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Inhaled Diesel Emissions Alter Atherosclerotic Plaque Composition in ApoE^{-/-} Mice

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Abstract

Recent epidemiological studies suggest that traffic-related air pollution may have detrimental effects on cardiovascular health. Previous studies reveal that gasoline emissions can induce several enzyme pathways involved in the formation and development of atherosclerotic plaques. As a direct comparison, the present study examined the impact of diesel engine emissions on these pathways, and further examined the effects on vascular lesion pathology. Apolipoprotein E-null mice were simultaneously placed on a high fat chow diet and exposed to four concentrations, plus a high concentration exposure with particulates (PM) removed by filtration, of diesel emissions for 6 h/d for 50 days. Aortas were subsequently assayed for alteration in matrix metalloproteinase-9, endothelin-1, and several other biomarkers. Diesel induced dose-related alterations in gene markers of vascular remodeling and aortic lipid peroxidation; filtration of PM did not significantly alter these vascular responses, indicating that the gaseous portion of the exhaust was a principal driver. Immunohistochemical analysis of aortic leaflet sections revealed no net increase in lesion area, but a significant decrease in lipid-rich regions and increasing trends in macrophage accumulation and collagen content, suggesting that plaques were advanced to a more fragile, potentially more vulnerable state by diesel exhaust exposure. Combined with previous studies, these results indicate that whole emissions from mobile sources may have a significant role in promoting chronic vascular disease.

Keywords

Particulate Matter; Vascular Inflammation; Air Pollution; Inhalation

Introduction

Exposure to traffic-related air pollution has been associated with the incidence of myocardial infarction and, more recently, proximity to highways has been associated with chronic vascular disease (Peters *et al.*, 2004; Hoffmann *et al.*, 2007). While controlled human studies of

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concentrated particulate matter (PM) exposure have provided evidence of mild vascular effects (Mills *et al.*, 2008; Shah *et al.*, 2008), the whole exhaust from diesel emissions appears to significantly impact vascular function in healthy and compromised individuals (Tornquist *et al.*, 2007; Mills *et al.*, 2007) and combined ozone and concentrated PM led to significant narrowing of arteries in healthy individuals (Brook *et al.*, 2001). In terms of chronic vascular effects, exposures to concentrated PM caused a promotion in the size of vascular lesions in a murine model of atherosclerosis (Sun *et al.*, 2005; Araujo *et al.*, 2008). Additionally, toxicological research with susceptible animal models has further suggested a strong role for non-particulate contaminants in mediating extrapulmonary health effects (Campen *et al.*, 2005; Lund *et al.*, 2008). As PM is invariably a component of an otherwise complex background of pollutants, there is concern that studies of PM, alone, may underestimate the cumulative health impact of urban air pollution.

Previous research on apolipoprotein E-null (ApoE^{-/-}) mice exposed to gasoline emissions identified several responsive transcriptional markers of vascular toxicity (Lund *et al.*, 2007). The mRNA coding for several matrix metalloproteinases (MMP) was found to be upregulated in aortas in a dose-dependent manner, along with that of endothelin-1 (ET-1) and heme oxygenase-1 (HO-1). Further characterization of short-term effects verified that protein and activity of MMP-9 increased due to gasoline emissions exposure, that ET receptors were crucial to this effect, and also identified parallel biomarkers in humans exposed to diesel exhaust (Lund *et al.*, 2009). The relevance of this pathway is primarily related to remodeling of the vascular wall, both in terms of chronic atherosclerotic lesion development and acute destabilization of advanced plaques (Newby, 2005). However, previous research efforts did not ascertain whether gasoline emissions induced growth or remodeling of plaques.

Perhaps of greater interest was the increased level of aortic lipid peroxides in gasoline-exposed mice, which was similarly unaltered by removal of PM from the pollutant atmosphere (Lund *et al.*, 2007). Oxidized lipids are known drivers of vascular disease (Leitinger, 2003), with demonstrated ability to induce atherosclerotic inflammation (Furnkranz *et al.*, 2005) and alter the phenotype of vascular smooth muscle cells (Pidkovka *et al.*, 2007). More advanced plaques, pathologically-speaking, are characterized as having higher levels of lipid peroxides (Nishi *et al.*, 2002). Recent *in vitro* studies note a synergistic relationship between specific oxidatively-modified lipids and diesel exhaust particulate matter, in terms of genomic responses (Gong *et al.*, 2007). In the present study, we revisit the profile of vascular toxicity assays with a model of diesel engine emissions, and further add histopathological assays of plaque composition with a focus on the impact on vascular lipids.

Materials and 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, 1.5g/kg cholesterol content) beginning on the first day of exposure, and subsequently exposed to one of four concentrations of diesel engine exhaust (DEE) or a filtered air atmosphere (controls) for 6 h/d × 7 d/wk for a period of 50 days. 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 housed outside of the exposure chambers until sacrifice. During the study period, all animals were exposed concurrently to either filtered air or to emissions at 100, 300, or 1,000 µg PM/m³ or a PM-filtered exhaust with gaseous concentrations matching those in the 1,000 µg PM/m³ concentration (n=10 for each group). 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 US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Upon completion of the 50-day exposure period, animals were sacrificed 16 hours after their last exposure.

Generation and Analysis of Atmospheres

Exhaust generation, dilution, exposure conditions, and exposure characterization methodology are described in detail in the protocol for the National Environmental Respiratory Center (NERC) Core Study I: Contemporary Diesel Emissions, on the NERC website (www.nercenter.org; McDonald *et al.*, 2004; Reed *et al.*, 2004). Briefly, DE was generated by either of two Cummins (2000 model) 5.9 L ISB turbo engines fueled by Number 2 Diesel Certification Fuel (340 ppm sulfur, 29% wt aromatics), lubricated with 15W-40 motor oil (Shell Rotella-T), and operated on a repeated (20 min) version of the EPA heavy-duty test cycle (EPA, U.S. Code of Federal Regulations, Title 40, Appendix 1). Exposure chamber temperature and humidity were monitored throughout exposures, and temperatures were maintained in the range of 20–26.7° C. Exposure atmospheres consisted of five levels: a clean air control and four dilutions of DE designed to achieve target concentrations. The clean-air control and diesel-dilution air were pre-treated by passage through a carbon-impregnated filter to remove volatile organics and through a HEPA filter to remove PM.

For analysis of the atmospheres, the particle concentration was monitored by sampling on 47-mm Pallflex (Pall-Gelman) filters. Pre-filter and post-filter weights were measured by using a Mettler MT5 microbalance. A static discharger was used before weighing to avoid any interference from electrical charge on the filters. Total NO_x was measured by using a chemiluminescent analyzer (API Model 200A NO_x Analyzer). CO was determined by using a Photoacoustic Gas Analyzer (Innova 1312). SO₂ was collected by using potassium carbonate-impregnated filters and was analyzed by ion chromatography (Dionex 500DX). Particle size was measured by using a 10-stage micro-orifice uniform-deposit impactor (MSP Corp.). The impactor was operated at a flow rate of 30 lpm, providing particle size resolution of 0.05–10 microns in aerodynamic diameter.

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. Blood was collected in an EDTA syringe (BD Vacutainer Systems, Franklin Lakes, NJ) through cardiac puncture, and immediately centrifuged (950 × 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 aortas were dissected, weighed and frozen in liquid nitrogen. Tissue was stored at –80°C until assayed.

Real time RT-PCR

Total RNA was isolated from the aortic arch as previously described (Lund *et al.*, 2007), with n=8–10 for each exposure group, using RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA). Real time PCR was performed using an iCycler^{TM} (Biorad, Hercules, CA) and an ABI 7500 (Applied Biosystems, Foster City, CA).

Aortic Lipid Peroxides (TBARS Assay)

To assess vascular oxidative stress, lipid peroxidation was assessed using a TBARS assay, as previously described (Lund *et al.*, 2007). The descending aorta (N=10 per group) was

resuspended by diluting 1:10 weight/volume in normal saline, and then homogenized and sonicated for 15 s at 40 V; homogenates were used to determine TBARS levels as described below. A TBARS assay kit (OXItek, ZeptoMetrix Corp, Buffalo, NY) was used to measure lipid peroxide levels in whole tissue homogenates, normalized to total protein in the homogenates. Duplicate samples were read on a spectrophotometer (Perkin Elmer Lambda 35, Boston, MA), and using a malondialdehyde (MDA) standard curve, and results expressed as MDA equivalents.

Plasma TBARS in LDL fraction

Butylated hydroxyl toluene (BHT: 15 μ M final concentration) was added to the plasma, which was then centrifuged for 10 min at 4°C in a microfuge. The infranant (100 μ l) was combined with 100 μ l of PEG-HDL reagent (Pointe Scientific) to precipitate the LDL/VLDL fraction. Following incubation on ice for 5 minutes, the sample was centrifuged again and the pellet was resuspended in 1.0 ml PBS containing 1 mM EDTA and 0.01 mM BHT. TBARS were measured and the results were normalized to the total cholesterol content of the fraction, measured with an enzymatic cholesterol determination reagent (Pointe Scientific).

Immunohistochemistry

Hearts were removed and the base containing the aortic outflow tract was immediately frozen in OCT. Cryostat-sections (8 μ m) from throughout the aortic valve (8–12 sections per mouse) were stained with Oil Red O (0.5% in propylene glycol; Polyscientific) and individually analyzed for total wall area and total lesion area by tracing individual leaflet areas using Metamorph (Universal Imaging Corp). Intervening sections were placed 3 per slide and stained for collagen (Sirius red; 0.0003125% in sat. aq. picric acid; F3B[CI:35780]; RALAMB), smooth muscle (mouse monoclonal anti- α -smooth muscle actin; 1:100; M0851, Dako), or macrophages (mouse monoclonal anti-MOMA-2, 1:25; MCA519, Serotec) content. Mouse monoclonals were biotinylated, blocked with normal mouse serum, and sections stained streptavidin-peroxidase using diaminobenzadine (Dako) as chromagen. The specific Oil Red O-, collagen-, smooth muscle- and macrophage-stained lesion areas were measured using Metamorph threshold function, which covered >95% of stained area in all sections. Total valve and total lesion areas were the sum of all individual areas measured throughout the valve (8–12 sections). Specific stained areas (e.g., collagen) were divided by the total lesion area for calculation of the ‘percentage stained area.’

Statistical Analysis

Analysis of variance (ANOVA) was used to evaluate systematic exposure-related variation in vascular remodeling parameters. For several parameters, there was substantial evidence of inequality of variances across experimental groups, which reflected positive associations between standard deviations and mean values. In these instances, logarithmic transformations were applied to the data prior to analysis. Dunnett’s multiple comparison procedure was used to compare treated group responses to control values. The linear term of the ANOVA was used to assess the significance of exposure-related trends across non-filtered exposure groups. To assess the effect of particle filtration, mean values in the PM-filtered high exposure group were compared to those of the unfiltered high exposure group based on a t-test employing the pooled variance estimate from the ANOVA. Summary statistics reflect scaling of the data by control mean values. Statistical significance was assessed at $p \leq 0.05$ and $p \leq 0.01$.

Results

Characterization of Atmospheres

As previously described, the diesel PM composition was ~60% elemental carbon, ~20% organic carbon, with smaller portions of ammonium, sulfate, and nitrate contributions (McDonald *et al.*, 2004; Reed *et al.*, 2004). The total mass of all emissions (minus water, carbon dioxide, and methane) in the high concentration DEE atmosphere was 91.7 mg/m³. While this seems high, it must be kept in mind that this is typical of engine exhausts and the gaseous portion is a far greater mass output of fossil fuel combustion. This mass was primarily attributable to the contributions of CO and NO_x in diesel, with PM contributing only a small fraction of the total mass (~1%). NO₂ concentrations range from ~350 ppb to 3.5 ppm, assuming the mix of NO_x was roughly 10% NO₂, as we have previously reported (Reed *et al.*, 2004) and CO concentrations ranged from 2.7 to 27 ppm. Details for all five concentrations are provided in Table 1, given in mass concentration units. It is worth noting that on a mass basis, urban pollution typically mimics this relationship, with PM being a small contributor by mass, although not necessarily in terms of biological relevance.

Measures of Aortic mRNA

Diesel emissions-exposed mice displayed an interesting panel of mRNA responses in the aortic arch. We first examined the changes in several MMPs, noting a concentration dependent increase in MMP-9 (~60% increase at the high concentration), and a significant decrease in MMP13 (Figure 1). This latter effect in the collagenase MMP13 (along with a similar albeit nonsignificant pattern in another collagenase MMP8) was interesting in that control levels were characterized by a high level of variability, perhaps due to the ongoing vascular pathology in ApoE^{-/-} mice, and exposure significantly dampened the expression. Other MMPs (2, 7, 8, and 12) showed no significant trends.

Examining complementary markers of vasculotoxicity, we observed a very clear dose-dependent upregulation for endothelin-1 (~100% increase at the high concentration; Figure 2). TIMP2 was significantly upregulated, between 2–3 fold over control, but not in a dose dependent manner with peak effects seen in the low and medium groups. Heme oxygenase-1 and TIMP1 (Figure 2), along with VEGF (not shown) showed no significant change from baseline. Removal of PM from the diesel atmospheres had no significant impact on any of these outcomes.

Plaque Alterations

We examined atheromatous lesions in the aortic leaflet region to ascertain alterations in plaque growth and composition. In diesel-exposed ApoE^{-/-} mice, a non-significant plaque area increase of approximately 13% was observed (Figure 3a). As mice were maintained on a high-fat diet for 50 days, it conceivable that the growth was close to maximal even in controls. However, in examining composition features of the plaques, several diesel-induced alterations were noted. Macrophage staining was significantly increased in diesel-exposed vessels at both medium and high exposures, with a greater than two-fold increase at the medium concentration, compared to controls ($p < 0.01$; figure 3b). Collagen staining showed a clear dose-dependent increase across non-filtered groups, with a significant linear trend ($p = 0.021$; Figure 3c); a subtle decrease was noted when PM was removed, but this effect was not statistically significant. A similar, albeit non-significant response pattern was observed for smooth muscle cell staining ($p = 0.072$; Figure 3d).

Interestingly, lipid staining by oil red o was reduced in the leaflet plaque regions of diesel exposed mice ($p < 0.01$; Figure 4a), contrary to the increased levels of lipid peroxides observed in the lower portion of the aorta (Figure 4b). Overall, the plaques from diesel-exposed mice

were characterized as having a less consolidated appearance, primarily in terms of lipid deposition (Figure 5a,b). Macrophage infiltration, assessed by the brown MOMA-2 staining, is relatively absent in control images (Figure 5c) but can be readily observed in diesel exposed leaflets (Figure 5d). Similarly, Sirius Red staining is not elevated above background adventitial levels in control animals (Figure 5e), but is significantly elevated in the plaque regions of diesel exposed mice (Figure 5f). Combined with the increased presence of macrophages, this manifestation is consistent with an overall advancement of plaque pathology.

Lipid Peroxides

A strong, dose-dependent induction of lipid peroxides (TBARS) was noted in aortas from the diesel-exposed mice (Figure 4b). The overall effect in the high concentration group was greater than three-fold increased over control mice. Interestingly, removal of particles by filtration caused a non-significant reduction in the TBARS levels (~ 30%), suggesting a role for PM in the systemic responses. When assessed in the LDL fraction of plasma, however, no significant changes in lipid peroxides were observed (Figure 4c).

Discussion

Diesel emissions had a clear impact on systemic vascular pathology in the pro-atherogenic ApoE^{-/-} mouse model. Compared to non-exposed control mice, aortas from diesel-exposed ApoE^{-/-} mice showed increased transcription of several markers of vascular remodeling, including MMP-9 and ET-1, as well as overall changes in plaque composition, including increased macrophage staining. Interestingly, while overall lipid content and consolidation appeared to decrease in the plaques from diesel-exposed mice, the levels of lipid peroxides, as measured by TBARS, increased substantially. The overall pattern of effects is consistent with the promotion of the plaque to a more vulnerable state, which may partially explain increased incidence of myocardial infarction. Filtration of particulate matter did not significantly reduce most responses to whole emissions, but the strength of several responses appeared reduced in magnitude by this permutation. Thus, the contribution to vascular remodeling of the particulate and vapor phases of the complex emissions cannot be easily dissected.

This study is directly comparable with our earlier work with gasoline engine emissions (Lund *et al.*, 2007), which were far more potent in inducing most of the transcriptional markers, but not quite as potent in terms of the lipid peroxidation. The total mass of emissions in the highest gasoline concentration of that study exceeded 140 mg/m³, which was predominantly CO. PM in the gasoline engine emissions represented a minute total mass portion of the emissions (60 µg/m³) and was, expectedly, not a significant contributor to the vascular outcomes. With a considerably greater mass concentration than the diesel emissions atmosphere in the present study (vapor plus particulate phase ≈ 90 mg/m³) and levels of carbon monoxide and certain volatile organic compounds were considerably lower in diesel, it is perhaps not surprising that the systemic vascular responses, for the most part, were more moderate following diesel exposure than in mice exposed to gasoline engine emissions.

Induction of ET-1 and MMP-9 mRNA was dose-dependent and less robust than was previously seen with gasoline emissions (Lund *et al.*, 2007). ET-1, which has been implicated in the vascular effects of air pollutants by a variety of groups (Calderón-Garcidueñas *et al.*, 2007; Campen *et al.*, 2006; Thomson *et al.*, 2005), has several potential roles in vascular pathology. ET-1 is a potent vasoconstrictive and mitogenic peptide (Yanagisawa *et al.*, 1988; Hirata *et al.*, 1989), but ET-1 may also aggravate atherosclerosis by its stimulatory activities on neutrophil adhesion and platelet activation (Luscher and Barton, 2000), as well as the ability to stimulate ECM degrading metalloproteinases (Ergul *et al.*, 2003). Recent studies with gasoline emissions confirm that the ET_A receptor is crucial to both the transcription and activity of MMP-9 (Lund *et al.*, 2009). While we assume that ET-1 is the upstream driver of this

response, there may conceivably be promiscuity of the ET_A receptor to other cytokines or even absorbed components of the emissions. Furthermore, while we empirically assume that mRNA transcription is elevated in vascular tissue, the increased recruitment of inflammatory cells in plaque regions suggests an alternative explanation, that PCR-related changes may reflect contributions from an altered cell population. As PM filtration appeared to reduce the macrophage staining but not mRNA changes, these pathways are likely unrelated.

Aortic macrophage infiltration, with concomitant changes in the composition of plaque regions, is perhaps the most remarkable observation from the present study. Several studies have pointed to either an activation of systemic immune cells or endothelial cells, leading to enhanced adherence to the vascular wall or extravasation into plaque regions. Repeated intratracheal instillation of PM₁₀ led to enhanced monocyte adherence and recruitment to aortic lesions in a rabbit model (Yatera *et al.*, 2008). Similarly, titanium dioxide particles can induce enhanced adherence and rolling of neutrophils that leads to deposition of myeloperoxidase along the vascular wall (Nurkiewicz *et al.*, 2006). While it is generally accepted that intraplaque inflammatory cells promote the pathology and affect a vulnerable state, the role of the infiltrating macrophages observed in the present study can only be speculated. Certainly, enhanced release of inflammatory cytokines, growth factors, and proteases would be expected to enlarge and destabilize lesions (Newby, 2005).

Despite the fact that mRNA markers were generally less responsive to diesel than shown previously with gasoline, the lipid peroxidation (measured by TBARS) in the aorta was greatly elevated by diesel emissions. Vascular lipid peroxidation was attenuated somewhat by the filtration of diesel PM, although statistically this effect was marginal. However, the net level of aortic TBARS in the PM-filtered group was reduced to approximately the level generated by the highest concentration of gasoline engine emissions (Lund *et al.*, 2007), further implicating an important, albeit partial, role for diesel PM. Interestingly, the overall lipid levels in the valvular lesions were significantly reduced and less consolidated as a result of diesel exhaust exposure. This may be due in part to infiltration of inflammatory cells and deposition of non-lipid components, thus relative volume of lipids becomes reduced and fibrotic deposits may interrupt lipid-rich regions.

We propose that the observed changes in plaque phenotype represent a promotion to an advanced, conceivably more vulnerable state. The ApoE^{-/-} mouse model has reasonable homology with human atherosclerosis pathogenesis, but is generally not a model of plaque rupture for several reasons. Most notably, due to the high fibrinolytic activity, small ruptures, or 'fissures' appear to readily resolve and heal, whereas in humans such events trigger myocardial infarction (Schwartz *et al.*, 2007). We did not examine indices of plaque rupture, such as fibrotic cap thickness, and so it is not appropriate to consider that the observed diesel-induced phenotype in the present study represents such an extreme pathological event. There is conflicting evidence regarding the use of oxidatively-modified lipids alone as a marker of plaque stability (Ravandi *et al.*, 2004; Torzewski *et al.*, 2004). However, it is becoming clear that the host immune response to the oxidatively-modified pool of lipids has a strong relationship with plaque vulnerability (Leitinger, 2003; Chou *et al.*, 2009). Antibodies against modified lipids and lipoproteins can drive a potent, chronic inflammatory response that leads to localized regions of infiltration and protease release, creating vulnerable regions. In the present study, we see clear evidence of a link between the oxidation of lipids and the extravasation of macrophages into the plaque regions of diesel-exposed mice. Collagen deposition may counter this conclusion, although more in-depth characterization is warranted for this endpoint.

What is lacking from these studies is a clear order of events. We do not yet understand if lipids are modified prior to scavenging into the vascular wall, if they are oxidized within the wall, or

if macrophages are activated elsewhere and bind to the wall of the plaque and cause the modifications. Currently evidence exists for all such hypotheses regarding the role of inhaled toxins and systemic vascular toxicity (Nurkiewicz *et al.*, 2006; Yatera *et al.*, 2008; Lund *et al.*, 2009). Another unresolved question relates to the reversibility of the phenotype. We did not address this in the present study, but while we generally assume the results of the 50-day exposure relates to chronic remodeling, there is every reason to consider that the inflammatory response is acute, and this would be consistent with recent studies showing similar effects as early as after a single day of gasoline emissions exposure (Lund *et al.*, 2009).

Filtration of PM from the atmospheres provided interesting insight into the components of the whole emissions that drive the observed effects. While not sufficiently powered to make strong conclusions, PM filtration appeared to reduce macrophage infiltration, collagen deposition and lipid peroxidation in aortas. However, no indication of PM effect was observed on the mRNA levels of ET-1, TIMP-2, or MMP-9. While it is conceivable that the $\sim 20 \mu\text{g}/\text{m}^3$ of residual PM in the filtered atmosphere was a selected portion of the PM that happens to be highly toxic, a more rational view would be that the $\sim 30 \text{ ppm CO}$ and $\sim 3 \text{ ppm NO}_2$, along with various other VOCs and copollutants, were able to drive these effects. The overall strategy of PM filtration was useful in this regard, and certainly could be useful on future studies. However, there are likely to be more complex interactions between PM and gases that may be missed by such approaches. Techniques to denude gases from combustion mixtures, leaving an enriched PM atmosphere, may similarly provide important insights into the respective toxic contributions of these components

Several caveats to the present study must be addressed. For one, the moderate vascular effects at exhaust concentrations in excess of environmental levels naturally draw into question the relevance of the findings. While the concentrations of PM in the various groups exceeded those used by Sun and colleagues (2005), the duration of exposure was less than a third of that 6-month protocol. Similarly, enlargement of plaques has been reported in mice exposed to ultrafine and fine PM at comparable mass concentrations to the present study, but for a slightly shorter period (Araujo *et al.*, 2007). In that study, a larger subject number was used for pathological endpoints, thereby strengthening the power for statistical conclusions. The present study used a potent atherogenic model that possibly obscured more subtle findings. Additionally, we only ascertained histological changes in one location, further diminishing our power to observe effects. However, unlike any other study of the atherogenic effects of air pollutants, the present study noted concentration-dependent effects, which strengthens the biological plausibility and forces us to consider that effects at lower concentrations may be observable in a study with greater statistical power. Despite these differences in study design, the interpretation of an advancement of atheromatous plaque remains consistent. While we were unable to discern a specific effect of diesel PM, several trends in the filtered emissions atmosphere indicated a likely biological contribution. However, the overall effect of the whole exhaust was substantial and exceeded the impact of PM, alone. As PM exposures are always accompanied by a milieu of co-pollutants, including carbon monoxide, oxides of nitrogen, and volatile organics, assessing risk of single component exposures would likely underestimate the overall health consequences related to ambient air pollution.

Thus, the findings from the present study demonstrate that inhaled air pollution, specifically diesel engine emissions, can alter the composition of advanced plaques. The increased lipid peroxides against a background of disrupted lipid deposits and increased macrophage invasion is consistent with a more vulnerable plaque. Observations of enhanced ET-1 and MMP9 expression may relate to these changes, although the extent to which these proteins mediate intraplaque alterations is uncertain. These data are consistent with patterns observed in numerous related studies of atherosclerotic advancement by traffic-related air pollution

exposure and offer potential pathways that may further explain the underlying mechanisms of air pollution-associated morbidity and mortality.

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References

1. Araujo JA, Barajas B, Kleinman M, Wang X, Bennett BJ, Gong KW, Navab M, Harkema J, Sioutas C, Lusis AJ, Nel AE. Ambient particulate pollutants in the ultrafine range promote early atherosclerosis and systemic oxidative stress. *Circ Res* 2008;102:589–596. [PubMed: 18202315]
2. Calderón-Garcidueñas L, Vincent R, Mora-Tiscareño A, Franco-Lira M, Henríquez-Roldán C, Barragán-Mejía G, Garrido-García L, Camacho-Reyes L, Valencia-Salazar G, Paredes R, Romero L, Osnaya H, Villarreal-Calderón R, Torres-Jardón R, Hazucha MJ, Reed W. Elevated plasma endothelin-1 and pulmonary arterial pressure in children exposed to air pollution. *Environ Health Perspect* 2007;115:1248–1253. [PubMed: 17687455]
3. Campen MJ, Babu NS, Helms GA, Pett S, Wernly J, Mehran R, McDonald JD. Nonparticulate components of diesel exhaust promote constriction in coronary arteries from ApoE^{−/−} mice. *Toxicol Sci* 2005;88:95–102. [PubMed: 16093524]
4. Campen MJ, McDonald JD, Reed MD, Seagrave J. Fresh gasoline emissions, not paved road dust, trigger alterations in cardiac repolarization in ApoE^{−/−} mice. *Cardiovasc Toxicol* 2006;6:199–210. [PubMed: 17347530]
5. Chou MY, Fogelstrand L, Hartvigsen K, Hansen LF, Woelkers D, Shaw PX, Choi J, Perkmann T, Bäckhed F, Miller YI, Hörkkö S, Corr M, Witztum JL, Binder CJ. Oxidation-specific epitopes are dominant targets of innate natural antibodies in mice and humans. *J Clin Invest* 2009;119:1335–1349. [PubMed: 19363291]
6. Ergul A, Portik-Dobos V, Giulumian AD, Molero MM, Fuchs LC. Stress upregulates arterial matrix metalloproteinase expression and activity via endothelin A receptor activation. *Am J Physiol Heart Circ Physiol* 2003;285:H2225–H2232. [PubMed: 12842810]
7. Furnkranz A, Schober A, Bochkov VN, Bashtrykov P, Kronke G, Kadl A, Binder BR, Weber C, Leitinger N. Oxidized phospholipids trigger atherogenic inflammation in murine arteries. *Arterioscler Thromb Vasc Biol* 2005;25:633–638. [PubMed: 15591214]
8. Gong KW, Zhao W, Li N, Barajas B, Kleinman M, Sioutas C, Horvath S, Lusis AJ, Nel A, Araujo JA. Air-pollutant chemicals and oxidized lipids exhibit genome-wide synergistic effects on endothelial cells. *Genome Biol* 2007;8:R149. [PubMed: 17655762]
9. Hirata Y, Takagi Y, Fukuda Y, Marumo F. Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Atherosclerosis* 1989;78:225–228. [PubMed: 2675859]
10. Hoffmann B, Moebus S, Möhlenkamp S, Stang A, Lehmann N, Dragano N, Schmermund A, Memmesheimer M, Mann K, Erbel R, Jöckel KH. Heinz Nixdorf Recall Study Investigative Group. Residential exposure to traffic is associated with coronary atherosclerosis. *Circulation* 2007;116:489–496. [PubMed: 17638927]
11. Leitinger N. Oxidized phospholipids as modulators of inflammation in atherosclerosis. *Curr Opin Lipidol* 2003;14:421–430. [PubMed: 14501580]
12. Lund AK, Knuckles TL, Obot Akata C, Shohet R, McDonald JD, Gigliotti A, Seagrave JC, Campen MJ. Gasoline exhaust emissions induce vascular remodeling pathways involved in atherosclerosis. *Toxicol Sci* 2007;95:485–494. [PubMed: 17065432]

13. Lund AK, Lucero JA, Lucas S, Madden MC, McDonald JD, Seagrave JC, Knuckles TL, Campen MJ. Vehicular emissions induce vascular MMP-9 expression and activity via endothelin-1 mediated pathways. *Arterioscler Thromb Vasc Biol* 2009;29:511–517. [PubMed: 19150882]
14. Luscher TF, Barton M. Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. *Circ* 2000;102:2434–2440.
15. McDonald JD, Barr EB, White RK, Chow JC, Schauer JJ, Zielinska B, Grosjean E. Generation and characterization of four dilutions of diesel engine exhaust for a subchronic inhalation study. *Environ Sci Technol* 2004;38:2513–2522. [PubMed: 15180045]
16. Mills NL, Robinson SD, Fokkens PH, Leseman DL, Miller MR, Anderson D, Freney EJ, Heal MR, Donovan RJ, Blomberg A, Sandström T, MacNee W, Boon NA, Donaldson K, Newby DE, Cassee FR. Exposure to concentrated ambient particles does not affect vascular function in patients with coronary heart disease. *Environ Health Perspect* 2008;116:709–715. [PubMed: 18560524]
17. Mills NL, Tornqvist H, Gonzalez MC, Vink E, Robinson SD, Soderberg S, Boon NA, Donaldson K, Sandström T, Blomberg A, Newby DE. Ischemic and thrombotic effects of dilute diesel-exhaust inhalation in men with coronary heart disease. *N Engl J Med* 2007;357:1075–1082. [PubMed: 17855668]
18. Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. *Physiol Rev* 2005;85:1–31. [PubMed: 15618476]
19. Nishi K, Uno M, Fukuzawa K, Horiguchi H, Shinno K, Nagahiro S. Clinicopathological significance of lipid peroxidation in carotid plaques. *Atherosclerosis* 2002;160:289–296. [PubMed: 11849650]
20. Nurkiewicz TR, Porter DW, Barger M, Millecchia L, Rao KM, Marvar PJ, Hubbs AF, Castranova V, Boegehold MA. Systemic microvascular dysfunction and inflammation after pulmonary particulate matter exposure. *Environ Health Perspect* 2006;114:412–419. [PubMed: 16507465]
21. Peters A, von Klot S, Heier M, Trentinaglia I, Hörmann A, Wichmann HE, Löwel H. Cooperative Health Research in the Region of Augsburg Study Group. Exposure to traffic and the onset of myocardial infarction. *N Engl J Med* 2004;351:1721–1730. [PubMed: 15496621]
22. Pidkovka NA, Cherepanova OA, Yoshida T, Alexander MR, Deaton RA, Thomas JA, Leitinger N, Owens GK. Oxidized phospholipids induce phenotypic switching of vascular smooth muscle cells in vivo and in vitro. *Circ Res* 2007;101:792–801. [PubMed: 17704209]
23. Ravandi A, Babaei S, Leung R, Monge JC, Hoppe G, Hoff H, Kamido H, Kuksis A. Phospholipids and oxophospholipids in atherosclerotic plaques at different stages of plaque development. *Lipids* 2004;39:97–109. [PubMed: 15134136]
24. Reed MD, Gigliotti AP, McDonald JD, Seagrave JC, Seilkop SK, Mauderly JL. Health effects of subchronic exposure to environmental levels of diesel exhaust. *Inhal Toxicol* 2004;16:177–193. [PubMed: 15204765]
25. Schwartz SM, Galis ZS, Rosenfeld ME, Falk E. Plaque rupture in humans and mice. *Arterioscler Thromb Vasc Biol* 2007;27:705–713. [PubMed: 17332493]
26. Shah AP, Pietropaoli AP, Frasier LM, Speers DM, Chalupa DC, Delehanty JM, Huang LS, Utell MJ, Frampton MW. Effect of inhaled carbon ultrafine particles on reactive hyperemia in healthy human subjects. *Environ Health Perspect* 2008;116:375–380. [PubMed: 18335106]
27. Sun Q, Wang A, Jin X, Natanzon A, Duquaine D, Brook RD, Aguinaldo JG, Fayad ZA, Fuster V, Lippmann M, Chen LC, Rajagopalan S. Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. *JAMA* 2005;294:3003–3010. [PubMed: 16414948]
28. Thomson E, Kumarathasan P, Goegan P, Aubin RA, Vincent R. Differential regulation of the lung endothelin system by urban particulate matter and ozone. *Toxicol Sci* 2005;88:103–113. [PubMed: 16081523]
29. Tornqvist H, Mills NL, Gonzalez M, Miller MR, Robinson SD, Megson IL, MacNee W, Donaldson K, Soderberg S, Newby DE, Sandström T, Blomberg A. Persistent endothelial dysfunction in humans after diesel exhaust inhalation. *Am J Respir Crit Care Med* 2007;176:395–400. [PubMed: 17446340]
30. Torzewski M, Shaw PX, Han KR, Shortal B, Lackner KJ, Witztum JL, Palinski W, Tsimikas S. Reduced in vivo aortic uptake of radiolabeled oxidation-specific antibodies reflects changes in plaque composition consistent with plaque stabilization. *Arterioscler Thromb Vasc Biol* 2004;24:2307–2312. [PubMed: 15528482]

31. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;332:411–415. [PubMed: 2451132]
32. Yatera K, Hsieh J, Hogg JC, Tranfield E, Suzuki H, Shih CH, Behzad AR, Vincent R, van Eeden SF. Particulate matter air pollution exposure promotes recruitment of monocytes into atherosclerotic plaques. *Am J Physiol Heart Circ Physiol* 2008;294:H944–H953. [PubMed: 18083905]

Figure 1.

Matrix Metalloproteinase transcriptional responses from the aortas of mice exposed to the diesel atmospheres or filtered air (control). Shown are data for MMP-3, MMP-7, MMP-8, MMP-9, MMP-12, and MMP-13. Experimental group means + s.e.m. are shown relative to control group means. Significant changes were noted for MMP9 and MMP13 with no alteration in all other markers. Asterisks (*) denote significant difference ($p < 0.05$) from control using Dunnett's multiple comparison procedure. Statistically significant concentration-dependent trends, as assessed using a linear trend test are indicated by the dagger symbol (†, $p < 0.05$).

Figure 2.

Transcriptional responses from the aortas of mice exposed to the diesel atmospheres or filtered air (control). Shown are data for ET-1, HO-1, TIMP-1, and TIMP-2. Experimental group means + s.e.m. are shown relative to control group means. Significant changes were noted for ET-1 and TIMP-2, with no alteration in all other markers. Asterisks (*, **) denote significant difference ($p < 0.05$, 0.01) from control using Dunnett's multiple comparison procedure. Statistically significant concentration-dependent trends, as assessed using a linear trend test are indicated by the dagger symbol (†, $p < 0.05$).

Figure 3. Quantitative results from aortic leaflet staining for total lesion area, macrophages (MOMA-2), collagen, and smooth muscle actin. Experimental group means + s.e.m. are shown relative to control group means. Asterisks (*, **) denote significant difference ($p < 0.05$, $P < 0.01$) from control using Dunnett's multiple comparison procedure. Statistically significant concentration-dependent trends, as assessed using a linear trend test are indicated by the dagger symbol (†, $p < 0.05$).

Figure 4.

Indices of alterations in aortic and plasma lipids. A) In plaques, a significant reduction in total lipids, assessed by oil red o staining in the aortic leaflet region, was noted with diesel-exposed groups. Filtration of PM had no effect on this parameter. B) Aortic lipid peroxides (TBARS), normalized to total protein, were increased in a dose-dependent manner with a moderate, but not significant reduction with removal of PM by filtration. C) No significant trend was noted for plasma oxidized LDL from diesel-exposed mice (n=9–10 per group). Experimental group means + s.e.m. are shown relative to control group means. Asterisks (*, **) denote significant difference ($p < 0.05$, 0.01) from control using multiple comparison procedure. Statistically significant concentration-dependent trends ($p < 0.05$) are indicated by the dagger symbol (†).

Figure 5.

Images of leaflet region staining for plaque compositional changes. Control mice (A) had relatively consolidated and dense lipid-rich regions (red staining) in the plaques, while mice exposed to diesel (B) were noted as having disrupted lipid deposits and a more complex pathological phenotype. Macrophage staining (MOMA-2; dark brown) was relatively light in control mice (C), while vascular lesions from mice exposed to diesel engine emissions exhibited substantial macrophage infiltration (D). Sirius red staining indicated the presence of collagen in control (E) and exposed mice (F).

Table 1

General composition of diesel exhaust atmospheres. Mean values for particulate matter (PM), oxides of nitrogen (NOx), carbon monoxide (CO), sulfur dioxide (SO₂) and non-methane hydrocarbons (NMHC) are shown for all exposure groups. Data are shown in mass concentration units; conversion to parts per million can be found at <http://www.cdc.gov/niosh/docs/2004-101/calc.htm>.

	Control	Low	Medium	High	High, Filtered
PM (μg/m ³)*	7.2	109.0	304.8	1012.3	27.5
NOx (mg/m ³)*	0.0	3.3	9.9	35.4	33.5
CO (mg/m ³)	0.3	3.6	10.2	30.9	30.9
SO ₂ (mg/m ³)	Not determined	141.1	299.9	955.2	955.2
NMHC (μg/m ³)	134.0	255.7	567.4	1578.6	1578.6

* An asterisk indicates actual measurements from the present study exposures. All other data are representative of the initial system characterization published by Reed et al. (2004).