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Effect of diesel exhaust inhalation on antioxidant and oxidative stress responses in adults with metabolic syndrome

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Abstract

Background—Traffic-related air pollution is associated with cardiovascular morbidity and mortality. Although the biological mechanisms are not well understood, oxidative stress may be a primary pathway. Subpopulations, such as individuals with metabolic syndrome (MeS), may be at increased risk of adverse effects associated with air pollution. Our aim was to assess the relationship between exposure to diesel exhaust (DE) and indicators of systemic antioxidant and oxidative responses in adults with MeS. We hypothesized that DE exposure would result in greater oxidative stress and antioxidant responses compared with filtered air (FA).

Methods—Ten adult subjects with MeS were exposed on separate days for two hours to FA or DE (at 200µg/m³), in a double blind, crossover experiment. Urinary 8-isoPGF_{2α} (F₂-isoprostanes), and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were assessed as markers of oxidative stress at 3 hrs and 22 hrs, respectively, after exposure initiation. To assess the short-term antioxidant response we analyzed plasma ascorbic acid (AA) 90 minutes after exposure initiation. All outcomes were compared to pre-exposure levels, and mean changes were compared between FA and DE exposures.

Results—Mean changes in urinary F₂-isoprostanes (ng/mg creatinine), (-0.05 [95% CI = -0.29, 0.15]), and 8-OHdG (µg/g creatinine) (-0.09 [-0.13, 0.31]), were not statistically significant. Mean changes in plasma AA (mg/dl) were also not significant (-0.02 [-0.78, 0.04]).

Conclusions—In this carefully controlled experiment, we did not detect significant changes in oxidative stress or systemic antioxidant responses in subjects with MeS exposed to 200µg/m³ DE.

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Keywords

Air pollution; diesel exhaust; oxidative stress; antioxidants; metabolic syndrome; controlled exposure experiment; crossover studies; vehicle emissions/toxicity; adult; biological markers; human; male; female

Background

Consistent associations between ambient fine particulate matter air pollution (PM_{2.5}, particles with aerodynamic diameter $\leq 2.5 \mu\text{m}$) and increased cardiovascular morbidity and mortality have been reported in epidemiologic studies (Dockery et al., 2005; Dockery & Stone, 2007; Laden et al., 2000; Pope & Dockery, 2006). Diesel exhaust (DE) represents a substantial fraction of ambient urban particle air pollution and traffic-related pollutants, and approximately 94% of diesel particulate matter is in the range of PM_{2.5} (Health Effects Institute, 1995). Traffic-related pollutants have been increasingly linked with cardiovascular disease (Dubowsky et al., 2006; Mills et al., 2005). Therefore, controlled exposure to DE is an excellent model for the assessment of health effects related to combustion-derived air pollution.

Although the underlying mechanisms are not well understood, oxidative stress has been observed in response to DE (or re-suspended DE particles) in cellular, animal, and occupational studies (Arbak et al., 2004; Kim et al., 2004; Lai et al., 2005).

Adults with diabetes are a population observed to be susceptible to the acute effects of PM, due to underlying metabolic characteristics (Dubowsky et al., 2006; Esposito et al., 2006; Ford et al., 2003; Resnick & Howard, 2002; Thomas et al., 2006). Likewise, patients with metabolic syndrome (MeS), a common “pre-diabetic” condition associated with increased cardiovascular risk, have similar underlying inflammation and oxidative stress (Dubowsky et al., 2006; Esposito et al., 2006; Ford et al., 2003; Palmieri et al., 2006).

Within the context of a larger study ($n = 22$), we conducted an intensive sub-study to ascertain whether systemic oxidative stress is a primary mode of action of traffic-derived air pollution. We performed a controlled experiment in which adult volunteers with MeS were exposed to DE. Plasma ascorbic acid (AA), urinary 8-isoPGF₂ α (F₂-isoprostane), and urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) were used as measures of antioxidant and oxidative response. In the overall study we examined vascular endpoints, including heart-rate variability, brachial artery reactivity, flow-mediated dilation, and coagulation markers (Carlsten et al., 2008; Peretz et al., 2008a; Peretz et al., 2008b; Gould et al., 2008; Scott et al., 2005; Lee et al., 1997). While we did detect acute vasoconstriction in the larger study, we did not have the power to detect health outcomes associated with oxidative stress in this smaller intensive sub-study; therefore, we do not report on those endpoints here. We designed a well-powered study to detect changes in systemic markers of oxidative stress, and we report here on this oxidative stress sub-study.

We hypothesized that short-term (90-min) exposure to DE would result in decreased AA, and that systemic markers of oxidative stress would be increased in the urine at 3 (F₂-

isoprostane), and 22 (8-OHdG) hr after exposure initiation. In order to establish a thorough kinetic measurement of oxidative stress, these variables were measured at multiple time points (90 min and 3, 6, 9, 12, and 22 h after exposure initiation).

Methods

Exposure Generation

Exposure sessions occurred at the University of Washington controlled exposure facility (Northlake lab). The DE generation and exposure system has been described elsewhere (Gould et al., 2008). Briefly, the DE generator consists of a 2002 turbocharged direct-injection 5.9-L diesel engine (model 6BT5.9G6 Cummins, Inc.) in a 100-kW generator. The generator-engine set is placed under load set at 75% of rated generator output.

The exhaust dilution system is a two-step process, which mimics a 4- to 5-min atmospheric dilution time. Dilution occurs in two sequential steps: First, the rapid mixing of diesel exhaust with filtered air is aided by an air blender. Second, the mixture flows through a 3-inch pipe for approximately 2 s, where it is diluted with secondary makeup air. All dilution air is heated and humidified (21°C, 50% relative humidity) and then passed through a HEPA filter (99.99% efficiency) and carbon filters.

Control over the final dilution mixture is achieved using a feedback-control system that continually adjusts the diversion fan based upon particle light scattering measurements at the intake plenum. Particle mass concentrations are assessed continuously in the subject area using a tapered element oscillating microbalance assessing particulate matter less than 2.5 micrometers in aerodynamic diameter (TEOM: Rupprecht & Patashnick model 1400a, Thermo Electron Corporation). In tandem with the TEOM measurements, 37-mm Teflon filter samples are collected using Harvard Impactors.

Subjects

We enrolled 10 adult subjects 18–49 yr old, nonsmoking at least 6 mo prior to recruitment, and with no history of ongoing medical care for heart disease, hypertension, asthma, diabetes, hypercholesterolemia, or other chronic condition. Women of childbearing age underwent a urine pregnancy test at screening and before each exposure, and were instructed to practice effective contraception during the study. All subjects gave written informed consent prior to exposures. The University of Washington Human Subjects Division approved the consent form and study protocol.

To qualify as metabolic syndrome, subjects needed to present with three of the following five criteria based on the updated ATP III criteria (Scott et al., 2005): waist circumference 102 cm for males and 88 cm for females; triglycerides 150 mg/dl; HDL cholesterol <40 mg/dl in males and <50 mg/dl in females; systolic blood pressure 130 mm Hg or diastolic blood pressure 85 mm Hg; and fasting glucose 100 mg/dl.

Exposure Session Protocol

On the morning of exposure days, subjects arrived fasting (at least 8 h) and were admitted to the University of Washington General Clinical Research Center (GCRC) at 7 a.m. First

morning urine samples were collected, measured, aliquoted, and frozen. Vital signs and pulmonary function tests were recorded at this time and an IV catheter was placed in the left forearm, blood was drawn, and a saline lock was placed. Participants had blood draws through the catheter serially during the exposure and at follow-up (90 min and 3, 6, 12, and 22 h after exposure initiation). Participants were driven (~5 min) to the exposure laboratory and began exposure sessions at 9 a.m. Participants had real-time physiologic monitoring throughout the duration of the exposure (heart rate, blood pressure, pulse oximetry), and subjects completed a symptom diary and pulmonary function test every 30 min. Exposure sessions lasted 2 h, after which participants were transported back to the GCRC for continuous monitoring and biological sampling for 18 h. On the following morning, interviews were conducted, vital signs were repeated, final blood draws and urine samples were collected, and participants were then discharged. Except for water, participants fasted until completing the exposure session. Following initial postexposure blood draws and pulmonary measures, subjects ate at the GCRC defined-composition meals, which were identical on each exposure day.

Antioxidant Determination

Ascorbic acid—The UW Clinical Nutrition Research Unit (CNRU) conducted all ascorbic acid (AA) analyses. Whole blood was collected in 7-ml Vacutainer tubes (containing lithium heparin). Samples were centrifuged at 2000 rpm, at 4°C, for 15 min to separate plasma; 500 µl heparinized plasma was pipetted into microcentrifuge tubes containing 50 µl metaphosphoric acid/dithiothreitol (MPA/DTT) immediately after centrifugation, and samples were stored at –70°C until analysis. Ascorbic acid was oxidized to dehydroascorbic acid by ascorbic acid oxidase, then converted to the quinoxalone derivative. This product absorbs at 340 nm, and the absorbance is directly proportional to the ascorbic acid concentration (Lee et al., 1997).

Oxidative Stress Determination

F₂-isoprostane—F₂-isoprostane assessments were conducted at the CNRU using liquid chromatography and tandem mass spectrophotometry (LC/MS/MS) (Saenger & Sadrzadeh, 2006). Urine samples were centrifuged at 2500 rpm for 5 min; 500 µl of the supernatant was pipetted into 1.5-ml microcentrifuge tubes to which was added 25 µl of internal standard. The sample was then centrifuged for 5 min at 1300 rpm, after which the supernatant was transferred to a high-performance liquid chromatography (HPLC) autosampler vial and a 30-µl aliquot was injected onto the analytical column at the start of the HPLC gradient run. F₂-isoprostane levels in duplicate samples were assessed by enzyme-linked immunosorbent assay (ELISA, Oxford Biochem), for internal comparison. Results from the ELISA (not reported) were inconsistent and had little correlation with HPLC results ($r = 0.2$), as has been previously reported (Pilger & Rudiger, 2006).

8-OHdG—Urine samples were analyzed for 8-OHdG by Genox Corp. (Baltimore, MD). Competitive ELISA was used for quantification. In brief, after thawing, 50-µl samples and standards were added to 8-OHdG conjugate plates, followed by the primary antibody solution. Samples were incubated for 1 h at 37°C. Plates were washed and a secondary antibody solution was applied for 1 h at 37°C. After washing, 100 µl of chromagenic

substrate (3,3',5,5'-tetramethylbenzidine) was added and allowed to react for 15 min. Optical density was assessed at 490 nm (Shigenaga & Ames, 1991).

Statistical Analysis

Data are presented as mean $\pm$ standard error (SE) unless specified otherwise. Statistical analyses were performed using STATA 9.2 (StataCorp LP). Mean changes in AA, F₂-isoprostane, and 8-OHdG after each exposure (FA and DE) were compared to respective pre-exposure levels for all time points, and exposure-specific changes were compared using two-tailed paired *t*-tests. Primary hypotheses were for specific time points for each variable (AA: 90 min, F₂-isoprostane: 3 h, and 8-OHdG: 22 h). Secondary hypotheses included the same variables at other time points. A two-way analysis of variance (ANOVA) model was used to test for period and carryover effect. Results were considered statistically significant if $p < .05$.

Results

All subjects ($n = 10$) completed exposure sessions to both DE and FA. The exposures were well tolerated and no adverse effects were reported. All subjects were nonsmokers. Participant characteristics (Table 1) illustrate a consistent elevation in body mass index (BMI). However, despite all individuals meeting clinical criteria for MeS, other parameters are robust and overall mean group values for other MeS parameters fall within the range of healthy adults. Our exposure system maintained consistent average exposure concentrations during the FA and DE exposure situations; values were for PM_{2.5} (4.8 ± 2.9 , 205.3 ± 6.2 $\mu\text{g}/\text{m}^3$), NO₂ (15.5 ± 2.5 , 25.5 ± 11.7 ppb), and CO (0.3 ± 0.3 , 0.7 ± 0.3 ppm), for FA and DE, respectively. Mean NO levels varied considerably between exposures (FA: 38.6 ± 30.3 ppb; DE: 1515.8 ± 417.3 ppb). Several unpaired data points exist due to nonsystematic errors in sample collection or handling. Missing data was not included in paired analyses. Absolute values of AA, F₂-isoprostane, and 8-OHdG reflect expected circadian variation. No changes in outcome measures were statistically significant between FA and DE in primary or secondary analyses (Tables 2 and 3). No period or carryover effects were observed.

Discussion

This study represents a carefully controlled experimental approach to assessing oxidative stress—a putative mechanistic pathway—as a result of diesel exhaust inhalation in humans; in contrast to expectations, we were unable to detect evidence of increased systemic reactive oxygen species (ROS) or change in antioxidant level. Though our sample size was small, our rigorous approach ensured that we were well powered to detect effect sizes comparable to or smaller than those detected in observational studies.

While there is no prior published evidence for systemic (ROS) production in controlled human DE exposures (Xia et al., 2006), in vitro, occupational, and epidemiological data point toward oxidative stress as an important mechanism of air pollution, traffic-related air pollution, and DE-related adverse effects. Experimental exposure studies have demonstrated increased exhaled CO (Nightingale et al., 2000) and nasal lining fluid ascorbate (Blomberg

et al., 1998) (both indicative of an oxidative response). Recent ozone controlled chamber studies have reported increased oxidative stress (Chen et al., 2007). Recent observational and semi-controlled studies have demonstrated increased thiobarbituric acid-reactive substances (TBARS) associated with ambient PM₁₀ exposure (Liu et al., 2007), elevated numbers of DNA strand breaks, and enhanced up-regulation of DNA repair enzymes associated with PM (Brauner et al., 2007). Still, the mechanistic role, and association with adverse health effects, of DE-induced oxidative stress remain unclear.

Our study was carefully executed to provide a robust assessment of DE effects on oxidative stress; the rigorous experimental design controls for most potentially confounding variables, and we would anticipate more precision in our results than in observational study designs. However, there are many potential reasons for why we might not have observed systemic oxidative effects of DE. Some possible explanations for the differences between our findings and those of other approaches include specific characteristics of our DE generating system, concentration and composition of gases and particles, subject population, exposure duration, other protocol differences, or the specific methods of and types of outcomes measured.

The DE concentration, gaseous co-pollutants, and collected filter sample metals from our exposure generating system are qualitatively and quantitatively different from those of other studies. For example, using a different DE inhalation system, Salvi et al. (1999) report NO₂ concentrations of 1.6 ppm, whereas we report significantly lower concentrations of 10-35 ppb (Carlsten et al., 2007). Since NO₂ directly initiates pulmonary inflammation (Mohsenin, 1994), higher NO₂ levels could be responsible for effects seen in other studies. In another study, Rudell et al. (1996) demonstrated in a controlled diesel exposure that by reducing particles, by 46% (using a particle trap), DE-induced bronchoconstriction was not attenuated, as compared with full PM dose, suggesting that certain gases—which might vary between exposure systems—and not particles are potentially associated with certain effects.

While study participants met the criteria for metabolic syndrome, they had only mildly abnormal metabolic and physiologic characteristics (see Table 1), which might indicate we had not identified the most sensitive subjects. Subjects' mean baseline AA levels were not reduced compared to population levels based on NHANES data (Ford et al., 2003). It is possible that, like smokers (Hiemstra, 2002; Lykkesfeldt et al., 2003), this population has adapted to an increased endogenous oxidant load and has an increased ability to neutralize an acute oxidant burden such as DE exposure. Severity of disease may be a factor linking adverse effects with exposure in observational studies. A recent case-crossover study demonstrated that short-term elevated ambient PM_{2.5} concentrations were only associated with adverse effects in individuals with angiographically demonstrated coronary artery disease (Pope et al., 2006).

There is no conventional experimental exposure duration, though our 2-hr exposure session is typical. Long-term repeated exposure studies have demonstrated adverse health effects associated with PM_{2.5}; however, effects of short-term exposures can be difficult to quantify in individuals with varied background exposure and susceptibility factors. Our subjects remained at rest throughout the exposure, while others have used intermittent exercise (Mills et al., 2005, 2007; Salvi et al., 1999). Activity level during exposures may be partly

responsible for results seen in other studies. Exercise increases ventilation rate, as well as generating inflammation and oxidative stress, independent of DE (Vincent et al., 2006).

There are numerous possible oxidative stress end-points, and the strengths and limitations of these have been reviewed in detail elsewhere.[44-46] We selected our endpoints based on data from observational studies, lack of invasiveness, and cost. We considered adding TBARS; however, recent reviews suggest that F₂-series isoprostanes are more sensitive, specific, and reproducible. In addition, we planned to examine plasma reduced and oxidized glutathione (GSH/GSSG); however, the analytic method we chose [47] and further developed in our laboratory was unable to provide reliable determination of these measures. [47]

Oxidative responses to PM may be tissue-specific, rather than systemic. For example, Loft et al. assessed PM-mediated changes in both lymphocyte and urinary 8-OHdG levels, with changes detected only in lymphocytes (Loft & Poulsen, 1999; Loft et al., 1999). Recently Araujo et al. (2008) observed hepatic oxidative stress (increased malondialdehyde) in mice exposed to ultrafine particles. Systemic oxidative stress biomarkers may not parallel tissue-specific changes; therefore, oxidative stress induced by DE may not be represented in systemic analyses, or may do so in a time period or biomarker not reflected in our study.

F₂-isoprostanes are the most readily tested of the isoprostane isomers. Although these are sensitive, specific, stable, and reproducible biomarkers, several other isomers increase from oxidative stress. Morrow et al. have demonstrated that the F₂-series isoprostanes are formed competitively with the E2 and D2 isoforms and that depletion of cellular reducing agents, such as glutathione (GSH) or alpha-tocopherol, favors the formation of E2/D2 isoprostanes over those of the F₂ series (Morrow, 2006). Our analysis included only the F₂-series isoprostane, 8-isoPGF₂α, in a population believed to be at increased risk for oxidative stress. Thus, it is possible that the lack of exposure-related effect could be due to GSH depletion-related shunting of arachidonic acid by-products toward the E2 and D2-isoprostanes. For this reason, future analyses for GSH and tocopherol should be coupled with F₂-series isoprostane assessment.

Methodological issues related to oxidative stress assessment remain an important area of inconsistency between studies. We were able to compare two methods of isoprostane assessment (ELISA and LC/MS/MS), and, consistent with the literature, there was little correlation (data not presented). With regard to 8-OHdG, we utilized ELISA, consistent with other exposure-related studies demonstrating air pollution-related effects (Kim et al., 2004). Although a number of large studies have used ELISA for 8-OHdG determination, uncertainty continues to exist with regard to specificity of the ELISA. Correlation studies between ELISA and HPLC and HPLC-EC have yielded mixed results; in nearly all cases the ELISA method produces higher levels than those measured by HPLC, indicating probable cross-reactivity in the assay (Pilger & Rudiger, 2006).

Our 8-OHdG results are consistent with results seen by Loft et al. (1999) where plasma 8-OHdG levels increased in response to traffic-related pollution, but no changes were observed in urinary levels. On the other hand, our results are contrary to elevated urinary 8-

OHdG levels observed in workers exposed to PM. (Kim et al., 2004) observed an effect of 1.67 ng/mg creatinine/($\mu\text{g}/\text{m}^3$ PM_{2.5}) (our findings can confidently exclude an effect of 0.31 in an experimental setting). Christiani et al. (Kim et al., 2004) also observed a strong exposure-response relationship in 8-OHdG with specific metal components of PM (vanadium, chromium, and nickel). These observational studies contrasted with our experiments by examining effects in workers (diesel bus drivers and boilermakers, respectively), and exposure duration was longer and had double the concentration of PM ($440 \mu\text{g}/\text{m}^3$) compared to our controlled study. In other studies, lymphocyte-specific 8-OHdG levels have been assessed. 8-OHdG levels have been shown to increase in parallel with an elevated metal fraction of PM, specifically vanadium and chromium (Sorensen et al., 2005). These increases were independent of PM_{2.5}, and no exposure-related changes were observed in urinary 8-OHdG.

Conclusion

Controlled experimental studies of this nature offer unique opportunities to study mechanisms of exposure-related responses, and we did not confirm that DE inhalation causes systemic oxidative stress in humans. Since observational studies have observed increased adverse human health effects, and since other experimental and observational studies suggest that oxidative stress may be a primary mechanism, the reasons for this discrepancy warrant further research. Future studies examining multiple oxidative stress and antioxidant markers, in multiple tissues and time points, will be needed to further clarify the role of oxidative stress in human health effects linked to traffic-related air pollution.

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Table 1

Subject characteristics.

Subject	Age(yr)	Gender(M/F)	Waist(cm)	BMI(kg/m ³)	TG(mg/dl)	HDL(mg/dl)	FBG (mg/dl)	BP (mm/Hg)
1	40	F	132	45	120	39	103	108/54
2	39	F	91	34	79	40	110	116/73
3	34	M	114	33	112	32	118	136/73
4	39	F	119	39	158	42	96	120/70
5	46	M	112	36	137	34	110	129/83
6	48	F	97	33	81	45	104	119/77
7	47	M	114	45	235	33	98	119/68
8	36	M	130	41	210	32	93	142/91
9	48	M	104	30	215	33	103	133/70
10	31	M	152	51	72	34	127	120/57
Mean ± SE	38.9 ± 8.3	F(4)M(6)	116 ± 18.0	40.3 ± 8.1	151.8 ± 66.0	35.90 ± 4.5	106.2 ± 2.4	124/72

Note. BMI: body mass index, TG: triglycerides, HDL: high-density lipoprotein, FBG: fasting blood glucose, BP: blood pressure.

Table 2

Changes in antioxidant and oxidative stress measures after exposures (mean $\pm$ SE).

	Ascorbate(mg/dl)		F ₂ -isoprostanes (μ g/g creatinine)		8-OHdG(ng/mg creatinine)	
	FA	DE	FA	DE	FA	DE
Pre	0.92 $\pm$ 0.14	0.92 $\pm$ 0.1	0.45 $\pm$ 0.04	0.36 $\pm$ 0.05	0.16 $\pm$ 0.06	0.16 $\pm$ 0.05
90 min	0.90 $\pm$ 0.14	0.96 $\pm$ 0.10	—	—	—	—
3 h	0.94 $\pm$ 0.14	0.98 $\pm$ 0.10	0.49 $\pm$ 0.05	0.44 $\pm$ 0.13	—	—
6 h	0.76 $\pm$ 0.11	0.81 $\pm$ 0.10	0.49 $\pm$ 0.05	0.29 $\pm$ 0.03	—	—
12 h	0.71 $\pm$ 0.10	0.75 $\pm$ 0.09	0.45 $\pm$ 0.05	0.32 $\pm$ 0.03	—	—
22 h	0.84 $\pm$ 0.12	0.91 $\pm$ 0.08	0.39 $\pm$ 0.03	0.30 $\pm$ 0.03	0.08 $\pm$ 0.01	0.07 $\pm$ 0.01

Table 3
Differences in mean change (post- minus pre-exposure), diesel exhaust versus filtered air.

	90 min	3 h	22 h	p Value	95% CI
Ascorbate (mg/dl)	-0.018	—	—	$p > .5$	-0.79, 0.04
F ₂ -isoprostanes (µg/g creatinine)	—	-0.05	—	$p > .6$	-0.29, 0.19
8-OHdG (ng/mg creatinine)	—	—	0.087	$p > .4$	-0.13, 0.31

Note. —, Samples not evaluated at this time or not a primary endpoint.