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Comparative effects of inhaled diesel exhaust and ambient fine particles on inflammation, atherosclerosis, and vascular dysfunction

Chunli Quan[1], **Qinghua Sun**[2], **Morton Lippmann**[1], and **Lung-Chi Chen**[1]

[1] Department of Environmental Medicine, New York University School of Medicine, Tuxedo, New York, USA

[2] Department of Environmental Health Sciences, College of Public Health, Ohio State University, Columbus, Ohio, USA

Abstract

Ambient air $PM_{2.5}$ (particulate matter less than 2.5 μm in diameter) has been associated with cardiovascular diseases (CVDs), but the underlying mechanisms affecting CVDs are unknown. The authors investigated whether subchronic inhalation of concentrated ambient $PM_{2.5}$ (CAPs), whole diesel exhaust (WDE), or diesel exhaust gases (DEGs) led to exacerbation of atherosclerosis, pulmonary and systemic inflammation, and vascular dysfunction; and whether DEG interactions with CAPs alter cardiovascular effects. $ApoE^{-/-}$ mice were simultaneously exposed via inhalation for 5 hours/day, 4 days/week, for up to 5 months to one of five different exposure atmospheres: (1) filtered air (FA); (2) CAPs (105 $\mu g/m^3$); (3) WDE (DEP = 436 $\mu g/m^3$); (4) DEG (equivalent to gas levels in WDE group); and (5) CAPs+DEG ($PM_{2.5}$: 113 $\mu g/m^3$; with DEG equivalent to WDE group). After 3 and 5 months, lung lavage fluid and blood sera were analyzed, and atherosclerotic plaques were quantified by ultrasound imaging, hematoxylin and eosin (H&E stain), and en face Sudan IV stain. Vascular functions were assessed after 5 months of exposure. The authors showed that (1) subchronic CAPs, WDE, and DEG inhalations increased serum vascular cell adhesion molecule (VCAM)-1 levels and enhanced phenylephrine (PE)-induced vasoconstriction; (2) for plaque exacerbation, CAPs > WDE > DEG = FA, thus PM components (not present in WDE) were responsible for plaque development; (3) atherosclerosis can exacerbated through mechanistic pathways other than inflammation and vascular dysfunction; and (4) although there were no significant interactions between CAPs and DEG on plaque exacerbation, it is less clear whether the effects of CAPs on vasomotor dysfunction and pulmonary/systemic inflammation were enhanced by the DEG coexposure.

Keywords

Air pollution; atherosclerosis; concentrated ambient PM2.5 (CAPs); diesel exhaust gases (DEGs); vascular dysfunction; whole diesel exhaust (WDE)

Address for Correspondence: Lung Chi Chen, PhD, Department of Environmental Medicine, New York University School of Medicine, Tuxedo, NY 10987, USA. lcc4@nyu.edu.

Declaration of interest

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Introduction

Epidemiological studies have consistently demonstrated that ambient air pollution is strongly associated with the increased morbidity and mortality caused by cardiovascular diseases (CVDs) (Dockery et al., 1993; Kunzli et al., 2005; Miller et al., 2007; Peters et al., 2001, 2004; Pope et al., 2004; Rossi et al., 1999). Human inhalation studies have found that exposure to concentrated ambient fine particles (CAPs) was associated with a small but statistically significant increase in brachiocephalic artery diameter (BAD) constriction (Brook et al., 2002), and small changes in levels of mediators of blood coagulability, inflammation, and heart rate variability (HRV) (Devlin et al., 2003; Gong et al., 2003). Animal inhalation studies find that short-term inhalation of CAPs resulted in decreases in heart rate (HR) and mean blood pressure in pulmonary hypertensive rats (Cheng et al., 2003), increases in the frequency of spontaneous supraventricular arrhythmias in old rats (Nadziejko et al., 2003), and increases in myocardial ischemia in a model of coronary arterial occlusion in conscious dogs (Godleski et al., 2000; Wellenius et al., 2003); Long-term exposures to CAPs has been shown to change HRV (Chen and Huang, 2005), exacerbate atherosclerosis progression, alter vascular tone, enhance macrophage infiltration into the vasculature, and disrupt nitric oxide regulation in an atherosclerosis-susceptible mouse model (Chen and Nadziejko, 2005; Sun et al., 2005). It also has been reported that exposure to fine PM induces molecular alterations associated with vascular disease progression (Floyd et al., 2009).

Despite the strength of the evidence from both epidemiological and controlled exposure studies, and despite the emergence of promising hypotheses (Brook et al., 2004; Pope et al., 2004), the biological mechanisms by which exposure to air pollution is associated with CVDs are still not clear. In addition, the important constituents in air pollution that are responsible for aberrant cardiovascular effects are largely unknown, and remain to be identified.

Diesel exhaust (DE), a major constituent of air pollution in urban and industrial areas, has been hypothesized to play an important role in air pollution-associated cardiovascular effects. Twenty percent to 70% of the ambient particulate matter (PM) is attributable to combustion-derived particles from traffic (Lanki et al., 2006; Geller et al., 2005), and up to 90% of traffic-generated PM in urban environments is from diesel exhaust particles (DEP) (Air Quality Expert Group, 2005, London). Epidemiological studies have noted that exposure to road traffic may increase the risk of acute myocardial infarction (Peters et al., 2004) and cardiopulmonary mortality (Hoek et al., 2002), and the associations between air pollution and cardiovascular effects may be particularly strong for vehicle-related pollutants (Adar et al., 2007; Riediker et al., 2004; Schwartz et al., 2005). Controlled exposure studies also have found that brief exposure to diluted whole diesel exhaust (WDE) by inhalation promotes electrocardiographic changes in animal models (Campen et al., 2005), exacerbates exercise-induced myocardial ischemia, and impairs endogenous fibrinolytic capacity in men (Mills et al., 2007a).

However, it remains uncertain whether or not chronic/subchronic exposure to occupationally relevant levels of WDE will lead to any increased risk of cardiovascular effects (including atherosclerosis, a chronic cardiovascular disease). Furthermore, even if exposure to WDE does increase the risk of cardiovascular effects, it is unclear to what extent that WDE contributes to the air pollution-associated cardiovascular effects. Could WDE be the major responsible source of air pollution leading to cardiovascular effects? Up until now, there have been no animal studies conducted to compare the cardiovascular effects of chronic/subchronic exposure of the ambient air PM with those produced by WDE. Moreover, even if exposure to WDE does increase the risk of cardiovascular effects, the following questions

remain unclear: are these effects elicited mainly by the particulate or the gaseous phase of DE? In other words, how much of the adverse effects could be attributable to each phase? In addition, even if diesel exhaust gases (DEGs) by themselves do not induce cardiovascular effects, could they still contribute to the air pollution-associated cardiovascular effects by showing interactive (additive, synergistic, or antagonistic) effects with ambient air $PM_{2.5}$?

The objectives of this study were to: (1) investigate whether subchronic inhalation of each of ambient air $PM_{2.5}$, WDE, or DEG leads to atherosclerosis exacerbation, pulmonary/systemic inflammation and vascular function in an atherosclerosis-susceptible mouse model; and (2) examine whether DEG, by interacting with ambient air $PM_{2.5}$, imposes synergistic/additive/antagonistic effects that alter cardiovascular effects imposed by ambient air $PM_{2.5}$.

Methods

Animals

Apolipoprotein E–deficient ($ApoE^{-/-}$) male mice (M&B Taconic, Germantown, NY), 12 weeks of age at the beginning of the exposures, were enrolled, and fed with normal chow throughout the experiment, food and water *at libitum*. Mice were housed one per cage in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited animal housing facility maintained under temperature- and humidity-controlled conditions. The animals were treated humanely and with regard for alleviation of suffering. All procedures were approved by New York University (NYU)'s Animal Care and Use Committee.

Experimental design

From July 9th, 2007 to December 6th, 2007, in Tuxedo, NY, a total of 100 mice (n = 20/group, based on randomized assignments) were exposed in whole-body inhalation chambers for an average of 5.2 hours per day, 4 days per week, to five different laboratory-generated atmospheres: filtered air (FA), CAPs, WDE, DEG (gases concentrations equivalent to the gases in the WDE atmosphere), or CAPs+DEG ($PM_{2.5}$ concentration similar to that in the CAPs atmosphere; DEG concentration equivalent to the gases in the WDE atmosphere). All exposures took place simultaneously. The schematic of the entire exposure system, including the ambient particle enrichment and diesel exhaust generation systems, is shown in Figure 1.

Right before the first exposure, as well as 3 and 5 months into exposure, noninvasive ultrasound scanning was performed on mice (n = 8/group/time point) in order to quantitatively assess plaque progression. At 3 and 5 months into the exposures, animals (n = 10/group/time point) were sacrificed; lung lavages and sera were collected for subsequent analyses; various tissues were collected for subsequent plaque analysis by en face Sudan IV staining and hematoxylin and eosin (H&E) staining. In addition, after 5 months of exposure, vascular function was also tested immediately after host sacrifice on five mice per group. All operation and analysis for serum and bronchoalveolar fluid (BALF) characterization, ultrasound, histology, and myograph experiment were performed in a blinded fashion without knowledge of animal exposure information.

Generation of five exposure atmospheres

$PM_{2.5}$ CAPs were produced using a versatile aerosol concentration enrichment system (VACES) that uses the principle of condensational growth of ambient particles followed by virtual impaction to concentrate the aerosol (previously described by Maciejczyk and Chen, 2005). A number of modifications were made to the system to fit with the specific needs of this study. Briefly, ambient air was first drawn through a $PM_{2.5}$ cyclone to remove particles

that are larger than 2.5 μm in aerodynamic diameter. The cyclone outflow was then pulled through a saturated water vapor and a cooler during which the particles grew larger due to condensation of the vapor onto the particles. Particles were then pulled through a virtual impactor (operated at a major flow of 95 L/min and a minor flow of 5 L/min) during which the particles become concentrated in the minor flow. The particles from the minor flow were diluted to 10 L/min with filtered clean air (room air passed through silica gel, carbon canisters, and a HEPA [high-efficiency particulate-arresting] filter to remove moisture, gaseous pollutants, and PM, respectively), or diluted with DEG for the generation of CAPs +DEG. The particles then were passed though diffusion dryers (Model 3062; TSI, St. Paul, MN) to return the particles to their original size, and then finally drawn to the exposure chambers. In order to eliminate the possibility that particles in the CAPs and CAPs+DEG atmospheres were different due to the potential variance in the performance and efficiency of the two identical VACES systems, the particles following the two minor flows out of two virtual impactors were combined together first, and then split equally before particles were diluted with FA or DEG and drawn into the two separate diffusion dryers.

WDE was produced by a 5500-watt single-cylinder diesel engine generator (Yanmar YDG 5500EE-6EI; Osaka, Japan) that contains a 418-cc displacement air-cooled engine (Model LE100EE-DEGY6). The engine was operated at 91% of the maximum engine load condition using Number 2 on-road ultra-low-sulfur diesel fuel delivered from a local gas station (SOS Fuels, Tuxedo Park, NY). Electrical current was pulled from the engine to provide a constant load (~91%) with a 5000-watt electronic heater. Engine oil (SAE, 15W/40, Delo400; Chevron Products, San Ramon, CA) was changed every 50 hours of engine operation. The diesel exhaust was diluted to a desirable level through a serial dilution system with HEPA-filtered ambient air.

The DEG in the WDE were separated by adding a high-efficiency inlet vacuum filter (Model 9179K17; McMaster-Carr Supply, Princeton, NJ) to remove DEP. The particle-free DEG was then introduced to the DEG only and CAPs+DEG exposure chambers. For the sham-exposed (control) group, the mice were housed, handled, and exposed in an identical fashion, except that their exposure atmosphere was the particle-free outdoor ambient air by utilizing a HEPA capsule filter (Gelman Sciences, Ann Arbor, MI) at the inlet to the exposure chamber.

Control of DEG and PM concentrations; PM characterization

To ensure that DEG concentrations were the same in the WDE, DEG, and CAPs+DEG exposure atmospheres, the concentrations of carbon monoxide (CO) in the DEG and CAPs +DEG exposure animal chambers were matched continuously (by adjusting the amount of dilution air for each exposure atmospheres) to that in the WDE exposure animal chamber. The CO levels in these three exposure atmospheres were measured every 20 min almost simultaneously using a TIF CO Analyzer (Model TIF8500A; TIF Equipment, Long Branch, NJ).

The mass concentrations of $PM_{2.5}$ in each of the five exposure atmospheres, as well as in the ambient air, were also recorded every 20 min using real-time Personal DataRam (PDR) aerosol monitors (Model PDR1000; MIE, Bedford, MA). By doing so, the following was monitored, and ensured (by adjusting the amount of dilution air): (1) there was no detectable $PM_{2.5}$ in the FA and DEG atmospheres; (2) the $PM_{2.5}$ mass concentrations in the CAPs and CAPs+DEG atmospheres were identical, and were at least 6 times of those in the ambient air; and (3) the $PM_{2.5}$ concentration target for the WDE atmosphere was 500 $\mu g/m^3$ so as to be comparable to that described by Campen et al. (2005), who showed that a 3-day 6-hour exposure to 500 μg PM/m^3 of WDE had minimum effects on cardiac function.

$PM_{2.5}$ in each of five exposure atmospheres, as well as in the ambient air, were also collected daily throughout the exposures on Teflon filters (Gelman Teflo, 37 mm, 0.2 μm pore; Gelman Sciences, Ann Arbor, MI) for subsequent gravimetric and elemental analyses. The filters were weighed before and after sampling on an electromicrobalance (Model MT5; Mettler-Toledo, Hightstown, NJ) located in a temperature-and humidity-controlled weighing room following the Environmental Protection Agency (EPA) PM filter weighing methodology (EPA 40 CFR). Particle size and number distributions were measured with an SMPS system (electrostatic classifier model 307100, CPC model 3010, SMPS software 2.3, CPC software 1.0, CP count software 1.02; TSI), and a wide-range particle spectrometer (0.01 to 10 μm, WPS; MSP, Shoreview, MN).

Serum and bronchial alveolar lavage fluid (BALF) characterization

At the end of 3 and 5 months of animal exposures, 18 hours after cessation of the final exposure, mice were euthanized via intraperitoneal (IP) injection of 10% ketamine, followed by a lethal dose of 10% pentobarbital. Blood was collected using left ventricle puncture and serum was isolated, aliquoted, and frozen for later analysis. Total cholesterol and triglyceride levels were assayed using diagnostic kits (Thermo Electron, Louisville, CO). Levels of interleukin (IL)-6, IL-10, matrix metallopeptidase-9 (MMP-9), membrane-bound vascular cell adhesion molecule-1 (mVCAM-1), membrane-bound intercellular adhesion molecule-1 (mICAM-1), mE-selectin, membrane-bound tumor necrosis factor-α (mTNF-α), resistin, and C-reactive protein (CRP) were measured using SearchLight Custom Multiplex Sample Analysis Service from ThermoFisher.

Following blood collection, the whole lungs of the designated mice were lavaged with 1.2 ml phosphate-buffered saline (PBS). Total nucleated cells were counted using a hemocytometer. Differential counts of nucleated cells were made using cytocentrifuge preparations stained with Wright-Giemsa. After the remaining BALF was centrifuged at 400 ×× *g* for 10 min, the resulting supernatant was then used in analysis of total protein (Bio-Rad, Hercules, CA) levels.

En Face Sudan IV stain

The aorta (from aortic valve to iliac bifurcation, including three branching arteries at aortic arch) was dissected away intact from the dorsal wall (n = 3/group/time point), immersed immediately in 2% glutaraldehyde, and held at room temperature until analysis. Any adventitial and adipose tissue attached to the aortic wall was removed carefully, and the aorta was then cut open longitudinally, splayed, and pinned flat on a black wax surface with the use of a dissecting microscope. The opened aorta was briefly rinsed with 70% ethanol for 5 min, then was immersed for 8 min with filtered Sudan IV stain solution containing 0.5% Sudan IV (Sigma), 35% ethanol, 50% acetone, and 15% water, then was de-stained in 80% ethanol for 5 min to eliminate background staining (Palinski et al., 1994; Chen and Nadziejko, 2005). The stained aorta was then immersed in PBS and images were captured through a microscope using a Canon S500 digital camera with Scopetronix microscope adapter. The total Sudan IV–stained lesion area was quantified using ImageJ software, version 1.38X (National Institutes of Health, Bethesda, MD) by manual drawing with polygon selection. The final data were expressed as percentage positive-staining area relative to total aortic area.

Hematoxylin and eosin (H&E) stain

Brachiocephalic arteries (n = 6/group/time point) were carefully isolated and embedded perpendicularly in Optimal Cutting Temperature compound, and were then snap frozen and stored in −80°C. Serial cross-sections were taken using a calibrated Microm HM500M cryostat (Microm, Walldorf, Germany). For each mouse, three successive sections (8 μm

thick) were collected onto each Fisher Superfrost Plus–coated slide and 10 slides were collected. The first (near entrance of artery) and tenth slides (around 300 μm away from entrance) were stained with H&E. Each image was digitized under a research microscope (Olympus, Model BH2) with a digital camera (SONY 3CCD color video camera; Model DXC-390). Plaque area was adjusted for the cross-sectional vessel cavity area and quantified with NIH ImageJ software. The final data were expressed as percentage plaque area relative to total cross-sectional vessel cavity area, and were the average of percent plaque area in two stained slides.

Quantification of plaque by ultrasound imaging

The ultrasound biomicroscope used in this study was a Vevo 770 (VisualSonics, Toronto, Ontario, Canada), with a single-crystal mechanical transducer (704) that has a central frequency of 40 MHz. Each mouse was anesthetized using continuous 1.5% isoflurane (EZ-Anesthesia; Euthanex, Palmer, PA), and was placed supine on a physiological platform. Chest hairs were removed and a layer of prewarmed sterile ultrasound gel was spread over the chest. Ten B-Mode movies (in cross-sectional position) were obtained along the brachiocephalic artery at around 333-μm gaps. From each of the second, third, and fourth movies, three pictures were selected based on following criteria: pictures were clear and representative of the entire movie, and all pictures selected were electrocardiogram (ECG)-synchronized. For each picture, plaque area was measured using NIH ImageJ freehand drawing, and expressed as percentage of plaque area relative to the cross-sectional vessel cavity area. For each artery, the percent area in each of three locations was then averaged, and expressed as the percent volume of the plaque in 1 mm length of each artery.

Vascular function evaluation

The following drugs were used to evaluate vascular function: phenylephrine (PE), acetylcholine (ACH), sodium nitroprusside (SNP), serotonin (5-HT), N^{ω}-nitro-L-arginine methyl ester (L-NAME), and Tiron (all from Sigma-Aldrich, St. Louis, MO). Briefly, immediately after mice sacrifice, the thoracic aorta was dissected away intact and immersed in ice-cold buffered physiological saline solution (PSS). Any adventitial and adipose tissue was removed under a dissecting microscope. Four segments of aortic rings (2 mm length each) per mouse were isolated and mounted on steel pins in the organ chambers of myograph (multi-Myograph 610 M; Danish Myo Technology, Aarhus, Denmark) containing 5 ml oxygenated PSS. The tension on the vessel segments were gradually adjusted to 800 mg, at which point the vessel segments were allowed to equilibrate for at least 1 hour. Each experiment was initiated by precontracting the vessels with 60 mM nonspecific depolarizing agent K^+-PSS, followed by contracting the vessels with 120 mM K^+-PSS. Vessels were then subjected to graded doses (1 nM to 3 μM) of 5-HT or PE. After the vessels being washed thoroughly and equilibrated for 30 min, vasorelaxation was tested by precontracting the segments with 1 μM PE and subsequently relaxing them with increasing concentrations (1 nM to 3 μM) of ACH or SNP. To test the role of NO and superoxide, L-NAME (1 mM, a nitric oxide synthase [NOS] inhibitor) and Tiron (1 mM, a superoxide inhibitor) were added into the different organ chambers to incubate with the tissues for 10 min, afterwards the vessels were precontracted with 1 μM PE and subsequently relaxed with increasing concentrations of ACH (1 nM to 3 μM).

Statistical data analyses

One-way analysis of variance (ANOVA), followed by Dunnett's test, were used to assess differences in the effects of each different exposure as compared to FA. The *t* test was used to test the difference in effects between any two exposure groups at a fixed time point, and to test the differences within a given group at different time points. Two-way ANOVA were used to assess any interactions between CAPs and DEG, with the CAPs and DEG as two

independent factors. Grubbs and Dixon tests were used to identify any outliers in a given data set. All p values were two-tailed; and statistical significance was set at $p < .05$. For en face Sudan IV stain, due to its limited sample size ($n = 3$), $p < .1$ was also examined. To be consistent, the other two plaque quantification methods (ultrasound and H&E stain) were also examined at both $p < .05$ and $p < .1$.

For vascular function evaluation, data from the cumulative dose-response of PE, 5-HT, ACH, and SNP were fitted to sigmoid dose-response curves using nonlinear regression analysis (GraphPad Prism Version 4; GraphPad Software, San Diego, CA). The half-maximal (either for dilation or constriction) dose for each experiment was obtained by logarithmic transformation. Vascular responses were compared using two-way ANOVA, with the half-maximal dose or responses at each dose as the dependent variables. $p < .05$ was considered significant. All statistical analyses were performed using GraphPad Prism Version 4.

Results

Exposure characterization

Table 1 summarizes the $PM_{2.5}$ concentrations, $PM_{2.5}$ size distributions, and gas concentrations in the five different exposure atmospheres. No $PM_{2.5}$ was detected in the FA and DEG atmospheres using real-time aerosol monitoring during the full course of the 5 months of exposure. $PM_{2.5}$ concentrations in the CAPs and CAPs+DEG were nearly identical, and both were approximately 7 times than those in the ambient Tuxedo air. $PM_{2.5}$ concentration in WDE was approximately 4 (for 5-month-exposed mice) or 3 times (for 3-month-exposed mice) than those in the CAPs or CAPs+DEG atmospheres. WDE was maintained at a relatively constant concentration during the 5-month exposures. Due to seasonal variations in ambient $PM_{2.5}$ concentrations, the mean PM concentrations in the CAPs and CAPs+DEG for the 3-month-exposed mice were slightly higher than those for the 5-month-exposed mice; the ambient and CAPs concentrations were highest in July and August, and declined significantly in the fall. CAPs had similar count median aerodynamic diameter (CMAD) as, but larger mass median aerodynamic diameter (MMAD) than, the $PM_{2.5}$ in WDE. Because mixing with gases would not likely change the particle size, it is reasonable to assume that the $PM_{2.5}$ size distributions in the CAPs+DEG were identical to those in the CAPs. The concentrations of CO in the DEG and DEG+CAPs were maintained identical to that in the WDE throughout the 5 months of exposures. The average CO level for each of these three groups was 5 ± 1.8 ppm. The continuous identical CO levels in WDE, DEG, and CAPs+DEG atmospheres indicate that the DEG levels in these three atmospheres were equivalent.

Overall animal health; characterization of BALF and serum

No animal death was observed throughout the course of the exposure. Although there were significant increases in body weight from the start to the end of the study, the weight gains were not statistically different among the exposure groups at any time point. For mice exposed for 5 months, both serum total cholesterol and triglyceride levels (data not shown) did not differ among the five exposure groups. Polymorphonuclear neutrophil (PMN) infiltration in the BALF was not significantly different among the five exposure groups after both 3 and 5 months of exposures. The total protein level in the BALF increased with exposure duration, perhaps due to animal aging; however, levels were not different among the five exposure groups at each time point (data not shown). Levels of various cytokines in serum were detailed in Table 2. Mice exposed to CAPs, CAPs+DEG, DEG, and WDE all showed elevated mVCAM-1 levels compared to those in the FA animals; however, there were no significant differences among the four non-FA groups.

Plaque progression evaluation by three methods

Figure 2A shows the representative en face aortic segments with plaques stained with Sudan IV in each of the five groups at two time points. Figure 2B depicts the plaque quantification results using en face Sudan IV staining. After the 5-month regimen, CAPs-exposed mice developed significantly ($p < .1$) more plaque than those in the FA and DEG groups, but were no different from those in the CAPs+DEG and WDE groups. Also after 5 month exposure, the plaque developments in WDE, DEG, and CAPs+DEG groups were not different from that in FA group, and were not different from each other. For mice exposed for 3 months, plaque that developed in CAPs+DEG-exposed mice was significantly ($p < .1$) greater than that developed in the FA- and CAPs-exposed mice, and the plaque developments in the WDE and DEG groups were not different from those in FA group and were not different from each other. Two-way ANOVA on FA, CAPs, DEG, and CAPs+DEG did not show interaction (neither synergistic, nor additive, nor antagonistic effects) of CAPs and DEG on plaque development at either time point.

Figure 2C shows two representative brachiocephalic artery cross-sections stained with H&E, including one clear artery with no plaque, and one artery with a large area of plaques. Figure 2D depicts the plaque quantification results using the H&E stain. For mice exposed for 3 months, there were no differences in plaque development as a function of treatment. For mice exposed for 5 months, both the CAPs+DEG-exposed and the WDE-exposed mice developed significantly larger area of plaques than FA mice; mice exposed to CAPs+DEG developed significantly more plaque than those exposed to WDE; whereas plaque development in DEG-exposed mice was not different from those in FA-exposed mice, and was significantly less than those in CAPs+DEG- and WDE-exposed mice. No significant difference (either at $p < .05$ or $p < .1$) in plaque areas was observed between CAPs- and FA-exposed mice, probably due to a large degree of variability of the H&E data in the CAPs-exposed mice. No interaction on plaque development between DEG and CAPs was observed at both time points.

Figure 2E shows a representative ultrasound image of brachiocephalic artery cross-section, with right side image showing how the plaques are identified and quantified for the left image. Figure 2F depicts the plaque quantification results using ultrasound-based method. At both time points, CAPs-exposed mice developed more plaque in the brachiocephalic area than did mice in the FA group and in the WDE group; plaque development in DEG- and WDE-exposed mice was not significantly different from that in FA-exposed control mice; whereas no interactive effects on plaque development between DEG and CAPs were observed. In addition, CAPs+DEG exposure induced more plaque development than DEG exposure at the 3-month time point, and more plaque development than FA at 5-month time point.

Vascular function evaluation

Constrictive responses to 120 mM KCl were not significantly different among all five groups (data not shown), which permits the use of 120 mM KCl responses for normalization of data observed in the constriction responses to PE and 5-HT. Figure 3A–D depicts responsiveness to graded doses of PE, 5-HT, ACH, and SNP in the thoracic aortic segments of animals exposed via inhalation to the five different exposure atmospheres. Table 3 details the maximum constriction (or relaxation) as well as the half maximum dose of constriction (or relaxation), EC_{50} (or ED_{50}), to PE, 5-HT, ACH, and SNP in all five groups. Comparing with FA-exposed animals, CAPs-, CAPs+DEG-, DEG-, and WDE-exposed mice showed significant increases in maximum constriction in response to PE. However, the levels of the enhancement of the PE-induced vasoconstriction were not different among four groups of CAPs, WDE, DEG, and CAPs+DEG (Figure 3A). There was no significant difference in

vascular responsiveness to 5-HT (or ACH, or SNP) between any of the exposure groups at any dose tested (Figure 3B–D). At its maximum dose, the response to 5-HT in the vessels from the CAPs- and CAPs+DEG-exposed animals seemed to be different from those of tissues from the FA controls, but the difference was nonsignificant ($p = .12$ and .11, respectively). At 10^{-9} M, response of WDE to SNP seem to be different to that of FA, but the difference is nonsignificant ($p = .101$). The sensitivity (i.e., half-maximal dose, EC_{50} or ED_{50}) was not significantly different among all five groups in response to each of PE, 5-HT, ACH, or SNP, although in response to SNP, WDE showed nonsignificant shift in EC_{50} (95% CI for WDE: $1.80–3.61 \times 10^{-9}$ to 3.61×10^{-9}, for FA: $0.75–1.81 \times 10^{-9}$). No interactions in vascular responsiveness were observed between the CAPs and DEG for any of the four testing agents. Pretreatment with either L-NAME (Figure 4A) or Tiron (Figure 4B) completely abolished the enhanced PE contraction observed in the aortas in CAPs, CAPs+DEG, DEG, and WDE groups (Figure 1A), indicating that the air pollutants (e.g., CAPs, CAPs+DEG, DEG, and WDE) enhanced vasoconstriction to PE through alterations in vascular superoxide and NO levels.

Discussion

This study has generated important findings. It is the first to demonstrate that subchronic inhalation of WDE, a major constituent of air pollution in urban and industrial areas, could impair vascular function, increase vascular adhesion molecules expression, and exacerbate the development/progression of atherosclerosis. Moreover, it also demonstrated that subchronic inhalation of DEG, an important component of WDE, could impair vascular function, increase vascular adhesion molecule expression, without significantly exacerbating atherosclerosis. It also confirmed that subchronic inhalation exposure to CAPs enhances adverse cardiovascular effects, as reported in our previous studies.

This study is the first to directly compare the cardiovascular effects caused by ambient air $PM_{2.5}$, WDE, and DEG exposures. The CAPs, WDE, and DEG enhanced adhesion molecule expression and potentiated vasoconstrictive responses to similar degrees, they had different impacts on plaque development. Although WDE exposure can exacerbate plaque formation, it is unlikely to be the most responsible source in ambient PM. DEG did not significantly affect plaque development, either by itself, or by interaction with $PM_{2.5}$. These findings inform our search for the responsible components in air pollution affecting cardiovascular health.

Exposure concentrations

The $PM_{2.5}$ and gas concentrations in the exposure atmospheres were within environmentally or occupationally relevant levels, therefore the cardiovascular effects observed in this study were relevant to susceptible populations in urban or occupational environments. Because the mice were exposed for 5.2 hours/day, 4 days/week, and were kept in particle-free chambers during the nonexposure periods, the longer-term average $PM_{2.5}$ concentrations for CAPs and CAPs+DEG atmospheres during the exposure period were ~13.1 and 14.3 $\mu g/m^3$, both below the US EPA's $PM_{2.5}$ National Ambient Air Quality Standard (NAAQS) in annual average of 15 $\mu g/m^3$ (US EPA NAAQS).

US EPA (2006) estimated a national average concentration of environmental exposure to DEP at 2.06 $\mu g/m^3$. However, historic occupational DEP exposures have ranged from 39 to, 191 $\mu g/m^3$ for railroad workers, 4–748 $\mu g/m^3$ for firefighters, and 7–98 $\mu g/m^3$ for public transit workers and airport crews (US EPA, 2002). The mean PM concentration of 438 $\mu g/m^3$ in the WDE atmosphere in this study was, therefore, within the occupational exposure range. Throughout the 5-month exposures, the average CO level in each of the WDE, DEG, and CAPs+DEG exposure atmospheres was 5 ppm, which is below the NAAQS for an 8-

hour average for CO of 9 ppm. The PM concentration used in this study was comparable to that used in studies performed by Campen et al. (2005), which showed that a 3-day 6-hour exposure to 500 μg (0.5 mg) PM/m^3 of WDE had minimum effects on cardiac function. In other previous studies, the WDE levels were much higher (Saber et al., 2009, 20 mg/m^3; Knuckles et al., 2008, 3.5 mg/m^3; Campen et al., 2006, 3.5 mg/m^3; Saber et al., 2006, 20–80 mg/m^3; Campen et al., 2005, 3.6 mg/m^3). Our results indicate that the WDE level that we used was high enough to produce significant effects, and yet low enough to be relevant to recent occupational exposures.

Interactive effects between DEG and CAPs were not observed

Interactive effects between gases and particles have been suggested in papers by Boren (1964), Kilburn and McKenzie (1978), and Madden et al. (2000). However, we did not observe any statistically significant interactive effects between CAPs and DEG on enhancing inflammation, altering vascular function, or on the exacerbation of atherosclerosis. This could have been due to the differences between the Madden et al. study and ours (i.e., DEP+O_3 versus CAPs+DEG, instillation versus inhalation, exposure for a few hours versus for 5 months, and higher concentration versus occupationally relevant concentration). We did test the potential interactions between DEG and ambient air $PM_{2.5}$ on plaque progression by exposing animals immediately after the mixing of DEG and $PM_{2.5}$ to investigate interactive health effects of gases and PM. However, it should be noted that allowing the mixtures of gases and PM to age, or treating the mixtures with UV light, etc., to alter the chemical composition, might lead to adverse health effects that differ from those arising from exposing subjects immediately following the mixing of the gases and PM.

Effects of each of CAPs, WDE, and DEG on atherosclerosis

Atherosclerosis is a major underlying cause of angina, heart attacks, strokes, and peripheral vascular disease, and it constitutes the single most important contributor to CVD (Libby, 2002). The consequence of plaque progression is complex, and the plaque size/volume is one critical factor on the outcomes. Other factors, such as macrophage contents in the plaques (to represent the inflammatory response), collagen content (to potentially indicate the maturity of the plaque), and fat deposition (to represent the characteristic of the plaque), also play important roles in evaluating the outcomes. In this study, we focused on evaluating plaque size/volume. We demonstrated that subchronic CAPs inhalation exposure exacerbated atherosclerotic plaque progression in a susceptible animal model, supporting previous observations that inhaled ambient $PM_{2.5}$ is an important stimulus for atherosclerosis. Using atherosclerosis-susceptible animal models, other studies found that repeated instillation of urban PM at very high doses (Goto et al., 2004; Suwa et al., 2002), or subchronic exposure to CAPs at environmental relevant levels (Chen and Nadziejko, 2005; Sun et al., 2005; Araujo et al., 2008) resulted in increased plaque size and greater inflammation. A human study (Kunzli et al., 2005) observed that a 10 μg/m^3 increase in $PM_{2.5}$ in Los Angeles was associated with an increase in carotid intima-media thickness, an indicator of carotid artery atheroma as measured by an ultrasound-based method.

With regard to the potential population impact of WDE on atherosclerosis progression, epidemiological studies have found that long-term residential proximity to high traffic is associated with the degree of coronary atherosclerosis (Hoffmann et al., 2007). In a recent study exposing ApoE mice to WDE or DEG for 6 hours/day for 50 days, no increase in lesion area of aortic leaflet sections was observed with either exposure (Campen et al., 2009). However, WDE induced doserelated alterations in gene markers of vascular remodeling and aortic lipid peroxidation, whereas DEG did not significantly altered these vascular responses. In addition, Lund et al. (2007) found increased vascular factors, i.e.. matrix metalloproteinase (MMP), endothelin (ET), and ROS levels in ApoE mice exposed

for 7 weeks to gasoline engine exhaust. The up-regulation of these circulating and vascular factors associated with progression of atherosclerosis was shown to be mediated in part through activation of ET-1–ETA receptor pathways (Lund et al., 2009). Our results suggest that subchronic WDE exposure could contribute to air pollution-associated atherosclerosis exacerbation, but subchronic exposure to DEG alone at an occupationally relevant exposure level did not significantly exacerbate atherosclerosis progression. In addition, because CAPs +DEG and CAPs exposures all enhanced plaque progression to a similar extent, whereas DEG appears to have had no effect, these outcomes indicate that DEG, either alone, or by interacting with ambient air $PM_{2.5}$, may not significantly contribute to air pollution-associated plaque exacerbation.

Comparison of the plaque exacerbation among CAPs, WDE, and DEG

The DEG and WDE atmospheres contained equal levels of DEG, with the difference being the presence of DEP. Although the WDE exposure resulted in significantly increased plaque size/volume, DEG exposure, either alone or mixed with ambient air $PM_{2.5}$, did not contribute to plaque exacerbation. Thus DEP, rather than DEG, plays a more important role in atherosclerotic plaque formation/growth.

Even at a PM concentration 4 times lower, subchronic exposure to CAPs alone, or to CAPs +DEG, resulted in more plaque development than WDE, indicating that some component(s) in ambient air $PM_{2.5}$, not present in WDE, are more potent in terms of exacerbation of plaque development. Thus, although WDE could enhance atherosclerosis plaque development in a susceptible population, it is not the most responsible constituent in ambient air $PM_{2.5}$ in exacerbating atherosclerosis progression.

In comparing CAPs+DEG and WDE atmospheres, note that both had equal levels of DEG, and that the ratio of DEG concentrations to those of CAPs was much higher than their ratio in ambient air. The PM in the CAPs+DEG had the same CMAD as the PM in the WDE, but a slightly bigger MMAD. The smaller particle size (in WDE) may produce more severe effects, but the PM size differences are small and might not be a major contributing factor for any observed differences in plaque development. Unlike the property of PM size, the PM chemical compositions were significantly different between the DEP and CAPs. The exposures in this study were conducted at the NYU laboratory located in the center of Sterling Forest (SF) State Park in Tuxedo, NY, a largely undeveloped woodland park without large power generators or industrial operations within 20 miles. Therefore, the composition of the ambient $PM_{2.5}$ in SF is representative of Northeastern regional background $PM_{2.5}$. The major components of Eastern United States ambient PM estimated by US EPA (1999) are ~4% elemental carbon (EC), ~21% organic carbon (OC), ~48% sulfate, ~4% metals, and 23% others. Because the VACES concentrator did not alter the particle composition (Maciejczyk et al., 2005), the PM compositions of CAPs are similar to those in the ambient $PM_{2.5}$. The DEP composition (generated in a system similar to that used in this study) had been characterized by McDonald et al. (2004). Their findings were consistent with US EPA's estimate for DEP compositions from a typical diesel engine, i.e., ~ 60% EC, ~ 20% OC, 1–4% sulfate, ~ 1–5% metals, and 7% unknown (US EPA,, 2002). These compositional differences between the CAPs+DEG and WDE exposure atmospheres might be major contributing factors underlying the observed differences in plaque progression level. From the data from this study, it is not possible to determine whether the sulfates, metals, or organic components were more responsible for the greater plaque development in the CAPs-exposed than in WDEexposed mice. It was also beyond the scope and capabilities of this study to find out the exact component(s) that are in the CAPs, but not in WDE, that exacerbate atherosclerosis in a greater extent.

Effects of each of CAPs, WDE, and DEG on vascular function and inflammation

Exposure to various air pollutants have been shown to be associated with perturbations in vasomotor function in both human studies (Brook et al., 2002; Mills et al., 2005; Peretz et al., 2008) and animal studies (Batalha et al., 2002; Campen et al., 2005; Sun et al., 2005). Our finding that CAPs exposure could affect vascular constriction function was consistent with those in previous studies. Subchronic exposure to CAPs has been reported to enhance vasoconstriction in mice (Sun et al., 2005). Human inhalation studies found that exposure to CAPs (at ~150 $\mu g/m^3$) was associated with a small, but statistically significant, brachiocephalic artery diameter (BAD) constriction (Brook et al., 2002).

Our findings demonstrated that subchronic inhalation of WDE, as well as DEG, at occupationally relevant levels, impaired vascular function. Several previous animal studies reported altered vasoconstriction or vasorelaxation after either in vitro DEP exposure or acute WDE inhalation exposure. Campen et al. (2005) reported enhanced vasoconstrictive effects to endothelin-1 and reduced dilatory responses to SNP after exposing the isolated coronary arteries from $ApoE^{-/-}$ mice to DE bubbled through a physiological saline solution. After exposing C57 mice to WDE (at 350 $\mu g/m^3$ for 4 hours) in a whole-body inhalation chamber, enhanced vasoconstrictions to endothelin-1 in the veins, but not in the arteries, were observed (Knuckles et al., 2008). In addition, a 5-hour exposure to WDE at 300 μg PM/m^3 increased ET-dependent coronary artery constriction by diminishing ET(b) receptor activation of endothelial NOS (Cherng et al., 2009). As for human studies, short-term whole-body exposure to WDE by inhalation at as low as 200 $\mu g/m^3$ elicited a decrease in BAD (0.11 mm; 95% CI, 0.02–0.18 mm), and it was concluded that short-term exposure to WDE is associated with acute endothelial responses and vasoconstriction in a conductance artery (Peretz et al., 2008). At levels encountered in an urban environment, inhalation of dilute WDE impaired two important (and complementary) aspects of vascular function in humans: the regulation of vascular tone and endogenous fibrinolysis (Mills et al., 2005; Tornqvist et al., 2007).

In 5-month-exposed animals, mVCAM-1 levels were significantly elevated (relative to the values in the control mice) in the sera of those that had been exposed to CAPs, CAPs+DEG, DEG, or WDE, suggesting that these exposures (CAPs, WDE, and DEG) could lead to a systemic inflammation. Systemic inflammatory responses upon PM exposure were reported by Salvi et al. (1999), Ishihara and Kagawa (2002), Upadhyay et al. (2008), and Utell and Frampton (2000). Nurkiewicz et al. (2004) found that exposure to residual oil fly ash (ROFA) by instillation in rats increased the number of adhering and rolling leukocytes in spinotrapezius muscle venules at 24 hours postexposure. Inhalation of carbon ultrafine particles also has been shown to alter leukocyte expression of adhesion molecules in the peripheral blood (Frampton et al., 2006).

It has been hypothesized that PM deposited in the lungs may initiate a local inflammatory response that may develop into a systemic inflammatory response (Donaldson et al., 2001). In our study, the CAPs, WDE, and DEG exposures did not change the levels of PMNs and total protein in BALF. However, each of these exposures enhanced the mVCAM-1 expression in serum. Because, in this study, these assays were only performed at a limited number of time points, it is possible that pulmonary inflammation may have been induced by these pollutants, but may have subsided before the measurements were made. It is also possible that subchronic exposure to CAPs, WDE, or DEG does induce a very low level of increased pulmonary inflammation that was below the detection limit of the assays used in this study. Such a persistent low grade of inflammation, although not detectable using current technology, may affect cardiovascular health, especially over a long-term exposure.

Comparison of the vascular dysfunction and inflammation among CAPs, WDE and DEG

One important novel finding of this study is that in terms of elevating vascular adhesion molecule expression and enhancing vasoconstriction, CAPs, WDE, and DEG, despite their very different gases and PM physicochemical characteristics, produced similar effects. It is well-known that mechanisms by which air pollution leads to vascular dysfunction and systemic inflammation are complex. It might be possible that all four exposure atmospheres had a similar potency with regard to inducing these changes. It might also be possible that the potential for maximal vasoconstriction responses may have already occurred in the presence of a lower pollution level or earlier in the exposure regimen timeframe, and that further responses to constricting agents could not be achieved.

Our findings that CAPs and WDE inhalation resulted in similar vasomotor functions in response to agonists are in contrast with those of Mills et al. (2005 Mills et al. (2008) and Törnqvist et al. (2007), who compared the effects of short-term inhalation of CAPs and of WDE on the impairment of vascular functions in humans. They exposed 12 male patients with stable coronary heart disease and 12 age-matched healthy volunteers to CAPs (in Edinburgh, UK) or FA for 2 hours and then measured peripheral vascular vasomotor and fibrinolytic function 6–8 hours after the exposures. They showed that exposure to Edinburgh CAPs—low in combustion- derived particles—did not affect vasomotor or fibrinolytic function in either middle-aged healthy volunteers or patients with coronary heart disease. In contrast, using a similar experimental design, the same group reported an impairment of vascular function in both healthy volunteers and patients with coronary heart disease after a 1-hour exposure to dilute WDE. They suggested that the trafficderived particles were especially important for inducing health effects. However, the differences in experimental designs between their studies and ours (e.g., human versus mouse, forearm blood flow versus isolated aortas, exposure duration, compositions of CAPs, etc.) make a direct comparison difficult.

Comparative effects of CAPs, WDE, and DEG are endpoint dependent

The impacts of CAPs, WDE, and DEG exposures were observed to be different on atherosclerosis progression, but to be similar with regard to the expression of VCAM-1 and alterations in vascular function. These findings indicated when comparing ambient $PM_{2.5}$, WDE, and DEG with regard to their potency in eliciting cardiovascular effects, depending on the endpoints and time points that are being studied, different observations and conclusions regarding which is more potent could arise. It might be possible that different endpoint have different mechanisms of how pollutants may lead to a change in this health index, and the contribution of the same chemical composition to each health index might be different. These data also suggest that atherosclerosis could be elicited by the pathways other than inflammation and vascular function. The composition of the PM may be especially important in affecting these pathways other than inflammation and vascular change.

One possibility is that smaller particles may translocate into the bloodstream, exerting direct effects on the vasculature. It is possible that this direct interaction of PM with vasculature do not change much in the levels of inflammation and vascular, but could lead to a significant change in plaque progression. More extensive studies are needed to elucidate the exact mechanism(s) of air pollutant–enhanced atherosclerosis and vascular dysfunction.

Some epidemiological studies have shown that associations between air pollutants and cardiovascular effects were particularly strong for vehicle-related pollutants (Adar et al., 2007; Riediker et al., 2004; Schwartz et al., 2005). These studies examined overall cardiovascular mortality and morbidity, or biologic endpoints not specific to atherosclerosis.

In addition, the findings from these studies were based on a general population that was exposed to aged WDE at an environmental exposure level. By contrast, the findings of our study were obtained under an experimental exposure to fresh WDE (as diluted emissions from the tailpipe). The different physical (PM size distribution, etc.) and chemical compositions between fresh and aged WDE might account for the differing observations between epidemiological studies on the role of WDE in air pollution–associated cardiovascular effects.

Conclusions

Our results suggest that in a susceptible animal model for atherosclerosis, under the exposure conditions used here: (1) subchronic inhalation of Northeastern regional $PM_{2.5}$, at environmentally relevant exposure concentrations, exacerbated atherosclerotic plaque progression, potentiated the expression of vascular cell adhesion molecules in serum, and significantly enhanced PE-induced vasoconstriction through potentiation of vascular superoxide levels and attenuation of NO levels; (2) subchronic inhalation of WDE, at an occupationally relevant exposure level, may have exacerbated plaque progression, but did potentiate the expression of systemic vascular cell adhesion molecules and significantly enhanced PE-induced vasoconstriction through increases in vascular superoxide levels and attenuation of NO levels; (3) subchronic exposure to DEG, at a level that could be encountered in an occupational setting, did not significantly exacerbate plaque progression, but did potentiate expression of systemic vascular cell adhesion molecules and significantly enhanced PE induced vasoconstriction through potentiation of vascular superoxide levels and attenuation of NO levels; (4) no interactive effects between DEG and ambient ambient air $PM_{2.5}$ were observed in this study on plaque exacerbation, vasomotor dysfunction, and pulmonary/systemic inflammation; (5) in terms of pulmonary/systemic inflammation and vasomotor function alteration, subchronic inhalation of CAPs, WDE, and DEG produced similar effects; (6) with regard to exacerbation of atherosclerotic plaque progression, the effects were CAPs > WDE > DEG ≈ FA, indicating that PM (rather than gases), especially some components of the ambient PM (not present in WDE), were more responsible for the plaque development; and (7) the impacts of CAPs, WDE, and DEG exposures were observed to be different on atherosclerosis progression, but to be similar in terms of the expression of VCAM-1 and alterations in vascular function. It is possible that atherosclerosis could be exacerbated through the pathways other than inflammation and vascular function. Thus, depending on the endpoints, time points, and concentration ratios that are being studied, the comparative potencies of ambient air $PM_{2.5}$, WDE, and DEG could differ.

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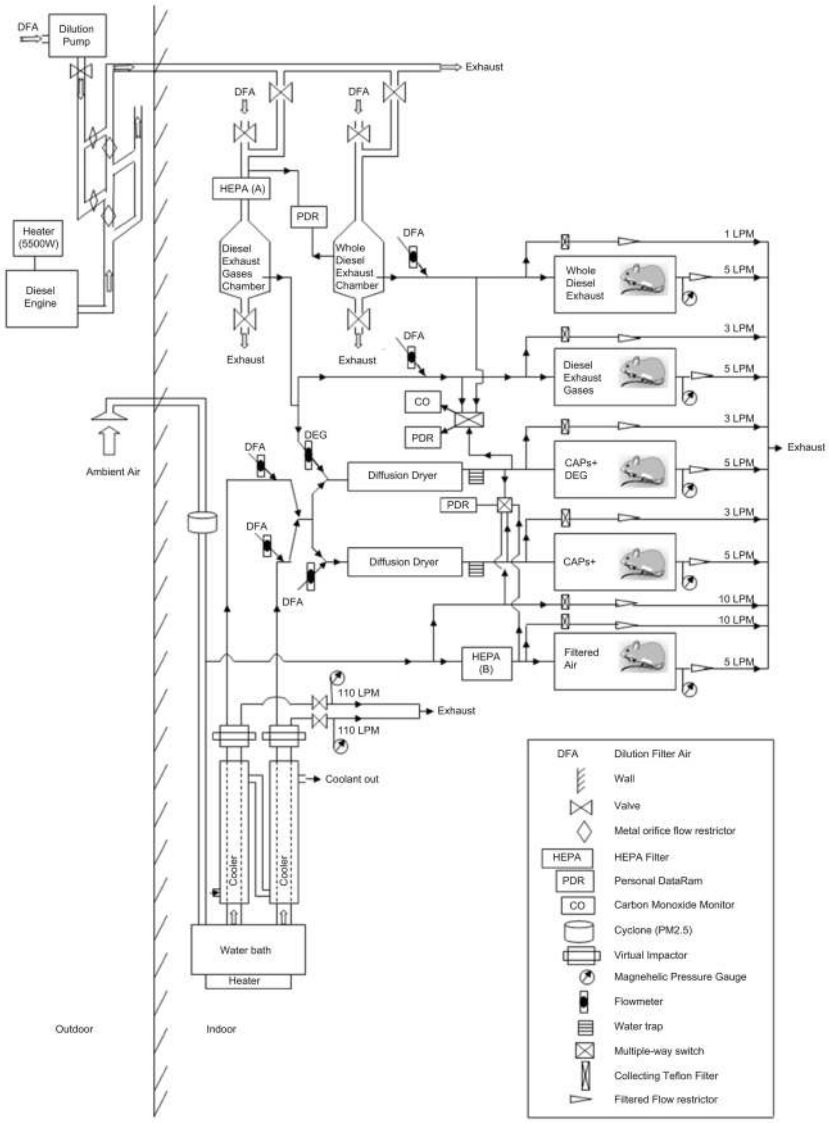
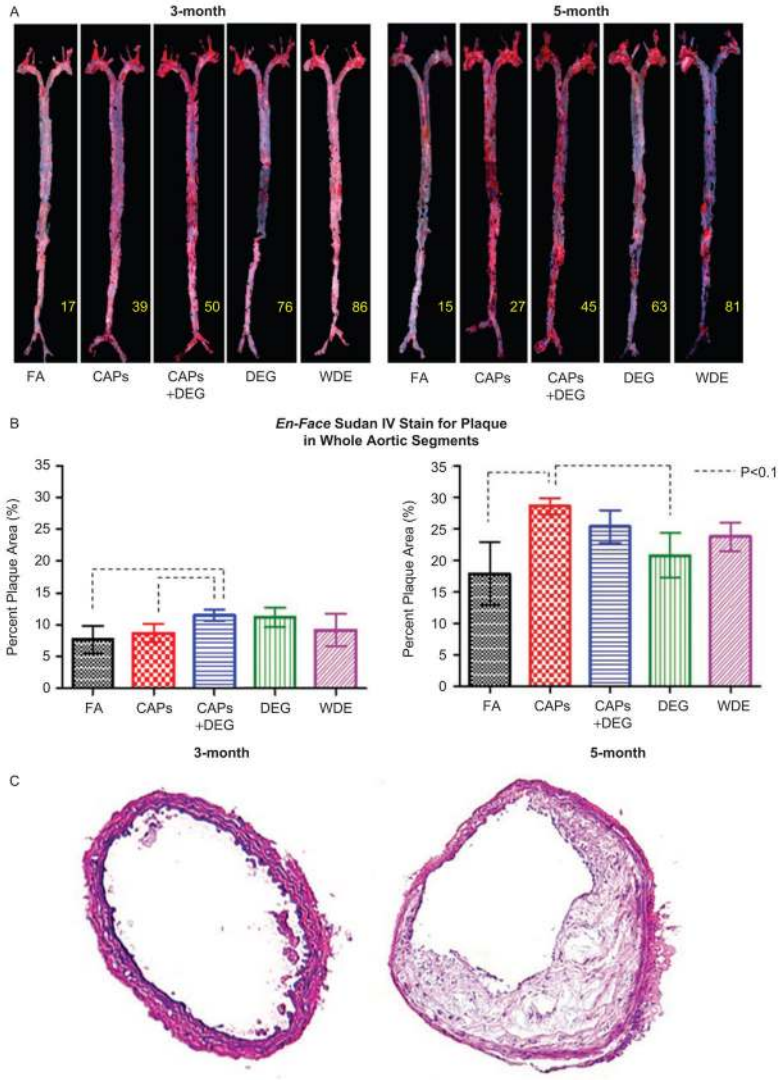


Figure 1.

A schematic diagram of the exposure system. Diagram includes the VACES CAPs exposure system, the diesel engine exhaust exposure system, and the design to allow for the mixture of the CAPs and diesel exhaust gases.



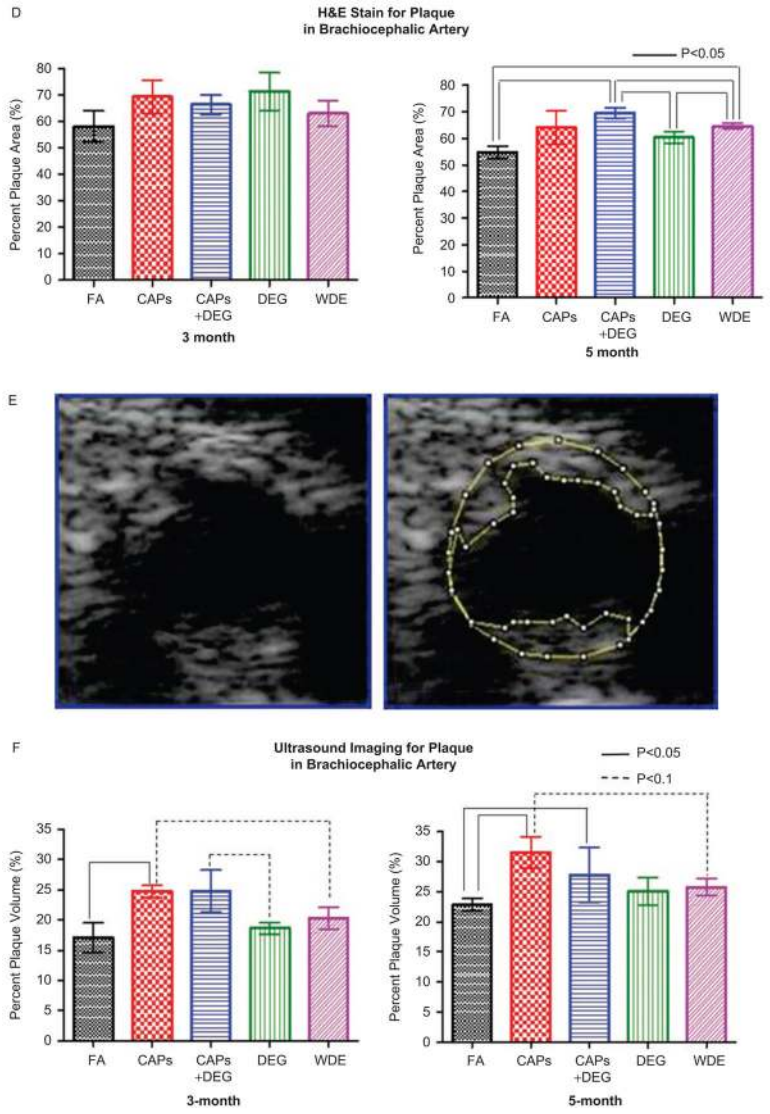


Figure 2.

(A) Representatives of the en face Sudan IV staining for plaque in whole aortic segments. Areas in red indicate plaque. (B) Plaque quantification by en face Sudan IV staining in whole aortic segments. Mean (*SEM*); $n = 3$/group. (C) Representatives of the H&E staining in brachiocephalic artery cross-sections. *Left*: Clear artery with no plaque. *Right*: Artery with large area of plaque in a cross-sectional view. (D) Plaque quantification by H&E staining in brachiocephalic artery cross-sections. Mean (*SEM*); $n = 5–6$/group. (E) Representatives of b-mode image of brachiocephalic artery by ultrasound biomicroscopy. *Left*: A representative ultrasound image of brachiocephalic artery cross-section. *Right*: Plaque identification and measurement for the left image. (F) Plaque quantification by ultrasound imaging in the brachiocephalic artery. Mean (*SEM*); $n = 6–8$/group.

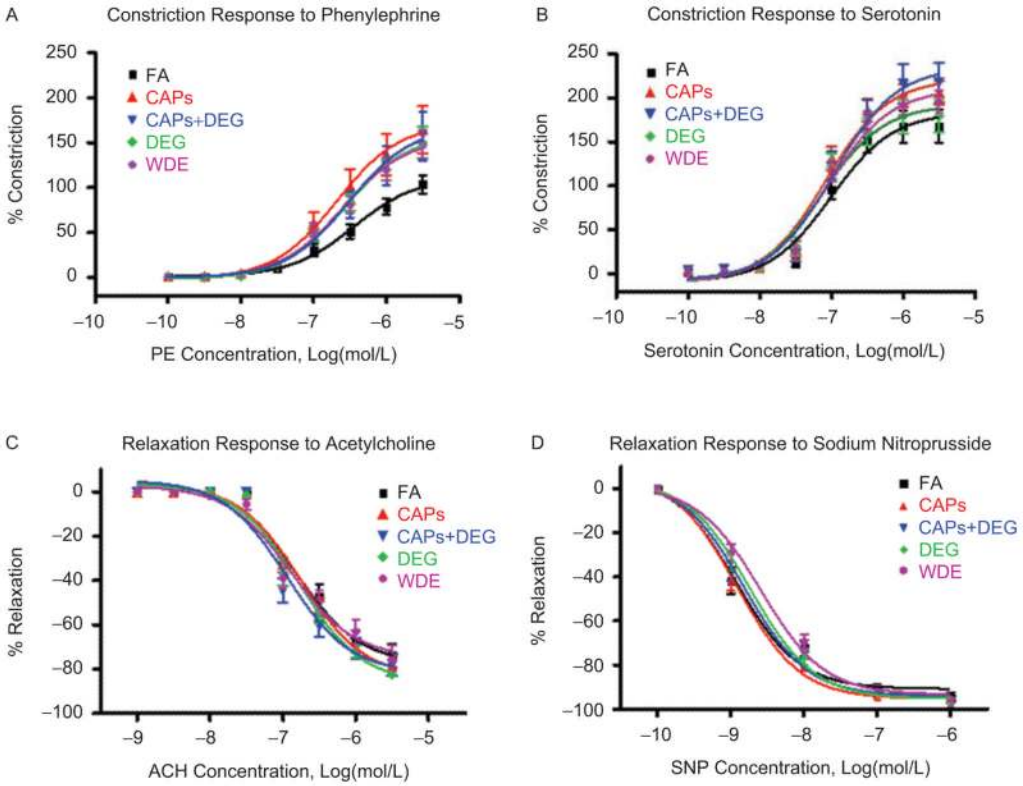


Figure 3.

Vascular responses to graded dose of (A) vasoconstrictor PE; (B) vasoconstrictor serotonin; (C) relaxation agonist acetylcholine; (D) relaxation agonist sodium nitroprusside. Five mice per group, two (for PE and 5-HT) or four (for ACH and SNP) aortic rings per mouse, were tested. Error bar represents *SEM*.

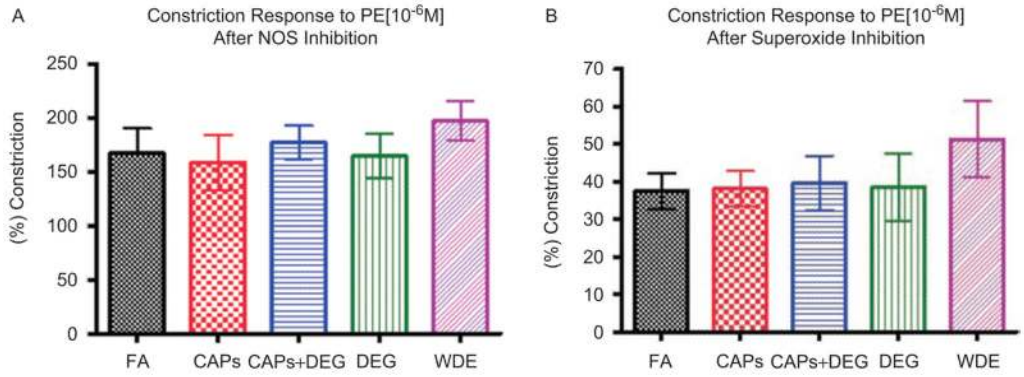


Figure 4.

(A) Vascular responses to 1 μM PE after NOS inhibition. (B) Vascular responses to 1 μM PE after superoxide inhibition.

Table 1

PM and gases characterizations in all five groups

				Ambient[a]	FA	CAPs	CAPs+DEG	DEG	WDE
PM conc. (μg/m^3)[b]	5-Months exposure (July to Dec. 07)	Mean ± *SD*		16.0 ± 13.9	Not detectable	105.4 ± 93.3	113.9 ± 95.0	Not detectable	437.5 ± 114.5
		Distribution	Median	12.0	/	70.1	79.1	/	430.1
			Max	78.0	/	432.1	445.7	/	715.5
			Min	0.6	/	12.0	20.3	/	157.3
	3-Months exposure (July to Oct. 07)	Mean ± *SD*		20.4 ± 14.8	Not detectable	135.4 ± 97.3	141.7 ± 101.2	Not detectable	434.8 ± 127.2
		Distribution	Median	16.7	/	116.7	120.0	/	432.0
			Max	78.0	/	432.1	445.7	/	694.4
			Min	1.6	/	17.6	21.8	/	157.3
PM size (nm)	CMAD[c] ± GSD[d]				/	79.7 ± 1.9	79.7 ± 1.9	/	78.2 ± 1.7
	MMAD[e] ± GSD				/	223.1 ± 1.6	223.1 ± 1.6	/	148 ± 1.5
Gases conc.	All gases						Equivalent to that in 438 μg/m^3 of DEP	Equivalent to that in 438 μg/m^3 of DEP	Equivalent to that in 438 μg/m^3 of DEP
	CO (ppm)						5.0 ± 1.8	5.0 ± 1.8	5.0 ± 1.8

Note: Data were expressed as mean ± standard deviation (SD).

[a] $PM_{2.5}$;

[b] by gravimetric measurements;

[c] count median aerodynamic diameter;

[d] geometric standard deviation;

[e] mass median aerodynamic diameter.

Table 2

Serum cytokine comparison of any two of the five exposure groups for animals exposed for five months

	mVCAM-1		mICAM-1		MMP9		E-Selectin		mResistin		mIL-10		mIL-6	
	Δ	*p*	Δ	*p*	Δ	*p*	Δ	*p*	Δ	*p*	Δ	*p*	Δ	*p*
CAPs vs. FA	98129.7	0.08	−9460.8	−0.58	2317.0	0.67	−943.0	−0.53	**10515.8**	**0.03**	−5.3	−0.46	−78.1	−0.24
CAPs+DEG vs. FA	**120494.7**	**0.02**	−19671.7	−0.27	624.0	0.90	−2151.6	−0.15	2061.5	0.40	**−14.6**	**−0.01**	**−124.9**	**−0.05**
DEG vs. FA	**115137.2**	**0.02**	**−29354.2**	**−0.02**	7520.8	0.19	618.5	0.69	2280.0	0.51	−6.8	−0.36	102.4	0.56
WDE vs. FA	**55244.0**	**0.00**	**−28182.5**	**−0.04**	4780.8	0.29	−140.2	−0.91	3987.5	0.23	**−11.8**	**−0.03**	−86.1	−0.27
CAP+DEG vs. CAP	22365.0	−0.71	−10210.8	0.61	−1693.0	0.76	−1208.5	0.33	−8454.4	0.06	−9.3	0.16	−46.8	0.26
CAP vs. DEG	−17007.5	−0.77	19893.3	0.18	−5203.8	−0.40	−1561.5	−0.29	8235.8	0.10	1.5	0.85	−180.5	−0.25
CAP vs. WDE	42885.7	0.42	18721.7	0.24	−2463.8	−0.61	−802.8	−0.39	6528.3	0.17	6.5	0.30	8.0	0.89
CAP+DEG vs. DEG	5357.5	0.92	9682.5	0.51	−6896.8	−0.25	−2770.0	−0.06	−218.5	−0.95	−7.7	−0.24	−227.3	−0.15
CAP+DEG vs. WDE	65250.7	0.16	8510.8	0.59	−4156.8	−0.36	**−2011.4**	**−0.05**	−1926.0	−0.52	−2.8	−0.42	−38.8	−0.48
WDE vs. DEG	−59893.2	0.18	1171.7	−0.87	−2740.0	0.59	−758.7	0.51	1707.5	−0.66	−5.0	0.44	−188.5	0.25

Note: For A vs. B, if A level is higher than B level, then Δ (difference) and *p* (significance) value is expressed as positive; if A level is lower than B level, then Δ and *p* value is expressed as negative. $|p| < .05$ (bold) is considered significant. All cytokine values were in pg/ml.

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Table 3

Effects of five different exposure atmospheres on vascular function in response to phenylephrine (PE), serotonin (5-HT), acetylcholine (ACH), and sodium nitroprusside (SNP)

		FA	CAPs	CAPs+DEG	DEG	WDE
PE	Maximum constriction, %	111.7 (8.0)	171.7 (15.4)*	168.6 (16.8)*	159.6 (9.9)*	155.7 (9.4)*
	Half maximal dose for constriction, 95%CI of EC_{50}, mol/L	$2.07–6.15 \times 10^{-7}$	$0.97–4.52 \times 10^{-7}$	$1.41–6.69 \times 10^{-7}$	$1.66–4.49 \times 10^{-7}$	$1.55–4.19 \times 10^{-7}$
5-HT	Maximum constriction, %	183.9 (9.9)	221.9 (8.4)	235.0 (12.7)	192.8 (10.2)	210.2 (7.7)
	Half maximal dose for constriction, 95%CI of EC_{50}, mol/L	$0.58–1.67 \times 10^{-7}$	$0.57–1.23 \times 10^{-7}$	$0.64–1.82 \times 10^{-7}$	$0.43–1.27 \times 10^{-7}$	$0.62–1.29 \times 10^{-7}$
ACH	Maximum relaxation, %	−77.46 (3.8)	−83.72 (3.1)	−81.69 (3.4)	−86.25 (3.1)	−74.87 (3.7)
	Half maximal dose for relaxation, 95%CI of EC_{50}, mol/L	$1.00–2.36 \times 10^{-7}$	$1.31–2.43 \times 10^{-7}$	$0.78–1.63 \times 10^{-7}$	$1.23–2.26 \times 10^{-7}$	$0.83–1.98 \times 10^{-7}$
SNP	Maximum relaxation, %	−90.90 (2.4)	−95.37 (1.8)	−94.17 (1.7)	−95.28 (1.9)	−93.47 (2.0)
	Half maximal dose for relaxation, 95%CI of EC_{50}, mol/L	$0.75–1.81 \times 10^{-9}$	$0.88–1.62 \times 10^{-9}$	$1.13–2.01 \times 10^{-9}$	$1.34–2.53 \times 10^{-9}$	$1.80–3.61 \times 10^{-9}$

Note: Data expressed as Mean (SEM). Significant difference comparing with FA was marked as *