA or DE; Post, combined (average) blood sample values of sLOX taken 30 min and 24 hr post exposure to either FA or DE, post- values are expressed normalized to pre- values ± standard deviation. M, male; F, female; BMI, body mass index [calculated from equation weight (kg) / height (m^2)]. Data represents values extracted from standard curve of known sLOX-1 concentrations/OD. #1 and #10 were excluded from analysis due to lack of sample for processing.

Table E3. Absolute Plasma sLOX Concentration Response to Filtered Air or Diesel Emission-Exposure.

Subject Number	Subject Gender	Plasma sLOX pg/ml					
		FA Pre	FA Post 30 min	FA Post 24 hrs	DE Pre	DE Post 30 min	DE Post 24 hrs
1	M	no sample	excluded	excluded	excluded	excluded	excluded
2	M	61	46	55	50	215	58
3	M	90	62	58	55	65	94
4	F	150	45	39	25	31	32
5	M	1352	1340	955	414	1050	856
6	F	215	192	169	173	194	160
7	F	267	103	115	640	725	1847
8	F	610	431	416	417	401	429
10	F	excluded	excluded	no sample	excluded	excluded	excluded
11	F	47	43	40	42	48	41

FA, HEPA-filtered air controls; DE, diesel exhaust exposure 100 μg/m^3 diesel for 2 hours. Pre, blood sample taken before exposure to FA or DE; Post, combined (average) blood sample values of sLOX taken 30 min and 24 hr post exposure to either FA or DE, post- values, expressed as absolute values. M, male; F, female. Data represents values extracted from standard curve of known sLOX-1 concentrations/OD. #1 and #10 were excluded from analysis due to lack of sample for processing.

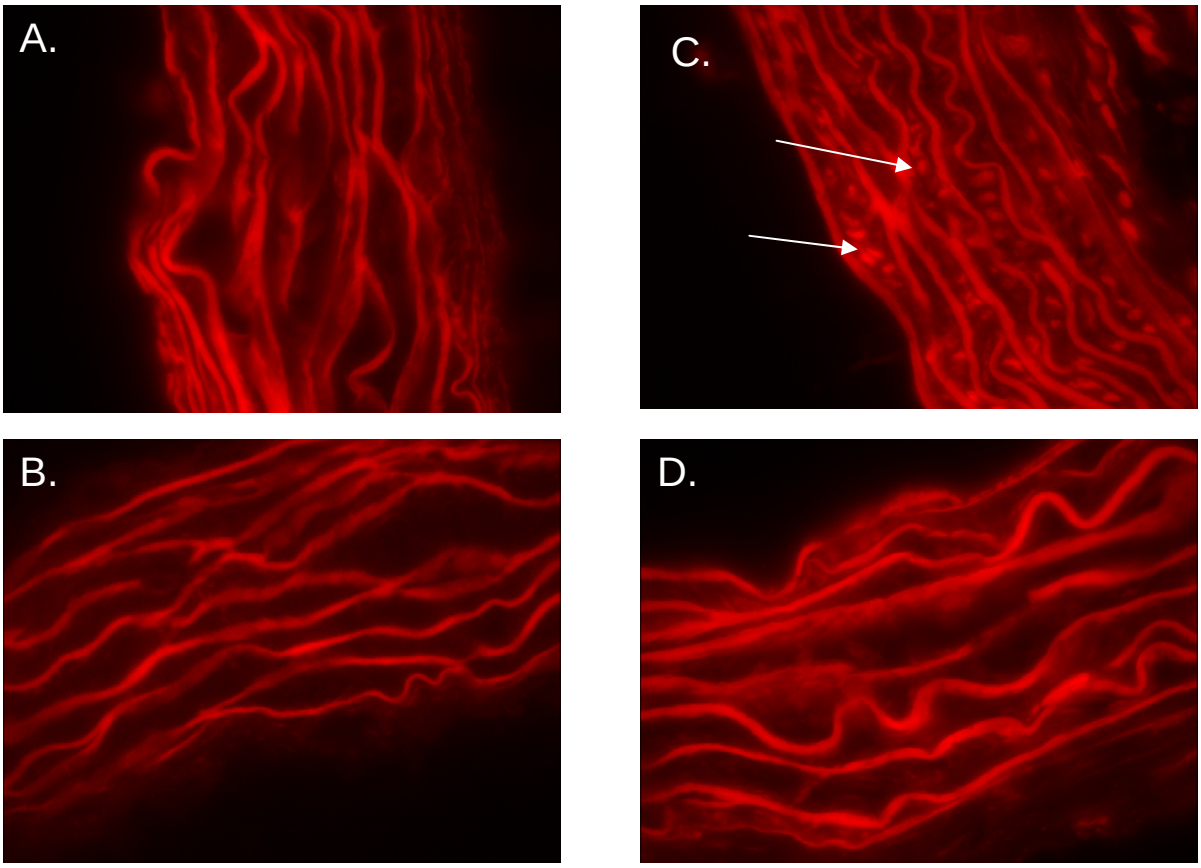

Figure E1. Mixed emissions – induced aortic reactive oxygen species. Representative photomicrograph of ethidium fluorescence in DHE-stained aortas, 7 day exposure (n=3 each group). A: Control, filtered-air + mouse IgG (IgG) B: FA + anti-LOX-1 Ab treatment (LOX-1); C: Exposed, ME + IgG: (50 µg PM/m^3 gasoline emissions + 250 µg PM/m^3 diesel emissions); D ME + anti-LOX-1 Ab treatment. DHE enters the cell and is oxidized primarily by superoxide to yield fluorescent products, such as ethidium (red fluorescence). Arrows indicate increased regions of ethidium fluorescence.

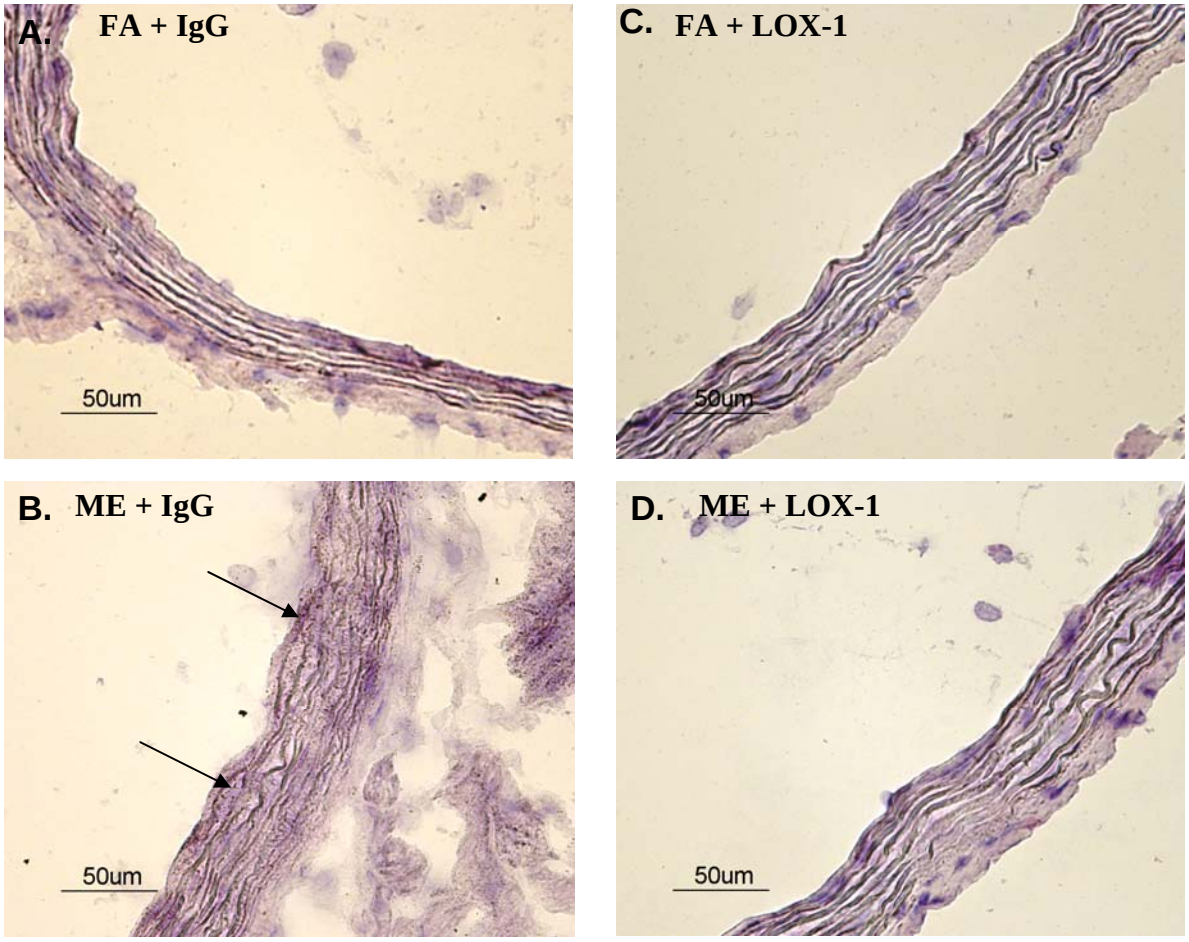

Figure E2. Vascular LOX-1 expression in ME-exposed ApoE$^{-/-}$ mice. Representative photomicrograph of LOX-1 immunohistochemistry in aortas of ApoE$^{-/-}$ mice exposed to ME (50 μg PM/m^3 gasoline emissions + 250 μg PM/m^3 diesel emissions) for 6 hr/day for 7 days (n=4-5 each group). A: FA, filtered-air with IgG treatment (IgG control); B: ME with IgG treatment; C: FA with concurrent anti-LOX-1 Ab treatment (LOX-1 Ab); D: ME + anti-LOX-1 Ab. LOX-1 is indicated by brown/red staining and is seen increased in the intimal layer of aortas from ApoE$^{-/-}$ mice exposed to ME (arrows). Slides are counterstained with hematoxylin. Total positive staining was calculated per unit area on each section (6 sections/animal, 4-5 animals/group) using Image J software. Arrows indicate areas of increased LOX-1 staining.

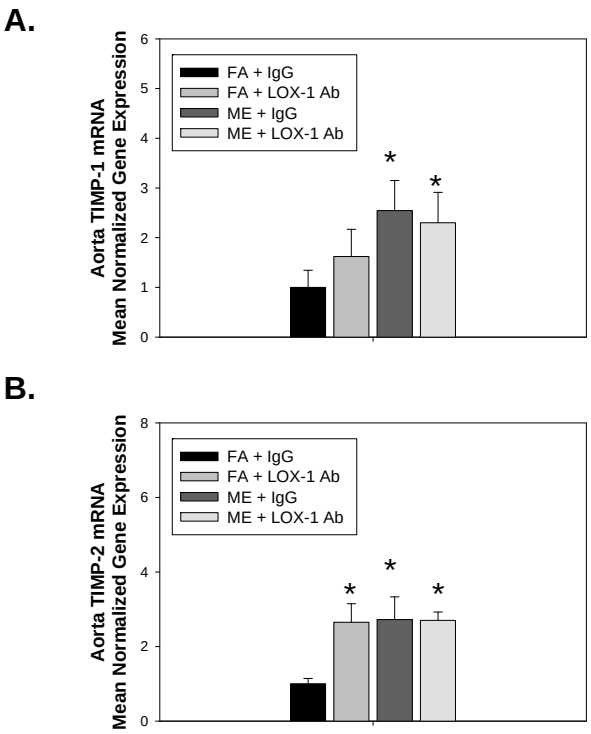

Figure E3. Transcriptional alterations in aorta TIMP-1 and TIMP-2 in ApoE$^{-/-}$ mice exposed to ME for 7 days. Aorta TIMP-1 (A) and TIMP-2 (B) mRNA expression, as determined by real time PCR, in ApoE$^{-/-}$ mice exposed inhalationally for 6 h/d for 7 days to either filtered air (FA, Control) + IgG (IgG control) or anti-LOX-1 antibody (LOX-1 Ab: R&D, anti-mouse LOX-1/SR-E1 antibody), or to mixed engine emissions (ME, 50 μg PM/m^3 gasoline emissions + 250 μg PM/m^3 diesel emissions) + IgG (IgG control) or anti-LOX-1 antibody (LOX-1). Data show mean normalized gene expression (to GAPDH) ± SEM, as determined by real time PCR. *$p \leq 0.050$ compared to FA + IgG controls.