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Exposure to diesel exhaust up-regulates iNOS expression in ApoE knockout mice

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ABSTRACT

Traffic related particulate matter air pollution is a risk factor for cardiovascular events; however, the biological mechanisms are unclear. We hypothesize that diesel exhaust (DE) inhalation induces up-regulation of inducible nitric oxide synthase (iNOS), which is known to contribute to vascular dysfunction, progression of atherosclerosis and ultimately cardiovascular morbidity and mortality.

Methods: ApoE knockout mice (30-week) were exposed to DE (at 200 μg/m^3 of particulate matter) or filtered-air (control) for 7 weeks (6 h/day, 5 days/week). iNOS expression in the blood vessels and heart was evaluated by immunohistochemistry and western blotting analysis. To examine iNOS activity, thoracic aortae were mounted in a wire myograph, and vasoconstriction stimulated by phenylephrine (PE) was measured with and without the presence of the specific inhibitor for iNOS (1400 W). NF-κB (p65) activity was examined by ELISA. The mRNA expression of iNOS and NF-κB (p65) was determined by real-time PCR.

Results: DE exposure significantly enhanced iNOS expression in the thoracic aorta (4-fold) and heart (1.5 fold). DE exposure significantly attenuated PE-stimulated vasoconstriction by ~20%, which was partly reversed by 1400 W. The mRNA expression of iNOS and NF-κB was significantly augmented after DE exposure. NF-κB activity was enhanced 2-fold after DE inhalation, and the augmented NF-κB activity was positively correlated with iNOS expression ($R^2 = 0.5998$).

Conclusions: We show that exposure to DE increases iNOS expression and activity possibly via NF-κB-mediated pathway. We suspect that DE exposure-caused up-regulation of iNOS contributes to vascular dysfunction and atherogenesis, which could ultimately lead to urban air pollution-associated cardiovascular morbidity and mortality.

Introduction

Numerous epidemiological studies have demonstrated an association between exposure to particulate matter air pollution with diameter less than 10 μm (or called PM_{10}) and increased cardiovascular morbidity and mortality (Kaiser, 2000; Miller et al., 2007; Peters et al., 2004; Pope et al., 2004; Samet et al., 2000). Furthermore, recent studies have also shown that reducing PM_{10} levels results in a declined in cardiovascular deaths by 10.3% (Clancy et al., 2002; Laden et al., 2006), suggesting a causal effect of PM_{10} and cardiovascular mortality. It has been well established that deposition of airborne particles in the lung provokes low-grade alveolar inflammation with a secondary systemic inflammatory response resulting in downstream cardiovascular dysfunction.

Atherosclerosis has been recognized as a chronic inflammatory disorder of blood vessels involving vascular, metabolic, and immune systems (Brook et al., 2004; Ross, 1999). We previously showed that exposure to PM_{10} caused progression of atherosclerosis of coronary arteries and aorta in rabbits that naturally developed atherosclerosis (Suwa et al., 2002). This sentinel finding was confirmed in ApoE knockout mice (Sun et al., 2005). In addition, human study also showed an association between progression of carotid atherosclerosis and the levels of ambient air pollution (Kunzli et al., 2005, 2010). This progression of atherosclerosis induced by PM_{10} exposure could

Abbreviations: CO, Carbon Monoxide; DE, Diesel Exhaust; ELISA, Enzyme-Linked Immunosorbent Assay; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; HPRT1, Hypoxanthine Phosphoribosyltransferase-1; iNOS, Inducible Nitric Oxide Synthase; IL-1, Interleukin-1; MIP, Macrophage Inflammatory Protein; NF-κB, Nuclear Factor-kappa B; NO, Nitric Oxide; NT, Nitrotyrosine; OxLDL, Oxidized Low-Density-Lipoprotein; PE, Phenylephrine; PM_{10}, Particulate matter air pollution with the diameter less than 10 μm; ROS, Reactive Oxidative Species; RT-PCR, Real Time Reverse Transcription Polymerase Chain Reaction; TNF, Tumor Necrosis Factor.

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contribute to the increased cardiovascular morbidity and mortality. The observations of PM_{10}-induced atherosclerosis progression are very compelling; however, the mechanisms whereby PM_{10} exposure causes progression in atherosclerosis, have not been fully elucidated.

Diesel exhaust (DE) is a mixture of fine particles and gases, and represents a useful model of traffic-related air pollutants, which accounts for up to 90% of the fine particulate mass in ambient air of many major cities, such as London (Maheswaran et al., 2005; Salvi et al., 1999). A recent study demonstrated an association between progression of atherosclerosis and living near major roads (Kunzli et al., 2010). In addition, evidence has shown that 6% of coronary heart disease deaths are linked to traffic-related pollution (Hoek et al., 2002; Maheswaran et al., 2005). Therefore, we selected to use DE to explore the underlying mechanisms of atherogenesis induced by ambient particulate matter exposure, using ApoE knockout mice that spontaneously develop atherosclerosis.

Inducible nitric oxide synthase (iNOS) is up-regulated in response to inflammatory cytokines as part of host defense responses (Stuehr and Marletta, 1987), and generates 100–1000 fold more nitric oxide (NO) than endothelium NOS(eNOS) (micromolar vs. nanomolar levels) does. NO derived from eNOS plays an important role in protecting vasculature from inflammation and atherosclerosis. However, excessive NO production from iNOS has detrimental effects on cardiovascular function. The large amount of locally released NO has been linked to the generation of harmful oxidative products, such as peroxynitrite, which is implicated in iNOS-mediated development of atherosclerosis (Ito et al., 2000; Sun et al., 2005; White et al., 1994). iNOS is undetectable under normal physiological conditions, but its expression can be detected in macrophages, endothelial cells and smooth muscle cells of human atherosclerotic plaques (Wilcox et al., 1997). The notion that iNOS plays a causative role in the progression of atherosclerosis is supported by the observation that atherosclerotic lesions were diminished in iNOS/ApoE double knockout mice, compared with ApoE knockout mice (Detmers et al., 2000; Kuhlencordt et al., 2001). iNOS overexpression was shown to be responsible for DE-induced lung inflammation (Ito et al., 2000; Takano et al., 1999), while iNOS knockout mice had a significant reduction of cytokines in the lung after exposure to ambient particles (Becher et al., 2007). iNOS expression is up-regulated by NF-κB, which is sensitive to inflammation and oxidative stress stimulation (Nathan and Xie, 1994). We recently have shown that DE exposure accelerates the progression of atherosclerosis in ApoE mice (Hirano et al., 2003). In this study, we test our hypothesis that DE exposure up-regulates iNOS expression and activity in vasculature via NF-κB-mediated pathway.

Material and methods

DE generation. Characteristics of the exposure system have previously been described (Gould et al., 2008). Briefly, DE was derived from a 2002 model turbocharged direct-injection 5.9-L Cummins B-series engine (6BT5.9 G6; Cummins, Inc., Columbus, IN) in a generator set. Load was maintained at 75% of rated capacity, using a load-adjusting load bank (Simplex, Springfield, IL) throughout the exposures. We used No.2 undyed, on-highway fuel and Valvoline 15 W-40 crankcase oil. All dilution air for the system was passed through HEPA and carbon filters, permitting a filtered air control exposure option with very low particulate and gaseous organic pollutant levels. The air entering the exposure room was conditioned to 18 °C and 60% relative humidity. During exposures, DE concentrations were continuously measured and maintained at steady concentrations using a feedback controller monitoring fine particulate levels. Multistage samples collected on a micro-orifice uniform deposition impactor (MOUDI; MSP, Shoreview, MN) indicated a mass median diameter of 0.104 μm.

Animals, exposure protocol, and sample collections. Male ApoE knockout mice were housed in a temperature- and humidity-controlled environment with a 12-h light/dark cycle with free access to water and standard rodent chow. At the age of 30 weeks, these mice were moved to a "Biozone" facility adjacent to the exposure chamber where exposure was controlled by opening or closing a valve to animal cages resulting in minimal stress for animals during the exposure period. We exposed ApoE knockout mice (10 mice/group) to DE for 7 weeks (5 days/week, 6 hrs/day) at the concentration of 200 $\mu g/m^3$ of particulate. Exposing mice to filtered air was the control. Animal procedures were approved by the Animal Care and Use Committee of the University of Washington.

After exposure, sodium pentobarbital (100 mg/kg, Abbott Laboratories, IL) and heparin sulfate (500 U/kg) were administered intraperitoneally. Upon the loss of all reflexes, the thoracic aorta, aortic root, and heart were carefully dissected from their connective tissues and kept at appropriate conditions until assay.

Measurement of vascular tone and iNOS activity. Thoracic aorta was carefully cleaned off all connective tissues without damaging the endothelium, and placed in ice-cold physiological salt solution (PSS). The vessels were cut to 2 mm rings and mounted on a wire myograph (Model 610 M; Danish Myo Technology, Aarhus, Denmark). Each vessel was bathed in oxygenated PSS at 37 °C for an hour during which the resting tension was gradually increased to 6 mN with three changes of PSS at 10 min intervals followed by stabilizing the vessels at resting tension (6 mN) for 30 min. Thereafter the vessels were stimulated with 80 mM KCl twice.

Smooth muscle contractility was studied by the addition of cumulative concentrations of phenylephrine (PE, 1nM–10 μM). To examine the impact of DE on iNOS activity, 1400 W, a specific inhibitor for iNOS, was used. Vessels were incubated with 1400 W (10 μM) for 30 min followed by addition of cumulative concentrations of PE (1 nM–10 μM). iNOS activity was determined by the fractional changes of maximal PE constriction with and without the presence of 1400 W.

Immunohistochemistry of analysis of macrophages, smooth muscle cells, the expression of iNOS, CD36, and nitrotyrosine. Thoracic aorta tissue adjacent to those used for functional studies, aortic root sections contained three complete valve leaflets, and the heart were fixed with 10% neutral formalin for 24 h, then embedded in paraffin. Sections (5 μm) were deparaffinized in xylene and hydrated by passing through a series of graded alcohol. Thereafter, these sections were treated with citrate buffer (Invitrogen) to unmask the antigenic sites, and incubated with 10% goat serum at room temperature for 30 min to block nonspecific binding proteins, followed by incubation with specific primary antibodies to: iNOS (1:100, Santa Cruz); macrophage (F4/80; 1:50, AbD Serotec); CD36 (1:50, Santa Cruz); smooth muscle cells (α-actin; 1:600, Abcam); and nitrotyrosine (1:400, Upstate Biotechnology) at 4 °C overnight. Negative controls were included with non-immune isotype antibody or omission of the primary antibody. Subsequently, sections were incubated with biotinylated goat anti rabbit IgG (1:800, Vector Laboratories) at room temperature for 30 min, followed by avidin-biotin conjugated alkaline phosphatase and Vector red (Vector Laboratories) to detect the antigen-antibody complexes.

Random images were captured by a spot digital camera (Nikon, Japan). We used Image Pro Plus software and a color segmentation method to identify positive staining of the antigens of interest, and examined without knowledge of experimental groups. The volume fraction (V/v%) of specific staining was determined and normalized to the area of atherosclerostic plaque (aorta roots), and the thickness of vascular wall (thoracic aorta), respectively.

Western blotting analysis of iNOS expression in the heart. Frozen heart tissues were homogenized in 10 volumes of ice-cold RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Roche Diagnostics). Supernatant was obtained following centrifugation of homogenates at 12,000 rpm for 15 min (4 °C). The protein concentrations of supernatant