Gasoline Exhaust Emissions Induce Vascular Remodeling Pathways Involved in Atherosclerosis

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Epidemiological evidence indicates that environmental air pollutants are positively associated with the development of chronic vascular disease; however, the mechanisms involved have not been fully elucidated. In the present study we examined molecular pathways associated with chronic vascular disease in atherosclerosis-prone apolipoprotein E-deficient (ApoE \(^{-/-}\)) mice, including markers of vascular remodeling and oxidative stress, in response to exposure to the ubiquitous environmental pollutant, gasoline engine emissions. ApoE \(^{-/-}\) mice, on a high-fat diet, were exposed by inhalation to either filtered air; 8, 40, or 60 mg/m\(^3\) particulate matter whole exhaust; or filtered exhaust with gases matching the 60-mg/m\(^3\) concentration, for 7 weeks. Aortas and plasma were collected and assayed for changes in histochemical markers, real-time reverse transcriptase–polymerase chain reaction, and indicators of oxidative damage. Inhalational exposure to gasoline engine emissions resulted in increased aortic mRNA expression of matrix metalloproteinase-3 (MMP-3), MMP-7, and MMP-9, tissue inhibitor of metalloproteinases-2, endothelin-1 and heme oxygenase-1 in ApoE \(^{-/-}\) mice; increased aortic MMP-9 protein levels were confirmed through immunohistochemistry. Elevated reactive oxygen species were also observed in arteries from exposed animals, despite absence of plasma markers. Similar findings were also observed in the aortas of ApoE \(^{-/-}\) mice exposed to particle-filtered atmosphere, implicating the gaseous components of the whole exhaust in mediating the expression of markers associated with the vasculopathy. These findings demonstrate that exposure to gasoline engine emissions results in the transcriptional upregulation of factors associated with vascular remodeling, as well as increased markers of vascular oxidative stress, which may contribute to the progression of atherosclerosis and reduced stability of vulnerable plaques.

Key Words: atherosclerosis; matrix metalloproteinases; reactive oxygen species; particulate matter; endothelin-1.

Recent epidemiological findings demonstrate associations between chronic exposure to ambient air pollution and increased incidence of ischemic heart disease (Pope \textit{et al}., 2004). With chronic cardiovascular disease being the leading cause of death in the United States and Europe (American Heart Association, 2003; Peterson \textit{et al}., 2005), such research suggests that the full public health impact of air pollution, even at levels within the limits set by the U.S. Environmental Protection Agency, may not yet be appreciated. Several recent epidemiological studies have reported a statistical relationship between exposure to particulate and gaseous air pollutants, at levels within attainment of current National Ambient Air Quality Standards (NAAQS), and increased rates of cardiovascular morbidity and mortality (Burnett \textit{et al}., 2000; Goldberg \textit{et al}., 2001; Pope \textit{et al}., 2004). Additionally, findings of systemic/cardiovascular health effects related to traffic exposure (Peters \textit{et al}., 2004) or components of vehicular origin (Riediker \textit{et al}., 2004) strongly suggests that (1) specific components of particulate matter (PM) or related combustion-source chemicals drive these responses and (2) vehicular emissions are rich with these putative toxicants.

We propose that air pollution–induced alterations of factors that mediate vascular remodeling process may explain, at least in part, both the chronic effects of atheromatous plaque growth as well as the acute effects on plaque instability, which may underlie associations with acute myocardial infarction. The matrix metalloproteinase (MMP) family represents a class of proteins that has been intimately linked to both plaque progression and plaque erosion or rupture in both humans and animal models of atherosclerosis, such as the ApoE \(^{-/-}\) mouse (Newby, 2005).

The role of dysregulated MMP activity in the development of obstructive lung disease has been clearly demonstrated (Elkington and Friedland, 2006), and exposures to various inhaled agents, such as environmental PM (Thomson \textit{et al}., 2005), can lead to increased expression and activity of various MMP isoforms in the lungs. The relationship of inhaled pollutants and systemic vascular MMP regulation is relatively unstudied.

In a recent study, Sun \textit{et al}.
(2005) examined the causative link between long-term (6-month) concentrated PM exposure...
and acceleration of vascular inflammation and atherosclerosis in susceptible mice. Despite the clear and robust findings from this study, there remains a great deal of uncertainty regarding molecular pathways that may be involved in the progression of vascular remodeling or plaque instability. Here, we investigate potential mechanisms related to systemic inflammation and vascular remodeling that may be involved in air pollution-mediated exacerbation of atherosclerosis, specifically the potential contribution of vascular MMPs, endogenous tissue inhibitors of MMP (TIMP), vascular endothelin-1 (ET-1), and markers of reactive oxygen species (ROS). To model vehicular air pollution exposure, we have utilized a two-engine system that enables two cold-starts per day for a 50-day exposure. Despite the fact that gasoline engines are a ubiquitous mobile source pollutant, there is little available information on the cardiovascular consequences of gasoline combustion emissions. Furthermore, while most studies that investigate adverse health effects as a result of air pollution exposure focus specifically on PM, here we examine the effects of both whole and particle-filtered engine emissions on the pathways associated with progression of atherosclerosis.

METHODS

Animals and inhalation exposure. 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, 1.5 g/kg cholesterol content) beginning on the first day of exposure, and subsequently exposed to varying concentrations of PM whole gasoline engine exhaust, filtered exhaust, or filtered air (controls) for 6 h/day \( \times \) 7 days/week for a period of 7 weeks. Mice were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International–approved rodent-housing facility that maintained constant temperature (20–24°C) and humidity (30–60% relative humidity) and provided with mouse chow and water ad libitum both during and between inhalational exposure periods throughout the study period, i.e., mice were not moved from exposure chambers until sacrifice. During the study period, all animals were exposed concurrently to filtered air (\( n = 16 \); 8 \( \mu \)g/m\(^3\) (\( n = 8 \)), 40 \( \mu \)g/m\(^3\) (\( n = 8 \)), or 60 \( \mu \)g/m\(^3\) PM whole exhaust (\( n = 16 \)); or filtered exhaust with gases matching the 60–80 ppm PM (\( n = 16 \))). Animals were monitored daily for health status (appearance, food and water consumption, activity levels, etc.) throughout the study period by the Animal Care staff. 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). Upon completion of the 7-week exposure period, animals were sacrificed within 16 h after their last exposure.

Gasoline emissions were generated using a common source: two 1996 6.5L General Motors V-6 engines equipped with stock exhaust systems (including muffler and catalyst), operated sequentially to enable two cold-starts per day. The engines were fueled with conventional unleaded, nonoxynated, nonreformulated gasoline blended to simulate a national average composition (ChevronPhillips Specialty Fuels Division, The Woodlands, TX). Exhaust was diluted approximately 1:12, 1:20, and 1:90 to produce the exposure atmospheres described in Table 1. At the 1:12 dilution, a separate treatment at the same dilution was conducted with an high efficiency particulate air filter in place to remove PM, while allowing the gas/vapor phase to pass through. 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 to 500 nm in 1 s time resolution.

**Table 1: Basic Characterization of Exposure Atmospheres Showing Average Daily Concentrations**

<table>
<thead>
<tr>
<th></th>
<th>PM (( \mu )g/m(^3))</th>
<th>NO(_2) (ppm)</th>
<th>CO (ppm)</th>
<th>HC (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (filtered air)</td>
<td>2</td>
<td>0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Low (1:90)</td>
<td>8</td>
<td>2</td>
<td>9</td>
<td>0.9</td>
</tr>
<tr>
<td>Mid (1:20)</td>
<td>39</td>
<td>12</td>
<td>50</td>
<td>8.4</td>
</tr>
<tr>
<td>High (1:12)</td>
<td>61</td>
<td>19</td>
<td>80</td>
<td>12.0</td>
</tr>
<tr>
<td>High-filtered (1:12)</td>
<td>2</td>
<td>18</td>
<td>80</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Note. PM, particulate matter; NO\(_2\), nitric oxide species; CO, carbon monoxide; THC, total hydrocarbons.

**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 of 0.1 ml per 30 g mouse) and euthanized by exsanguination. Blood was collected in a heparinized syringe (BD Vacutainer Systems, Franklin Lakes, NJ) through cardiac puncture, and immediately centrifuged (950 \( \times \) g, 10 min, 4°C) to separate plasma. Plasma was stored at \(-80°C\) for plasma thiobarbituric acid reactive substances (TBARS) analysis, until assayed. Additionally, the aorta and lung tissues were dissected, weighed, and frozen in liquid nitrogen. Tissue was stored at \(-80°C\) until assayed. \( N = 8 \) aortas from each exposure group for real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis and TBARS analysis; \( n = 8 \), control, \( n = 8 \), 60 \( \mu \)g/m\(^3\) PM whole exhaust, and \( n = 8 \), 60 \( \mu \)g/m\(^3\) PM-filtered exhaust for histochemical analysis.

**Real-time RT-PCR.** Total RNA was isolated from the aortic arch (ascending thoracic, aortic arch and small portion of descending thoracic), \( n = 8 \) for each exposure group, using RNaseasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA in a 60-\( \mu \)l final reaction volume containing 250 ng of sample RNA, 12.5 nM of 18S RT primer, 0.005 \( \mu \)g oligo dt, 0.0004 U RNAasin, 0.006 U M-MLV RT enzyme, 25 mM deoxynucleoside triphosphate, 12 \( \mu \)l 5× RT buffer, and water to attain 60 \( \mu \)l. The mixture was heated at 42°C for 1 h. Real-time PCR was performed using an iCycler (BioRad, Hercules, CA) and an ABI 7500 (Applied Biosystems, Foster City, CA). Control reactions without reverse transcriptase or without RNA were run to verify the absence of contaminated DNA and primer dimerization, respectively. PCR amplification was carried out in a 25-\( \mu \)l volume containing 0.25 ng of cDNA, 500 nM each forward and reverse primers (sequences below), 5× SYBR green Supermix (BioRad), and 9.5 \( \mu \)l water. PCR consisted of 95°C for 10 min, and 40 cycles of 95°C for 30 s and 60°C for 30 s. The primer sequences used for the genes analyzed were MMP-3 (forward 5′-AGAGAGCAGGCAAGACAGAA-3′, reverse 5′-GCACTTGGGATGATGGAATG GCA-3′), MMP-7 (forward 5′-CTATGCAAGTCACCCTGTGTACG-3′, reverse 5′-GGCTGTTCCCACTATGTCG-3′), MMP-9 (forward 5′-GACAGGGCATT CACCAC-3′, reverse 5′-GCTTGGCCACCCCCATTGGTG-3′), MMP-12 (forward 5′-TTGGAACAAGATTGATGCTAC-3′, reverse 5′-GAGAGAGCAGGCAAGACAGAA-3′), MMP-13 (forward 5′-CTATGCAAGTCACCCTGTGTACG-3′, reverse 5′-GGCTGTTCCCACTATGTCG-3′), TIMP1 (forward 5′-CACTGATACCTCCAGTAAGG-3′, reverse 5′-CTTATGACCAGTGGCAGGG-3′), TIMP2 (forward 5′-CTTCAAGACATCGAAGGTGC-3′, reverse 5′-CTCAATTTTGTTGACAAAGAGGA-3′), ET-1 (forward 5′-AGAGACCCTGTTGTGTCGTA-3′, reverse 5′-CAGGTTGTTTATCTGAGGC-3′), heme oxygenase-1 (HO-1) (forward 5′-TTGCTTGTGGGGGATTACC-3′, reverse 5′-TGGGTTGTTTATCTGAGGC-3′), and housekeeping gene 18S (forward 5′-GCTTGGTTTATCTGAGGC-3′, reverse 5′-GCTTGGTTTATCTGAGGC-3′). To confirm the presence of a single amplification product, PCR products were subjected to a melt curve analysis. Samples were run in triplicate, and mean normalized gene expression was calculated as previously (Lund et al., 2003).
**TBARS assay.** Aortic tissue (thoracic descending + abdominal sections of aorta), n = 6 randomly chosen from each exposure group, was resuspended by diluting 1:10 wt/v in normal saline. Tissue was homogenized and sonicated for 15 s at 40 V and homogenates were used to determine TBARS levels as described below. For plasma TBARS assay, plasma was pooled from three mice in each study group to obtain sufficient volumes prior to analysis (final n value, n = 2 for each group). The plasma was fractionated to separate the various lipid and lipoprotein fractions to attempt identification of specific oxidation in the low-density lipoprotein (LDL) fraction. Plasma samples were mixed with 3 ml of Optiprep (1 part of Optiprep:4 parts 10% HEPES in 0.9% NaCl with 1 mM EDTA and 1 mM butylhydroxytoluene BHT). This mixture was underlayered with 20% Optiprep in the same diluent and was overlaid with 1 ml of HEPES-buffered saline with 1 mM EDTA and 1 mM butylhydroxytoluene (BHT) in an ultracentrifuge tube. The gradients were then centrifuged for 22 h at 33,500 rpm at 16°C and fractionated in 0.4-ml fractions. Fraction 1 was at the top, with a density of 1.00 g/ml, while fraction 20 was at the bottom of the gradient, with a density of approximately 1.12 g/ml. The density of each fraction was determined gravimetrically. Plasma TBARS was measured using equal volumes of 3.75 mg/ml thiobarbituric acid in 10% trichloroacetic acid, heated to 95°C for 15 min. The product was extracted into butanol and read at 532 nm in a microtiter plate reader (Versamax, Molecular Devices Inc., Sunnyvale, CA), using tetramethoxypropane for the standards. A TBARS assay kit (OXItek, ZeptoMetrix Corp, Buffalo, NY) was used to measure TBARS levels in whole-tissue homogenates. Duplicate samples were read on a spectrophotometer (Perkin Elmer Lambda 35, Boston, MA) and using a malondialdehyde (MDA) standard curve, and results were expressed as MDA equivalents.

**Nitrotyrosine and MMP-9 immunohistochemistry.** Aortas, n = 8 each for control, 60 µg/m³ PM whole exhaust, and 60 µg/m³ PM-filtered exhaust were fixed, paraffin embedded, and sectioned at 6 µm thickness. Sections were taken from the aortic arch of each sample. For MMP-9, six sections were taken per subject. Due to errors in preparation or staining, the number of acceptable samples for data analysis ranged from three to six per subject, although most subjects had six sections for analysis. For nitrotyrosine, only one section per animal was stained with averages of four image fields were captured per slide for analysis. Sections were deparaffinized, rehydrated and heated to 90°C for 5 min in 10 mM sodium citrate. Slides were incubated with PeroxO-Block (Zymed, San Francisco, NY) for 60 s and blocked with 5% goat serum (Sigma-Aldrich, St Louis, MO) phosphate-buffered saline for 1 h. Sections were incubated for 12 h with rabbit polyclonal anti-nitrotyrosine (NT) antibody (1:81; Upstate, Atlanta, GA) at 4°C and goat anti-rabbit secondary antibody HRP (1:1000; Upstate) for 40 min. Sections were visualized with a diamino-phenylindole (DAPI) for nuclear staining and with hematoxylin, and coveredslipped with Permount (Fisher Scientific, Denver, CO). Slides were imaged by light microscopy at 400×, digitally recorded, and analyzed by image densitometry using Image J software (NIH, Bethesda, MD). Briefly, RGB images of stained vessels were split to eliminate the counterstain (blue) signal, leaving primarily the HRP signal. Since both NT and MMP-9 are diffusely released into the extracellular space, analyses were based on intimal, medial, and plaque regions. Quantification was conducted by mean values from histogram analysis; data reported are normalized by area, and histograms of staining density were determined for all areas present.

**Lung cytokine multiplex assays.** Whole lungs were homogenized and diluted to protein levels (determined by Bradford assay) as recommended by the manufacturer (Luminex, Austin, TX). Luminex combines the principle of a sandwich immunoassay with the fluorescent bead-based technology, allowing multiplex analysis of several different analytes in a single microtiter well (Vignali, 2000). The Mouse Cytokine I multiplex was obtained from BioSource International (Camarillo, CA) to measure a panel of mouse cytokines (IL-4, IL-6, IL-12, IFN-γ, and TNF-α). Assays were performed in a 96-well microplate format according to the protocol by BioSource International. Briefly, an appropriate mix of beads were added to a filter-bottom, 96-well microplate (Millipore) and washed with wash buffer. All washes were vacuum filtered at 2 psi. To generate a standard curve, 4-fold dilutions of appropriate standards were prepared in assay diluent. The standards and samples were added to the filter plates containing the bead mix in 50 µl in duplicate and diluted to a total of 150 µl with standard diluent. Analytes and beads were allowed to shake on an orbital shaker at 500–600 rpm for 2 h at room temperature. Wells were washed with wash buffer and aspirated by vacuum filtration. A biotinylated detector antibody (100 µl) was added and allowed to incubate for 1 h with shaking. After washing, 100 µl of streptavidin-(R-phycocerythin)-conjugated secondary antibody was added to each well and incubated for 30 min with shaking in the dark at room temperature. Wells were washed three times, wash buffer was added to each well, and plates were allowed to shake gently until analysis. The fluorescence intensity of the detection antibody was determined using a BioFlex System (BioRad Laboratories). Fluorescence intensity readings for 100 beads per cytokine were collected for each standard and sample. A four-parameter algorithm was used to generate the standard curve.

**Statistical analysis.** Data are expressed as mean ± SEM. For statistical comparisons between multiple groups, a one-way analysis of variance was used with Holm-Sidak testing for post hoc testing between groups (Sigma Stat Version 3.1, Point Richmond, CA). A value of p < 0.05 was considered statistically significant.

**RESULTS**

**Gasoline Exhaust Characterizations**

Throughout the exposures, levels of PM, carbon monoxide (CO), nitrogen oxide species (NOx), and total hydrocarbons (THC) were measured (Table 1). Exposure concentrations followed a highly consistent daily profile, with repeatable average concentrations of the primary components. At the highest concentration (1:12 dilution of the exhaust), mice were exposed daily for 50 days to 60 µg/m³ PM, 19 ppm NOx, 80 ppm CO, and 12 ppm THCs. Filtration by HEPA removed the vast majority of PM (essentially down to control levels), while matching the high whole exhaust level for NOx, CO, and THC. For NOx less than 10% of the species was nitrogen dioxide. Additional exposures to lower dilutions (1:20, 1:90) were also included for most assays. The levels of CO and NOx exceeded NAAQS at the high levels but were within allowable limits in the low-concentration exposures. It is important to note that the filtered-air control has an actual measurement of 2 µg PM/m³, not 0, due to measurement of particles present from sources such as mouse dander and particles generated from bedding contained within the animal housing during the exposure; this PM concentration does not represent contamination from engine emissions. More detailed characterization of gaseous and particulate fractions of the gasoline engine emissions was conducted and will be communicated in a forthcoming manuscript. Currently, such details may be obtained by visiting the National Environmental Respiratory Center Web site (http://www.nercenter.org/) or by contacting the authors.

**Lung Cytokines and Pathology**

To assess pulmonary inflammation, lung tissue from the filtered air, 8-µg/m³, 60-µg/m³ and 60-µg/m³ PM-filtered concentrations were assayed in a multiplex cytokine assay. No effects were observed on the whole-lung levels of IL-4,
IL-6, IL-12, IFN-γ, or TNF-α (Table 2). No lesions or pathological signs were observed in the lungs macroscopically at sacrifice, and no edema was apparent. Exposure groupings were matched for body weight; by the completion of the study, no significant changes in body weights or growth rate had occurred (final weights in mean ± SE: 31.7 ± 0.9, 32.6 ± 0.9, 33.3 ± 1.5, 32.5 ± 1.0, and 30.9 ± 1.3 for control, low, mid, high, and high-filtered groups, respectively, N = 8 per group). Additionally, no changes in lung:body weight or heart:body weight ratios were observed.

Effects of Gasoline Emission Exposure on Vascular MMP and TIMP Transcription

To determine whether MMP expression was affected by gasoline engine exhaust exposure, RNA was isolated from aortas of animals from each of the study groups. Real-time RT-PCR was used to quantitatively measure expression of MMP-3, -7, -9, and -12, which are associated with both human and murine atherosclerosis. Exposure to gasoline engine exhaust resulted in a 2-fold induction at both the 60-µg/m³ PM and PM-filtered concentrations in aortic expression of MMP-3 (Fig. 1A). Similarly, we observed a concentration-related increase in aortic expression of MMP-7 (2.5-fold at the highest level; Fig. 1B) and MMP-9 (4-fold at the highest level; Fig. 1C). Aortic expression of MMP-12 (Fig. 1D) was not significantly altered by gasoline exhaust exposure. Interestingly, expression of each induced MMP at the 60-µg/m³ PM-filtered concentration showed at least an equal, if not higher, increase in expression compared to the 60-µg/m³ PM exposure. MMP-9 protein expression was additionally confirmed by immunohistochemistry, where the increase in MMP-9 expression was localized to the endothelial cells and plaque regions (Fig. 2).

In addition to MMP expression, we also quantified aortic expression of the endogenous inhibitors of MMPs, namely, TIMP1 and TIMP2. Using the methods described above, we observed that aortic TIMP1 mRNA expression was slightly decreased (Fig. 1E), whereas TIMP2 mRNA expression was significantly elevated in a concentration-dependent manner (Fig. 1F). Again, no significant difference in either TIMP expression was observed between whole or PM-filtered gasoline engine emission exposures.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Cytokine Levels from Whole-Lung Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered air</td>
<td>8 µg/m³</td>
</tr>
<tr>
<td>IL-4</td>
<td>49.7 (4.7)</td>
</tr>
<tr>
<td>IL-6</td>
<td>85.1 (57.2)</td>
</tr>
<tr>
<td>IL-12</td>
<td>404 (58)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>16.8 (3.3)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>19.2 (21.6)</td>
</tr>
</tbody>
</table>

Note. Values represent mean (SD) (in pg/ml). n = 6 for each group. No significant changes were measured between samples.

Gasoline Engine Emission Exposure Increases Expression of Vascular Peptides

To determine which pathways may be involved in the upregulation of MMP expression, we examined the expression of compounds that are associated with vascular disease states, namely, the vasoactive and mitogenic peptide ET-1 and the stress-induced protein HO-1. Using real-time RT-PCR, we found aortic ET-1 (2.3-fold at the highest level; Fig. 1G) and HO-1 (2.8-fold at the highest level; Fig. 1H) mRNA expression to be significantly elevated in an exposure concentration-dependent manner. There was no significant difference in vascular expression observed in either ET-1 or HO-1 in whole gasoline engine emission exposures compared to PM-filtered exposures.

Effects of Gasoline Engine Emission Exposure on Vascular and Circulating Indicators of Oxidative Damage

To assess ROS levels in aortas from gasoline exhaust–exposed ApoE−/− mice, we first examined NT staining of histology sections from aortas of mice in the control group and 60-µg/m³ PM and PM-filtered concentration groups, as an indicator of peroxynitrite (·ONOO) generation and subsequent nitrosylation of proteins (indicated by brown staining). Compared to control samples (Fig. 3A, negative antibody control, and 3B, filtered-air control), our results show an elevation in NT staining in aortas from ApoE−/− mice exposed to both 60 µg/m³ PM and PM-filtered gasoline exhaust (Fig. 3C). The pattern of staining was diffuse and while similar trends were noted in intimal and plaque regions, only the medial assessments demonstrated a significant effect of gasoline exhaust exposure. There was no significant difference in NT staining found between aortas from whole versus PM-filtered gasoline engine emission exposure groups.

We next assayed aortic and plasma TBARS levels as an indicator of lipid peroxidation. Gasoline engine exhaust exposure caused a significant elevation in aortic TBARS levels in the 8-µg/m³ PM dose, with further increases observed at the 40- and 60-µg/m³ PM, and PM-filtered concentrations (Fig. 4A). TBARS and lipid peroxidation end points in the density-fractionated plasma, on the other hand, were largely negative, and we found no statistical difference in control versus gasoline engine emission exposure TBARS levels at any exposure concentration (Fig. 4B).

DISCUSSION

Our results describe alterations in several pathways involved in vascular remodeling which are associated with the progression of atherosclerosis, in response to subchronic inhalational exposure to gasoline engine exhaust. These results included transcriptional increases in MMP-3, -7, and -9,
TIMP2, ET-1, and HO-1 mRNA, along with increased markers of oxidative stress. Unlike recent findings by Sun et al. (2006), our results indicate that the gaseous portion, not the PM fraction, of the whole exhaust mediated the observed endpoints; however, both studies confirm that environmentally relevant levels of inhaled toxicants can induce vascular effects in a susceptible model.

Atherosclerosis is an inflammatory disease characterized by the presence of arterial lesions that contain fat-filled macrophages (foam cells), endothelial dysfunction, and remodeling of the arterial wall (Ross, 1999). The primary mediators of extracellular matrix (ECM) degradation are included in the MMP family of proteins (Galis and Khatri, 2002; Galis et al., 1994), which have been shown to influence cell differentiation, migration, and proliferation in the vasculature (Newby, 2005). Vascular MMP activity is regulated at multiple levels including gene transcription, posttranslational activation, and interactions with TIMPs (Brew et al., 2000). Furthermore, both ROS

FIG. 1. Transcriptional changes in aortic MMP, TIMP, HO-1, and ET-1 mRNA, as determined by real-time RT-PCR. Expression of aortic MMP-3 (A), MMP-7 (B), MMP-9 (C), MMP-12 (D), TIMP1 (E), TIMP2 (F), ET-1 (G), and HO-1 (H) mRNA in ApoE−/−/C255 mice exposed for 6 h/day × 7 weeks to filtered air (controls, 0); 8, 40, and 60 μg/m³ PM; or 60 μg/m³ PM-filtered (60 PM-F) concentrations. N = 8 for each group represented. Data show mean normalized gene expression (to 18S) ± SEM. *p ≤ 0.050 compared to filtered-air controls; †p ≤ 0.050 compared to 8 μg/m³ PM; ‡p ≤ 0.050 compared to 40 μg/m³ PM.
Rajagopalan et al. (1996) and vasoactive peptide ET-1 (Ergul et al., 2003) have been associated with the upregulation of vascular MMP expression and activity.

Our results show that exposure to gasoline engine emissions increased expression of vascular MMP-3, -7, and -9 mRNA in ApoE−/− mice; measurement of MMP-9 protein levels by immunohistochemistry was confirmatory, albeit not as robust, statistically. It is hypothesized that the balance between MMPs and TIMPs is crucial in regulating ECM homeostasis, which is supported by findings that show upregulation of MMP expression and activity in a number of disease processes, including atherosclerosis (Newby, 2005). Specifically, MMP-2 and -9 (Galis et al., 1994; Li et al., 1996), MMP-1 (Galis et al., 1994), MMP-3 (Galis et al., 1994), MMP-7 (Halpert et al., 1996), and MMP-12 (Li et al., 1996) have been detected at the protein or mRNA level in atherosclerotic lesions, but not in normal human vessels (except MMP-2). In agreement with these findings, MMP-9, but not MMP-12, has also been reported to be involved in atherosclerotic plaque growth in ApoE−/− mice (Luttun et al., 2004). Our findings of exposure-related changes in vascular TIMP expression are analogous to previous findings in both human and murine studies, which report that TIMP1 is found to be relatively unchanged in atherosclerotic remodeling (Galis et al., 1994), while TIMP2 expression is highly upregulated (Tabibiazar et al., 2005; Woessner, 1991). It is not known whether the upregulation of TIMP2 is a feedback mechanism in response to the overexpression of MMPs, although logic would suggest that such a negative feedback mechanism in the vasculature would be essential for homeostasis.

Subchronic exposure to gasoline engine emissions resulted in increased vascular levels of ET-1 and HO-1 mRNA, in association with elevated MMP expression. We have previously observed that circulating plasma ET-1 is significantly elevated in ApoE−/− mice exposed to the 8-μg/m³ concentration (with dose-related elevations observed for the 40-μg/m³ PM, 60-μg/m³ PM, and PM-filtered concentrations) after only an acute 3-day exposure (Campen et al., 2006), which is consistent with other PM health effect studies (Thomson et al., 2005). A key feature of atherosclerosis is the imbalance of vasoactive factors produced by the endothelium, including ET-1 (Ross, 1999). In agreement with this premise, previous studies have demonstrated elevated ET-1 expression in atherosclerotic vessels, compared with normal vessels (Ihling et al., 2001). ET-1 is reported to aggravate atherosclerosis by its stimulatory activities on smooth muscle cell mitogenesis,

![FIG. 2. MMP-9 staining in aortas from ApoE−/− mice exposed to filtered air or gasoline engine exhaust. Staining for MMP-9 shown in (A) a negative control (no antibody), (B) a control exposure (filtered air) animal, and (C) whole exhaust-exposed subject; representative images for the PM-filtered exhaust-exposed mice were similar to those of the high whole exhaust–exposed mice and are therefore not included. Arrows indicate increased MMP-9 staining (brown) in the intimal region of aortas from animals exposed to whole 60-μg/m³ exhaust concentrations (C) (statistically similar results were seen in the PM-filtered vessels), compared to control (filtered air) vessel. Regions are denoted as luminal (L) and medial (M); intimal regions were defined by the cells along the luminal border, except for plaque regions (*p < 0.05 by analysis of variance). N = 6 per group; plaque regions also showed increasing trends but too few samples were available for appropriate statistics.]
neutrophil adhesion, and platelet activation (Luscher and Barton, 2000). Conversely, HO-1 is transcriptionally upregulated as a sensitive anti-inflammatory protein by various types of oxidative stress, such as oxidized LDL (Wang et al., 1998), and is highly expressed in atherosclerotic lesions (Ishikawa et al., 2001). Thus, ET-1 is believed to play a role in the progression of atherosclerosis through induction of MMP expression (Ergul et al., 2003) and stimulation of ROS (Duerrscheidt et al., 2000), while HO-1 induction likely confers protection against vascular injuries associated with elevated ROS.

While MMPs promote vascular oxidative stress (Hao et al., 2006), the converse has also been shown, such that ROS upregulates most MMP isoforms (Newby, 2005). We therefore investigated whether vascular and systemic ROS levels were similarly elevated in response to gasoline engine emission exposure. Vascular content of •OONO was increased in the exposed animals, while TBARS was significantly elevated in a concentration-dependent manner. Interestingly, TBARS levels in the plasma showed no significant changes and were potentially decreased by exposure at the higher fractions. These results suggest that the lipid peroxidation products measured by the TBARS assay may be specifically generated in the diseased aortas or that lipid peroxide by-products generated elsewhere (presumably the lung) may be selectively scavenged by macrophages present in the atherosclerotic regions of the vascular wall.

For the studies described herein, we used an animal model of atherosclerosis, ApoE<sup>−/−</sup> mice, which develop atherosclerotic lesions when fed a high-cholesterol diet (Zhang et al., 1994). Cholesterol was not specifically measured in the present study, but previous reports of ApoE<sup>−/−</sup> mice on a similar diet report values around 1300 mg/dl (Sun et al., 2006). The exposure concentrations used in the present study, which range from environmentally to occupationally relevant, did not result in observable pulmonary inflammation in ApoE<sup>−/−</sup> mice either subchronically (as reported here) or acutely, as previously demonstrated (Campen et al., 2006). Previously, we had investigated more conventional assays of bronchoalveolar lavage cytology and chemistry and seen no effects in ApoE<sup>−/−</sup> mice exposed to gasoline exhaust for 3 days. In complementary studies with short-term diesel exhaust exposures, pulmonary inflammation was only observed at PM concentrations of 3.5 mg/m<sup>3</sup>, with no differences between ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup> mice.

**FIG. 3.** Nitrotyrosine (NT) staining in aortas from ApoE<sup>−/−</sup> mice exposed to filtered air or gasoline engine exhaust. Staining for NT shown is in (A) a negative control (no antibody), (B) a control exposure (filtered air) animal, and (C) whole exhaust–exposed subject; representative images for the PM-filtered exhaust–exposed mice were similar to those of the high whole exhaust–exposed mice and are therefore not included. Increased NT staining (brown) was observed diffusely in aortas from animals exposed to both whole and PM-filtered 60 µg/m<sup>3</sup> exhaust concentrations, compared to control (filtered air) vessel. Nonsignificant trends were observed in the intimal and plaque regions, but only medial regions showed a statistically significant effect of exhaust exposure (*p < 0.05 by analysis of variance). Regions are denoted as luminal (L) and medial (M); intimal regions were defined by the cells along the luminal border, except for plaque regions (AP). N = 6 per group for intima and medial regions; N = 3, 5, and 3 for control, whole, and PM-filtered exhausts, respectively, for plaque imaging.
C57BL/6J mice, suggesting that the ApoE<sup>−/−</sup> mouse model is not unusually sensitive to pulmonary insults (Campen et al., 2005).

At the lowest exposure level used in these studies, gasoline engine exhausts were diluted 1:90, with PM levels at approximately 8 µg/m<sup>3</sup> for the 6-h exposure. The net contribution of gasoline exhaust to ambient PM has been estimated at approximately 10% (Kim and Hopke, 2006; Zheng et al., 2002). Thus, even on days when PM is within allowable NAAQS limits, the contribution from gasoline engines may plausibly achieve 5–15 µg/m<sup>3</sup> for a 24-h average, which is well within our dose-response range. To test the role of specific components in the gasoline engine emissions, we conducted concurrent exposures with PM-filtered concentrations that were matched by CO, NO<sub>x</sub>, and THC levels. No statistical differences were observed in any of our measured end points in the vasculature of animals exposed to the 60-µg/m<sup>3</sup> PM or PM-filtered concentrations. We found previously that gasoline exhaust PM can specifically alter ECG acutely in the ApoE<sup>−/−</sup> mouse model, although changes in circulating ET-1 appeared dependent on the gas phase (Campen et al., 2006).

The finding that particles in our study were unrelated to changes in any of the end points assayed was somewhat surprising, given the numerous reports of PM effects on cardiovascular health. It should be noted, however, that a primary criticism of the ecological epidemiological studies has been the associative nature of the analyses and the ever-present inability to account for all potential confounders. PM, therefore, has been suggested to be a surrogate for some other pollutants (Moolgavkar, 2005). However, the present gasoline exhaust atmosphere, representing a major mobile pollutant source, was relatively lower in PM than in other important pollutants including CO, which at the high level was roughly seven times the 8-h average allowable limit. NO<sub>x</sub> species and hydrocarbons were also relatively high in comparison to the overall PM mass. The impact of the levels of these copollutants on the interpretation of our results will, necessarily, be subjective pending further study, and while PM filtration was highly efficient even at the nanometer range, the possibility of off-gassing of semivolatile compounds from the PM on HEPA filters cannot be discounted.

In conclusion, our findings show a significant increase in aortic expression of ET-1, HO-1, MMPs (MMP-3, -7, -9), and TIMP2, associated with elevated ROS, in response to subchronic exposure to the ubiquitous environmental air pollutant, gasoline engine exhaust. These end points are factors involved in mediating vascular remodeling, inflammation, and the progression of atherosclerosis. Our results also indicate that these observations are not a direct effect of the PM in the emissions since animals exposed to the PM-filtered atmosphere exhibited similar inductions to those in the 60-µg/m<sup>3</sup> whole exhaust group. The present study results infer a role for the MMP isoforms in the chronic vascular remodeling process, but acute induction of MMPs may contribute to adverse health effects, as well. Recent findings implicate upregulation of macrophage MMP-9 or lesion-associated MMP-9 with acute plaque destabilization (de Nooijer et al., 2006; Gough et al., 2006). Further investigations into the acute outcomes of air pollutant exposure should help to confirm such hypotheses. Considering the socioeconomic ramifications of cardiovascular disease on health care today, it is critical to determine the role that common environmental factors may contribute to the onset and/or progression of such diseases.

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FIG. 4. Lipid peroxides in plasma and aortas from ApoE<sup>−/−</sup> mice exposed to filtered air or gasoline engine exhaust. Aortic (A) and plasma (B) TBARS levels were quantified by comparing samples from each treatment group, 0 (control); 8, 40, 60 µg/m<sup>3</sup> PM; or a 60-µg/m<sup>3</sup> PM-filtered (60 PM-F) concentrations (n = 6 for each group), to a MDA standard curve. For plasma, TBARS levels were determined for the control, high whole exhaust (High), and 40-µg/m<sup>3</sup> (Mid) concentrations only. Data show mean ± SEM and results are expressed as MDA equivalents. *p ≤ 0.050 compared to filtered-air controls; †p ≤ 0.050 compared to 8 µg/m<sup>3</sup> PM; ‡p ≤ 0.050 compared to 40 µg/m<sup>3</sup> PM.
REFERENCES


atherosclerosis in mice heterozygous and homozygous for apolipoprotein E 

apportionment of PM2.5 in the Southeastern United States using solvent-