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Controlled Exposure to Diesel Exhaust Causes Increased Nitrite in Exhaled Breath Condensate among Subjects with Asthma

Sabiha Hussain, MD¹, Robert Laumbach, MD³, Jakemia Coleman, MD¹, Hatim Youseff, MD², Kathie Kelly-McNeil, BA³, Pamela Ohman-Strickland, PhD⁴, Junfeng Zhang, PhD⁵, and Howard Kipen, MD³

¹University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School-Department of Pulmonary and Critical Care Medicine, New Brunswick, New Jersey, USA

²University Medical Center at Princeton, Department of Pulmonary and Critical Care Medicine, Princeton, New Jersey, USA

³University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School-Department of Environmental and Occupational Medicine and Environmental and Occupational Health Sciences Institute, Piscataway, New Jersey, USA

⁴University of Medicine and Dentistry of New Jersey-School of Public Health, Department of Biostatistics, Piscataway, New Jersey, USA

⁵Keck School of Medicine, University of Southern California, Department of Preventive Health, Los Angeles, California, USA

Abstract

Objective—We aimed to determine if oxidative/nitrosative stress plays a role in the acute effects of diesel exhaust (DE) on asthmatics.

Methods—Crossover study design, 16 subjects with mild to moderate asthma were exposed to clean filtered air (CA) or diluted DE (300 μ g/m³ as PM_{2.5}) for 1 hour with intermittent exercise.

Results—Airway hyperreactivity increased 24 hrs after exposure to DE as compared to CA (PC20 14.9 mg/ml vs. 19.7 mg/ml, $p=0.012$). Nitrite in EBC was elevated immediately after diesel exposure ($p=0.052$), and remained elevated 4 and 24 hrs after exposure.

Conclusions—After exposure to DE, subjects with asthma demonstrated increased airway hyperreactivity and obstruction. Increased nitrite in EBC, in the absence of increased eNO, suggests a non-inflammatory oxidative stress mechanism by which DE affects the lung.

Corresponding Author: Sabiha Hussain, MD, University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School, 1 Robert Wood Johnson Place, Medical Education Building – Room 568, New Brunswick, New Jersey 08901, hussaisa@umdnj.edu; Phone: (732) 235-7840; Fax: 732-235-7048.

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Keywords

asthmatics; diesel exposure; hyperreactivity; nitrite; exhaled breath condensate

INTRODUCTION

Epidemiological studies have associated asthma morbidity with exposure to traffic-related air pollution.[1–2] Diesel exhaust (DE) is a major constituent of traffic pollution that has been used experimentally in a number of controlled animal and human exposure studies. DE is a complex mixture of gas and particle-phase compounds known to cause respiratory tract irritation and inflammation in animal models and among healthy humans.[3, 4–5] However, in three published controlled exposure studies, DE did not consistently produce acute airway inflammation among human subjects with asthma.[6–9]

The lack of significant airway inflammation among asthmatics in prior controlled studies of DE led us to speculate that nitrosative and/or oxidative stress without inflammation may play a direct role in DE-induced asthma exacerbation. The formation of reactive nitrogen and oxygen species plays a role in asthma pathogenesis. [10–11] Nitric oxide (NO) and its metabolites in exhaled breath condensate (EBC) may be markers of oxidative/nitrosative stress, in addition to inflammation.[12–13] The stable oxidation products of NO, nitrite and nitrate, are elevated in the EBC of persons with asthma, especially during acute exacerbations.[14] To date, there are no published reports of changes in nitrite/nitrate in EBC following controlled exposure to DE among healthy subjects or subjects with asthma.

Individuals with lowered antioxidant capacity may be at increased risk for development of asthma as well as exacerbations.[15–16] Experimental studies suggest that responses to DE may follow a hierarchical model in which low levels of exposure stimulate antioxidant responses, with inflammation and tissue injury occurring only if innate anti-oxidant defenses are exceeded.[17] The glutathione S-transferase (GST) superfamily of antioxidant and detoxifying enzymes appears to play an important role in antioxidant responses and respiratory health. Asthmatics with polymorphisms of the GST genes such as glutathione S-transferase mu-1 (GSTM1) null and glutathione S-transferase P-1 (GSTP1) 105 Ile/Ile have demonstrated heightened nasal allergen responsiveness, bronchial hyperreactivity, pulmonary oxidative stress and inflammation after exposure to DE.[18–19]

We aimed to expand the limited data on airway effects of DE among individuals with asthma by measuring markers of nitrosative/oxidative stress in EBC, along with a marker of inflammation in exhaled breath, exhaled nitric oxide (eNO). We hypothesized that inhalation of DE would result in increased nitrosative and oxidative stress, and that effects on lung function and airway hyperresponsiveness would be modified by genetic polymorphisms of the GST superfamily of genes. To test these hypotheses, we conducted a controlled DE exposure study in subjects with mild or moderate asthma. We demonstrated significant increases in airway obstruction, airway reactivity, and markers of nitrosative/oxidative stress with the latter demonstrating an interaction with GSTM1 genotype.

METHODS

Subjects

We recruited 16 non-smoking subjects, (9 females; 7 males) with a mean age of 29.9 years (range 20–48). All were diagnosed to have either mild/moderate asthma according to the American Thoracic Society (ATS) guidelines.[20] Asthma was defined clinically as a chronic airway disease that may cause wheezing, breathlessness, chest tightness and nighttime or early morning coughing. All subjects underwent baseline methacholine challenge tests (MCTs) 3–7 days prior to exposure to determine the provocative concentration of methacholine causing a 20% reduction in forced expiratory volume in one second (PC20). Six subjects had PC20 $\leq$ 8 mg/ml. All subjects were free of respiratory tract infections for at least 6 weeks prior to the study period. Subjects continued their routine inhaled corticosteroids and long and short acting beta agonists during the study. Informed consent was obtained, and the study was approved by The University of Medicine and Dentistry of New Jersey Institutional Review Board.

Study Design

The study was a single-blind crossover trial. All subjects were exposed to clean filtered air (CA) and diesel exhaust (DE) for 1 hour on 2 separate occasions from 1 to 3 weeks apart. During exposure, the subjects alternated at 15-minute intervals between seated rest and moderate exercise on a cycle ergometer, targeted to achieve 65% of maximal heart rate, approximating a minute ventilation of 20 L.min⁻¹/m². Lung function and EBC were assessed before and at, 0, 4, and 24 hrs after exposure. Exhaled NO was measured before and at, 0, and 24 hrs after exposure. MCT was performed at 24 hrs after each exposure. Symptom surveys were completed before 0, 4 and 24 hrs after exposure. Medication history was taken at enrollment and use of rescue inhaler during the course of the study was documented in the symptom survey.

Exposure—The exposure was conducted in the controlled environment facility at the Environmental and Occupational Health Sciences Institute.[21] Exposures were to DE, generated by a one-cylinder 5500W Yanmar electrical generator and diluted with filtered air, and filtered air only (CA condition). The actual PM_{2.5} concentrations for the experimental sessions were $294 \pm 24.2 \mu\text{g}/\text{m}^3$ for DE and $4 \pm .3 \mu\text{g}/\text{m}^3$ for CA. Similar exposure conditions used in our previous studies have been reported in detail.[22]

Exhaled Nitric Oxide (eNO)—We collected whole exhaled breath in mylar balloons using a controlled-flow apparatus described by Paredi. et al.[23] Subjects inhaled to total lung capacity without a nose clip, and then immediately exhaled for 15 seconds against resistance into the balloon while targeting a pressure of 20mm Hg. Within 2 hrs of collection, the exhaled NO concentration in the balloon was measured with a chemiluminescence analyzer (Model 42 C, Thermo Electron, Franklin, MA).

Exhaled Breath Condensate (EBC)—EBC was collected with RTubes™ (Respiratory Research Inc, Austin TX). Subjects were asked to breathe tidally without a nose clip for 10 minutes and 1–3 ml of specimen was collected and stored at -80C and later analyzed for

nitrite and nitrate, after reduction to NO, using a chemiluminescence technique (NOA28i, Sievers, Boulder, CO). EBC pH was measured using a Micro-Combination pH electrode (Thermo Electron, Beverly, MA).

Lung Function Test—Spirometry was conducted using a SpiroPro handheld spirometer (Sensorimedic, Minneapolis, MN) that was calibrated with a standard 3L syringe, according to ATS recommendations, accuracy $\pm 3\%$. Tests were interpreted and performed according to ATS guidelines.[24]

Methacholine Inhalation Test (MCT)—All MCT's were performed using the 5-breath dosimeter method by the same technician in accordance with the method described by ATS guidelines.[24] Airway responsiveness was defined by the latest ATS guidelines: PC20 ≤ 8 is recommended to be used clinically as it is associated with fewer false positive results.[24]

Genotyping—Single nucleotide polymorphisms of three genes from the GST superfamily were evaluated. GSTM1 (null or present), GSTP1 105 (Ile/Val or Ile/Ile), and GSTT1 (null or present) were assayed in white blood cells or buccal epithelial cells via allelic discrimination using Taqman chemistry with probes and primers designed using Primer Express v.2.0 (ABI, Foster City, CA). Ten μ l polymerase chain reactions were run in an ABI 7900HT (ABI, Foster City, CA) using default cycling and end plate detection protocols. All data were analyzed using the Sequence Detection Software v 3.0 (ABI, Foster City, CA).

Statistics—Means and standard deviations were calculated for all outcomes at each time point. Mixed linear models were used to evaluate the effect of exposure (DE versus CA), with post-exposure measures as the response, exposure as the predictor of interest and pre-exposure level of the corresponding measure as an additional covariate. A random effect for subject accounted for correlation of measurements between sessions (CA and DE exposures). P-values from the type III F-tests are reported to assess the significance of exposure effects.

RESULTS

Subject Characteristics

Characteristics of the individual subjects are presented in Table 1. Two subjects were former smokers, having quit 8 and 2 years previously. Individuals were selected based on clinical diagnosis of asthma and no allergy or skin prick testing was performed. Out of the 16 subjects, 6 (37.5%) had PC20 ≤ 8 at baseline prior to exposure.

Lung function effects

Spirometry demonstrated progressive reductions in percentage of predicted forced expiratory volume in 1 sec (FEV1%) after DE exposure, with decrements of 0.8%, 1.8% and 3.3% at 0, 4, and 24 hrs after exposure compared to before exposure. (Figure 1-Mean change in percentage of predicted FEV1 from baseline, immediately post, 4 and 24 hours post DE and CA exposure). In contrast, after exposure to CA, there were post-baseline increases in FEV1 of 0.8%, 2.8% and 3.1%, respectively. The difference in mean percent change in

FEV1% from baseline between DE and CA exposures was statistically significant at 24 hrs post exposure ($p=0.043$).

Airway Hyperresponsiveness (AHR)

Mean PC20 24 hrs after DE exposure (14.9 mg/ml) was significantly decreased compared to CA (19.7 mg/ml), (CI 95 1.23–8.35, $p=0.012$). Only three individuals were MCT positive after CA whereas seven were MCT positive after DE. Two individuals with negative MCT when exposed to CA had positive MCT with DE. The single line at 25 represents thirteen MCT negative subjects when exposed to CA and eight after DE exposure. (Figure 2 – PC20 24 Hrs Post Exposure CA and DE). Individuals with PC20 <16 at baseline (6) had increase AHR after exposure to DE ($p=0.0078$).

Exhaled Nitric Oxide

Exhaled nitric oxide was increased immediately and 24 hrs after exposure to DE (Figure 3- Exhaled nitric oxide before, post and 24 hours post DE and CA exposure). However, eNO levels increased 24 hrs after CA as well. The differences between DE and CA were not statistically significant at any time point.

EBC Markers

EBC pH did not demonstrate any clear pattern of change after exposures, and there were no statistically significant differences between DE and CA at any time point (Figure 4-pH of EBC collected immediately post, 4 and 24 hours post DE and CA exposure).

Compared to before exposure, EBC nitrite was increased after exposure to DE, but not after CA (Figure 5-EBC nitrite change from before exposure, immediately post, and 4 and 24 hours post DE and CA exposure). The difference between DE and CA approached significance ($p=0.052$) only at immediately post exposure.

Analysis by Genotype

In a subsample of our subjects ($n=7$) GSTM1 genotype did not modify the effect of exposure on FEV1 at any time point; however, it did modify the effect of DE on EBC nitrite with GSTM1 null subjects demonstrating a larger increase immediately post diesel exposure compared to GSTM1 present subjects ($p=0.009$). There was no significant effect modification by GSTT1 or GSTP1 (Figure 6-Glutathione S-transferase genotype influence on EBC nitrite concentration. Change is the difference between immediately post- ad pre-exposure value comparing DE and CA exposure).

DISCUSSION

Subjects with mild/moderate asthma demonstrated decreased FEV1% predicted and increased AHR along with increased nitrite in EBC following a 1-hour controlled exposure to DE at a concentration reflective of levels encountered in occupational and extreme urban environments.[25–26] The lack of increase in eNO, is consistent with previous studies that did not find increased inflammation among subjects with asthma after controlled exposure to DE. These findings support previous determinations that DE does not cause decrements in

lung function and increased AHR by increasing airway inflammation⁵. Our novel finding of an immediate increase in EBC nitrite after DE exposure may suggest an alternative biological mode of action for changes in airway function and responsiveness.

The clear increase in acute airway obstruction, measured as a decrease in FEV1% predicted, is also a novel finding not reported in previous studies of controlled exposure to DE. The FEV1% decreases of 0.8, 1.8 and 3.3 are of unclear clinical significance for an individual, but may be important when applied to larger populations. The decrease at 24 hrs was statistically significant and comparable to the 4% decrease at 24 hrs associated with real-world diesel exposure [26]. Nordenhall et al, and Stenfors et al, reported no change in FEV1 among asthmatics after exposure to DE at 300 µg/m³ for 1 hr and 108 µg/m³ for 2 hrs, respectively.[6–7] Nordenhall et al. did find an increase in specific airways resistance (Raw).[9] These different outcomes across studies of controlled exposure to DE may be attributable to differences among the exposure conditions; as, chemical and physical properties of DE can vary with engine type, load, other operating conditions, and dilution conditions.[25] They can also be related to severity of asthma and allergy status and a subject's treatment regimen. . Following a yet-more-complex, real-world exposure to diesel traffic on a London street, McCreanor et al also observed decreased FVC and FEV1 among asthmatics, but no change in eNO.[26]

We also found increased mean AHR at 24 hrs after exposure to DE compared to CA. Each of the six subjects who had PC20 of 8 at baseline had a decrease in PC20 following DE exposure. In prior studies, Nordenhall et al, found a decrease in PC20 at 24 hrs after exposure to DE at 300 µg/m³ for 1 hr, but Behndig reported no significant change in AHR at 40 hrs after 100 µg/m³ for 2 hrs.[7–8] Our finding of increased AHR at 24 hrs is in agreement with the results of Nordenhall et al, who used the same concentration and duration of DE exposure. There were ten clinically asthmatic individuals who had PC20 >8mg/ml prior to exposure. There is substantial support in the literature for MCT-negative asthma reportedly due to lack of current symptoms or steroid treatment.[27–28] In addition methacholine challenge testing is insensitive for those with mild disease (6 of our subjects). Methacholine testing has many false negatives and positives related to technical errors, and underlying comorbidities such as sarcoidosis and heart failure. Does having increased hyperresponsiveness necessarily indicate increased airway inflammation? The relationship between airway hyperresponsiveness and airway inflammation was recently examined. [27, 29] Two components of AHR were postulated: a persistent, structural, component (subendothelial thickening, smooth muscle hypertrophy) and a variable component (acute effects of pollutants, allergens, respiratory infections). The effect of repeated exposures to DE on lung disease, as occurs in the real world, is beyond the scope of our single, short-term DE exposure. However, the data presented here reinforces an acute role for diesel exhaust and other pollutants in asthma exacerbation, with a novel detailed mechanism hypothesized below.

In general, biological modes of action underlying acute changes in lung function and airway hyperresponsiveness include inflammation and/or neuronal pathways.[²⁸] Three earlier controlled exposure studies of DE showed an unexpected lack of evidence for increased airway inflammation measured as cell counts and soluble markers (IL-8, ICAM-1,

RANTES, GM-CSF) in induced sputum or bronchoalveolar lavage among subjects with asthma, in contrast to marked inflammation among healthy subjects.[6–8] Consistent with these findings, we found no increase in eNO, a marker of inflammation, after exposure to DE compared to CA. Extensive evaluations of markers of inflammation have been done in prior studies; referenced above, found a general lack of evidence of inflammation among subjects with asthma after exposure to DE. Absent demonstrable inflammation, neuronal pathways are an alternative explanation for our findings of decreased lung function and increased AHR following exposure to DE. Diesel exhaust contains a number of known irritants, most notably various aldehydes. The capacity of irritants to induce exacerbation of asthma is well known.[29] Recently, members of the Transient Receptor Potential (TRP) family of ion channels that are expressed on sensory nerves and other cell types have been implicated in airway responses to specific irritating compounds.[30] In particular, TRPA1 (Transient Receptor Potential Ankyrin 1) is activated by alpha-beta unsaturated aldehydes such as acrolein and crotonaldehyde, both found in DE. Vanilloid receptor TRPV1 also plays a role in airway responses to irritants. [31]

Interestingly, stimulation of TRPA1 and TRPV1 receptors in the airways may also explain the increase in EBC nitrite that we observed immediately following DE exposure. This increase in EBC nitrite appears too quickly to result from induction of nitric oxide synthase (NOS) II, which is regulated primarily at the level of transcription, and is usually associated with activation of cytokine receptors and inflammation.[11] In contrast, regulation of NOSI and NOSIII, expressed in sensory nerves and epithelial cells of the lung, as well as other cell types, is primarily calcium-dependent and occurs rapidly.[32] Activation of TRP receptors increases intracellular calcium.[33] Although there is no direct evidence that TRPA1 or TRPV1 receptors cause increased production of NO in the airway epithelium, activation of TRPV1 channels caused release of NO from vascular endothelium in the rat.[34] Our lack of increase in measured eNO remains to be explained; however others have reported a lack of correlation between levels of eNO and NO metabolites in EBC, possibly due to different anatomic sources.[15]

In addition to inhaled irritants, agonists of TRPA1 receptors include endogenous aldehydes that are by-products of oxidative stress and lipid peroxidation. DE contains redox active compounds, such as metals, polycyclic aromatic hydrocarbon (PAH), and PAH-quinones that are believed to contribute to formation of the free radicals that can initiate lipid peroxidation.[21] A role for oxidative stress in the production of EBC nitrite following exposure to DE is consistent with our observation that subjects who were null for GSTM1, an important antioxidant enzyme, had greater immediate increases in EBC nitrite as compared to GSTM1 present subjects in this small sample. .

This study confirms and expands understanding of the acute effects of exposure to DE on mild-to-moderate asthma, but it has several limitations. The small size of the study, incomplete at-home collection of samples, and limited genotype data resulted in low statistical power for some of the outcome analyses. Despite these limitations, we did find statistically significant differences in lung function, responsiveness, and EBC nitrite after exposure to DE compared to CA, with a suggestive result that GSTM1 polymorphism may modify the latter response. The controlled exposure design allowed us to control potentially

confounding variables, and facilitated comparison to previous studies that used this approach, but limits our ability to generalize our findings to real-world exposure conditions, in which the physical, chemical, and temporal characteristics of exposures vary greatly.

CONCLUSIONS

This study adds to our understanding of the acute effects of DE exposure on subjects with mild-to-moderate asthma, while suggesting a possible alternative pathway that explains effects on lung function in the absence of inflammation. Taken together, our findings of increased EBC nitrite, along with the consistent absence of DE-induced inflammation in studies of subjects with asthma, suggest that acutely increased bronchial hyperresponsiveness and decreased lung function following DE exposure may occur via an irritant pathway, hypothetically through activation of TRPA1 receptors. Testing of this hypothesized mechanism will require new experimental animal models, before one can directly measure activation of, or block, TRPA1 in human subjects.

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Figure 1.
Mean Change in Percentage of Predicted FEV1 from Baseline, Immediately Post, 4 and 24 Hours Post DE and CA Exposure

Figure 2.
PC20 24 Hours Post CA and DE Exposure

Figure 3.
Exhaled Nitric Oxide Before, Post and 24 Hours Post DE and CA Exposure

Figure 4.
pH of EBC Collected Immediately Post, 4 and 24 Hours Post DE and CA Exposure

Figure 5.
EBC Nitrite Change from Before Exposure, Immediately Post, And 4 and 24 Hours Post DE and CA Exposure

Figure 6. Glutathione S-transferase Genotype Influence on EBC Nitrite Concentration. Change is the Difference Between Immediately Post- and pre-exposure Value Comparing DE and CA Exposure

Table 1**Baseline Characteristics**

Subject	Gender	Age	Race	Medications	Baseline MCT (PC20)	GSTM1	GSTT1	GSTP1 105
1	F	20	Caucasian	Fluticasone/Salmeterol (100/50mcg)	1.02	N/A	N/A	N/A
2	F	21	Caucasian	Albuterol PRN	>25	Null	Null	Ile/Val
3	M	28	Caucasian	Loratadine, flonase nasal spray	>25	Null	Present	Ile/Val
4	F	48	Caucasian	Albuterol PRN	3.76	Null	Null	Ile/Val
5	M	33	Caucasian	Albuterol	6.11	Present	Present	Ile/Val
6	F	44	Caucasian	Fexofenadine, Montelukast	6.75	Present	Present	Ile/Ile
7	F	20	African American	Albuterol Inhaler	>25	Present	Present	Ile/Ile
8	F	43	Hispanic	Albuterol Inhaler PRN	>25	Null	Present	Ile/Ile
9	M	22	Caucasian	Albuterol PRN	>25	Null	Present	Ile/Val
10	M	42	Caucasian	Albuterol	0.754	Null	Present	Ile/Val
11	M	20	Asian	Albuterol every 1–2 weeks	>25	N/A	N/A	N/A
12	M	22	Caucasian	Albuterol twice weekly	15.71	N/A	N/A	N/A
13	M	28	Caucasian	Albuterol PRN	1.78	Present	Null	Ile/Val
14	F	42	Caucasian	Fluticasone/Salmeterol (250/50mcg), Montelukast, Albuterol, Levalbuterol, Triamcinolone nasal spray	>25	Present	Null	Ile/Ile
15	F	21	Asian	Albuterol	>25	Present	Present	Ile/Val
16	F	24	Caucasian	Montelukast, Albuterol PRN	>25	N/A	N/A	N/A