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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

were quantified by Bradford method using Bio-Rad DC Protein Assay kit according to the manufacturer's instructions (Biorad). Same amount of protein samples (40 μg/lane) from different mice was separated on 7.5% SDS-PAGE gel, subsequently electrotransferred to polyvinyldifluoride membranes (Biorad) and blocked with 5% BSA dissolved in 0.1% TBS-Tween 20 for 60 min at room temperature. The membrane was incubated with specific primary antibody for iNOS (1:500, Santa Cruz) at 4 °C overnight, followed by 1-hour incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). Immunoreactive bands were visualized by enhanced chemiluminescence kit (Amersham Life Sciences). Densitometry was carried out using Image J (NIH), and the results were expressed in optical intensity per mg protein.

*ELISA of NF-κB (p65) activity.* Nuclear extracts from frozen heart tissues were obtained using nuclear extract kit (Cayman Chemical). Protein concentrations of nuclear extract were quantified. NF-κB (p65) activity in the nuclear extracts from heart tissues was measured by enzyme-linked immunosorbent assay (ELISA) using NF-κB (p65) transcription factor kit according to the manufacturer's instructions (Cayman Chemical). This kit is a non-radioactive, sensitive method for detecting NF-κB (p65) DNA binding activity in nuclear extracts as a replacement for the radioactive electrophoretic mobility shift assay. Results were expressed as absorbance (450 nm) per milligram protein.

*Real-time reverse transcription polymerase chain reaction (RT-PCR) of iNOS and NF-κB mRNA expression.* Total RNA was extracted from heart tissues using RNeasy fibrous tissue mini kit (Qiagen). RNA concentrations were measured by Nanodrop (Thermo Scientific). Reverse transcription was performed using SuperScript III first-strand synthesis system for RT-PCR (Invitrogin). The same amount of RNA was loaded in triplicates for each assay, and RT-PCR was performed using TaqMan universal PCR master mix and Taqman gene expression assay system (Applied Biosystems) according to the manufacturer's instructions. The mRNA expression of iNOS, NF-κB, β-actin, and hypoxanthine phosphoribosyltransferase-1 (HPRT1) was determined by RT-PCR using ABI Prism 7900HT sequence detection system (Applied Biosystems). Gene expression values were calculated based on the comparative threshold cycle method, normalized to the expression values of β-actin and HPRT1, and displayed as ratio relative to β-actin.

*Solutions and chemicals.* The PSS consisted of the following (in mM): NaCl 119, KCl 4.7, $KH_2PO_4$ 1.18, $NaHCO_3$ 24, $MgSO_47H_2O$ 1.17, $CaCl_2$ 1.6, glucose 5.5 and EDTA 0.026. All reagents were purchased from Sigma.

*Statistical analysis.* Data are shown as mean ± SEM. The statistical significance was evaluated using the unpaired Student's *t* test for simple comparison between two values. Non-parametric analysis (Kruskall–Wallis test) was used to analyze data that were not normally distributed and Bonferroni correction was used for multiple comparisons. Linear regression modeling was used to assess the relationship between parameters. In all experiments, n equals the number of mice from which samples were obtained. $P<0.05$ was considered to be significant.

## Results

*iNOS expression in the thoracic aorta*

Immunohistochemical staining of thoracic aorta sections demonstrates that iNOS was predominantly expressed by smooth muscle cells and macrophages in the vessels (Figs. 1A, B, C). Immunohistochemistry analysis shows that iNOS expression was significantly elevated in the thoracic aorta after DE exposure (11.9 ± 3.2% vs. 40.2 ± 6.5%; V/v% of staining in filtered air vs. DE; $p<0.002$) (Figs. 1D, E, F).

*iNOS expression in heart tissues*

Western blotting analysis shows that DE exposure significantly augmented iNOS expression in heart tissues (0.7 ± 0.1% vs. 1.0 ± 0.1%; filtered air vs. DE; $p<0.03$) (Figs. 2A, B).

*Similarly localized expression of iNOS with CD36 and nitrotyrosine in aortic root lesions*

iNOS was predominantly expressed by macrophages in aortic root sections (Figs. 3B, C). Serial sections of aortic root from the same mouse show that the expression of iNOS, CD36 and nitrotyrosine was similarly localized and predominantly expressed by foam cells (Figs. 3D,E,F). In addition, iNOS expression was positively associated with CD36 expression ($R^2=0.4580$, $p<0.01$) (Fig. 3G), and CD36 expression correlated with nitrotyrosine formation ($R^2=0.4629$, $p<0.01$) (Fig. 3H), suggesting a close relationship between iNOS and these oxidative stress markers.

*iNOS activity in the thoracic aorta*

PE-stimulated vasoconstriction of the thoracic aorta was significantly suppressed after DE exposure (9.5 ± 0.6 mN vs. 7.4 ± 0.6 mN, filtered air vs. DE; $p<0.02$) (Figs. 4A,B). In the presence of 1400 W, the fractional change of PE-elicited maximal constriction was significantly increased in DE exposure group, compared with the control (5.2 ± 3.0% vs. 24.1 ± 7.6%; filtered air vs. DE; $p<0.04$) (Figs. 4C,D,E), suggesting an increase in iNOS activity after DE exposure. To verify whether the reduced vasoconstriction was due to morphological abnormalities, we stained the thoracic aorta adjacent to the segments for functional studies with hematoxylin and eosin, and examined the thickness and the structure of the vessels using morphologic analysis. We found no difference in the thickness and structure of the vessels after DE exposure (data not shown).

*The mRNA expression of iNOS and NF-κB (p65)*

The mRNA expression of both iNOS (76 ± 3% vs 90 ± 6%; filtered air vs. DE; $p<0.004$) (Fig. 5A) and NF-κB (83 ± 1% vs 88 ± 3%; filtered air vs. DE; $p<0.01$) (Fig. 5B) was significantly higher in heart tissues after DE exposure.

*NF-κB (p65) activity*

To assess whether iNOS was regulated at the transcription level via NF-κB activation, we extracted nuclear protein from frozen hearts, and NF-κB activity was evaluated using ELISA. DE exposure up-regulated NF-κB (p65) activity (0.014 ± 0.0004 vs. 0.029 ± 0.006 absorbance of 450 nm/mg protein; filtered air vs. DE; $p<0.01$) (Fig. 6A). iNOS expression was positively correlated with NF-κB activity ($R^2=0.5998$, $p<0.0005$) (Fig. 6B), indicating that DE exposure up-regulated iNOS expression at the transcriptional level via NF-κB activation (p65).

## Discussion

Studies from our (Bai et al., 2011; Suwa et al., 2002) and other (Kunzli et al., 2005, 2010; Sun et al., 2005), laboratories have shown that exposure to $PM_{10}$ that was derived from different sources is associated with development of atherosclerosis. In this study, we show that exposure to DE, (urban particulate matter) increased iNOS expression in the thoracic aorta and heart (Figs. 1, 2). DE inhalation also enhanced iNOS activity in blood vessels, which resulted in decreased vascular contractility (Fig. 4). In this study, we show that iNOS was expressed by smooth muscle cells, macrophages and cardiac myocytes, and the expression of iNOS, CD36 and nitrotyrosine was

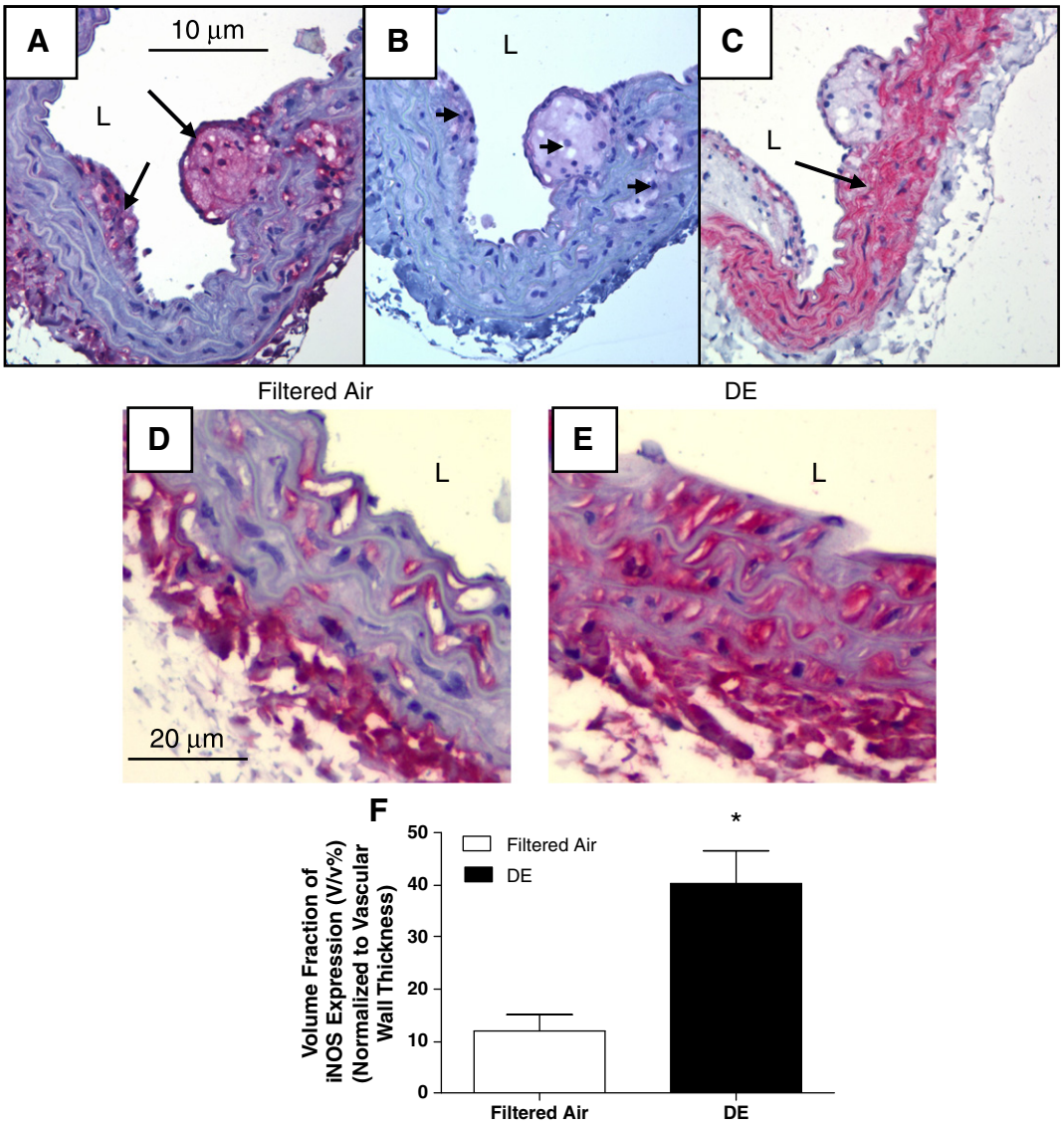


**Fig. 1.** Immunohistochemical analysis of iNOS expression in the thoracic aorta. Representative photomicrographs of thoracic aorta sections stained for A) iNOS (400X) (arrow); B) macrophages (F4/80) (400X) (arrowhead); C) smooth muscle cells (α-actin) (400X) (arrow); representative photomicrographs of thoracic aorta sections stained for iNOS exposed to filtered air (D) or DE (E) (600X); F) Exposure to DE increased iNOS expression in the thoracic aorta, n = 6 (filtered air) and n = 8 (DE), *p<0.002. L: lumen. Values are mean ± SEM.

similarly localized in aortic lesions (Figs. 3D,E,F). The positive association between enhanced NF-κB activity and iNOS expression (Fig. 6B) suggests that activation of transcription factor NF-κB played a key role in this enhanced iNOS activity. The augmented mRNA expression of iNOS and NF-κB (Fig. 6) also indicates that iNOS was up-regulated at both transcription and translation levels. To our knowledge,

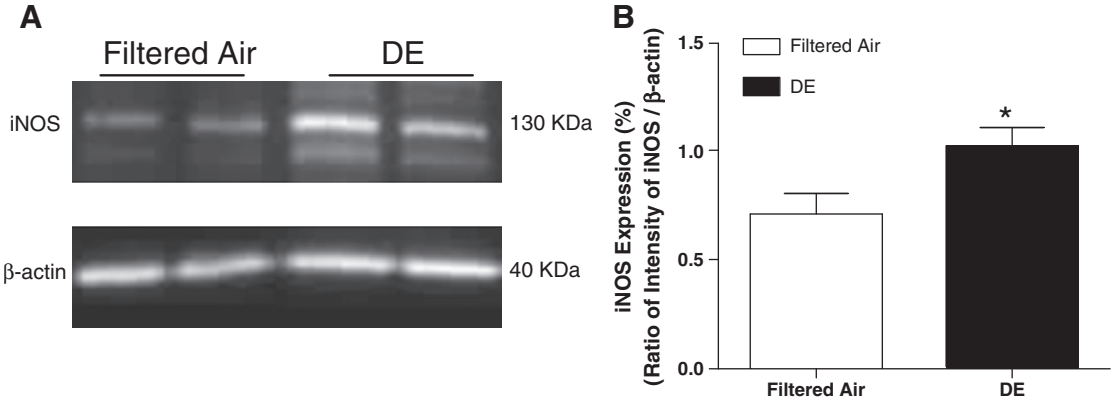


**Fig. 2.** Western blotting analysis of iNOS expression in the heart. A) Representative photomicrograph of western blotting of iNOS expression in the heart; B) Western blotting analysis demonstrates that iNOS expression was enhanced following DE exposure, n = 8, *p<0.03. Values are mean ± SEM.

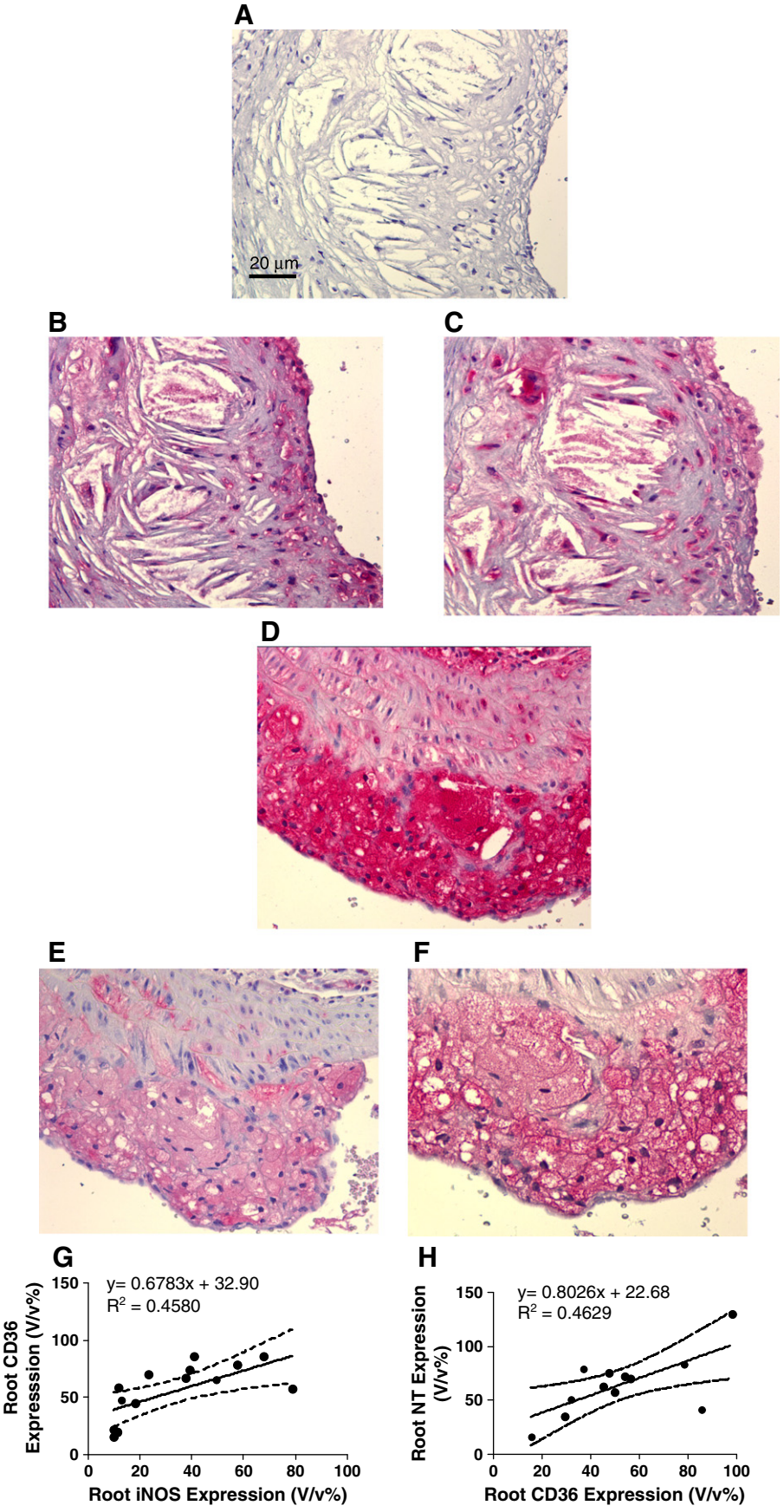


**Fig. 3.** iNOS expression in atherosclerotic plaque in aortic root and its relationship with the expression of CD36 and nitrotyrosine (NT). Representative photomicrographs of aortic root sections stained for A) negative control, B) iNOS, and C) macrophages (F4/80) (400X); Representative photomicrographs of aortic root sections showing similarly localized expression of D) iNOS, E) CD36, and F) NT; G) Positive correlation of the expression between iNOS and CD36, $R^2 = 0.4580$, $p < 0.01$; H) Positive correlation of the expression between CD36 and NT, $R^2 = 0.4629$, $p < 0.01$.

this is the first study demonstrating that DE exposure up-regulates iNOS activity and expression in the vasculature and heart, and we speculate that oxidative stress induced NF-κB activation contributes to the increased iNOS expression and activity.

*The level of DE exposure*

In this study, we exposed ApoE knockout mice fed with regular chow to DE for 7 weeks using a well-controlled inhalation system that mimics real-world exposure. Our average DE concentration throughout a 24-hour period is around 35 μg/m$^3$ (averaged concentration by 30 h/week exposure of DE at the concentration of 200 μg/m$^3$ for 7 weeks), which is environmentally relevant and within the National Ambient Air Quality Standard (Brook et al., 2004).

*DE exposure and up-regulation of iNOS*

Nitric oxide (NO) is an essential biological mediator protecting the vessels from inflammation and atherosclerosis; however, overproduction of NO catalyzed by iNOS is cytotoxic. iNOS is not expressed under normal physiological conditions, but can be up-regulated in response to pro-inflammatory mediators, and oxidative stress stimulation. The up-regulation of iNOS is implicated in the pathogenesis of a number of cardiovascular diseases, including the progression

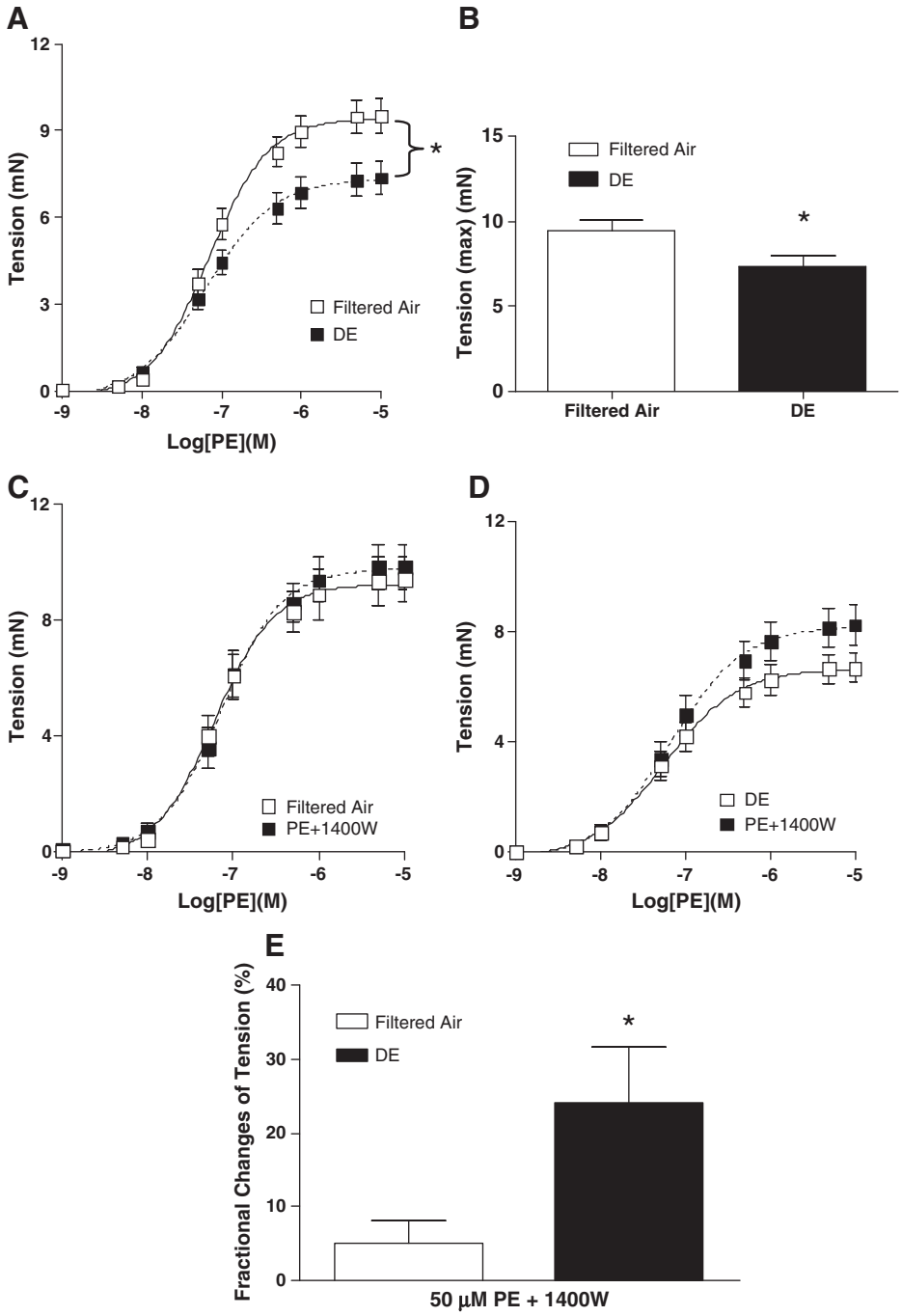


**Fig. 4.** Reduced vasoconstriction and increased iNOS activity in the thoracic aorta. A) Dose-response curves of PE-elicited constriction show that exposure to DE attenuated vasoconstriction, n = 9, *p<0.02; B) DE exposure caused significant attenuation of maximum vasoconstriction, compared with the filtered air exposure, n = 9, *p<0.02; C) In the control group, the presence of iNOS blocker (1400 W) had no effect on PE-elicited constriction; D) In DE exposure group, the reduced vasoconstriction was partly restored by 1400 W; E) Inhibition of iNOS by 1400 W caused a significant elevation of maximal vasoconstriction in DE exposure group, n = 9, *p<0.04. Values are mean ± SEM.

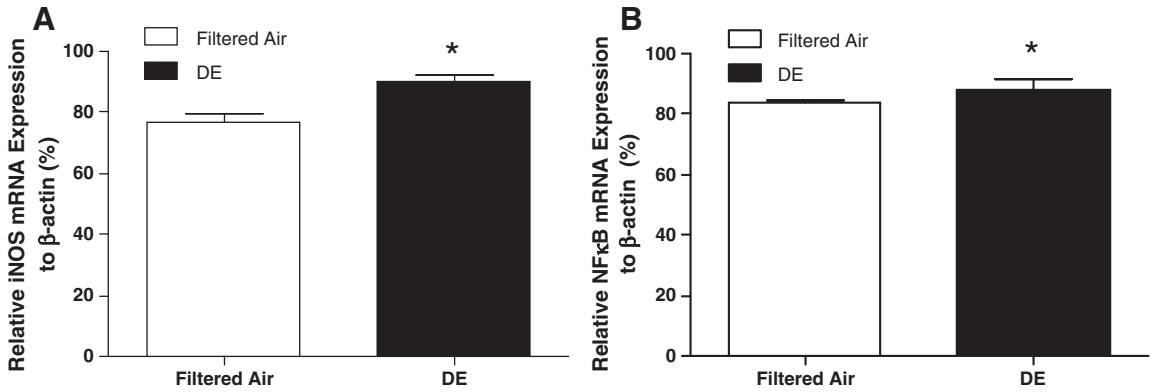


**Fig. 5.** Increased mRNA expression of iNOS and NF-κB in the heart. A) Increased mRNA expression of iNOS in the heart after DE exposure, n = 7 (filtered air) and n = 8 (DE), *p < 0.004; B) Exposure to DE increased NF-κB mRNA expression, n = 7 (filtered air), n = 8 (DE), *p < 0.01. Values are mean ± SEM.

in atherosclerosis (Cromheeke et al., 1999). In this study, we show that DE exposure increased iNOS activity (Fig. 4), and enhanced iNOS expression in blood vessels (Fig. 1) and heart (Fig. 2) at both protein and mRNA levels (Fig. 6A). We also found a positive correlation between iNOS expression with foam cell formation and the number of smooth muscle cells in aortic lesions (Supplementary Fig. A.1). These associations suggest that iNOS contributes to the progression of atherosclerosis induced by DE inhalation.

Similar to the finding of Sun and colleagues in which ApoE knockout mice were exposed to concentrated ambient particles for 6 months (Sun et al., 2005), we found that iNOS expression was up-regulated at both protein and mRNA levels after exposure to DE for a shorter exposure period (7 weeks vs. 6 months), suggesting that the constituents of DE may be more active, compared with concentrated ambient particles (Hirano et al., 2003). Sun and colleagues found that exposure to concentrated ambient particles enhanced vasoconstriction to phenylephrine, which likely resulted from reduced NO production or endothelial dysfunction. In contrast, we found suppressed vasoconstriction to phenylephrine, which was likely due to excessive NO production derived from iNOS, because the inhibition of iNOS enhanced the constriction (Figs. 4D,E).

Diesel exhaust is a complex mixture of gases and diesel particulate matter. The gas-phase pollutants include ozone, sulphur dioxide, carbon monoxide (CO), and nitrogen oxides (NO and $NO_2$), and the particulate components consist of a central carbon core nucleus with an estimated 18,000 combustion products adsorbed, including organic chemicals, such as polycyclic aromatic hydrocarbons, and transition metals. The composition of DE (gas- and particulate-phase) is different from other ambient particulate (such as concentrated ambient particles) exposed in different studies, and the variation may explain the discrepancy of vascular effects. In our study, the concentrations of CO and NO were lower than those in other studies (Campen et al., 2010; Quan et al., 2010), despite that NO in the DE exposure group (1515.80 ppb) was higher than filtered air group (38.63 ppb) in the present study. Our study did not address the differential effect of DE composition on vascular function, because a number of studies have examined the impact of different composition of gasoline and diesel exhaust on cardiovascular function. For example, Campen and colleagues reported that CO and NO, but not $NO_2$, had greater effect on ET-1 mRNA expression in thoracic aorta (Campen et al., 2010). Furthermore, organic compounds are not only potential occupational carcinogens (Mauderly and Chow, 2008), but can also generate oxidative stress in rat heart microvessel endothelial cells (Hirano et al., 2003), and induce pro-inflammatory responses in human aortic endothelial cells (Li et al., 2010), which may contribute to myocardial ischemia associated with non-particulate matter component of DE (Campen et al., 2005).

We found no impairment of ACh-stimulated endothelium-dependent relaxation (unpublished data). These data indicate that effect on smooth muscle cells is prominent after DE inhalation for 7 weeks. Sun and colleagues also observed that mice fed with regular chow did not develop endothelial dysfunction after exposure to concentrated ambient particles (supporting our finding), suggesting that high fat diet-mediated modifications on vasculature are important elements

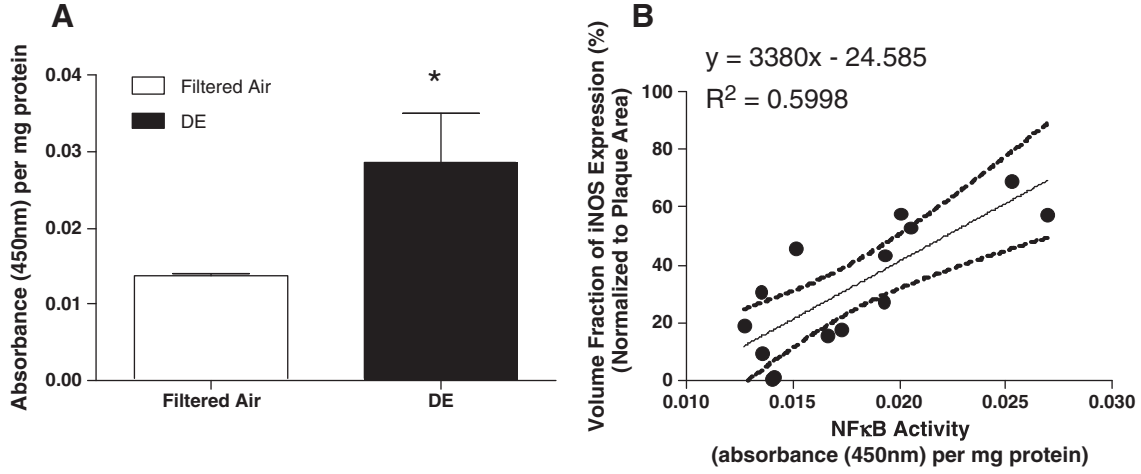


**Fig. 6.** DE exposure increased NF-κB activity of heart nuclear extracts. A) Exposure to DE increased NF-κB activity of heart nuclear extracts, n = 9 (filtered air), n = 10 (DE), *p < 0.01; B) Positive correlation between NF-κB activity and iNOS expression, $R^2 = 0.5998$, p < 0.0005. Values are mean ± SEM.

and may have synergistic effect on ambient particle exposure-induced vascular dysfunction. Data from a recent human study have implied that increased lipid levels contribute to atherosclerosis associated with ambient particle exposure (Brüske et al., 2011).

iNOS activation is mainly regulated at the transcriptional level by NF-κB (Taylor et al., 1998). In this study, we found that NF-κB activity and mRNA expression were significantly increased (Figs. 5B & 6A). The positive correlation between iNOS and increased NF-κB activity (Fig. 6B) suggests that NF-κB plays an important role in the up-regulation of iNOS following DE exposure. NF-κB is known to regulate the transcription of various genes participating in oxidative stress responses. NF-κB dimers (p50/65) are sequestered in the cytoplasm by a family of inhibitors, called inhibitors of κB (IκB). Activation of NF-κB is initiated by degradation of IκB proteins triggered by oxidative stress. NF-κB complex enters into the nucleus in which it can turn on the expression of specific genes by binding to their promoters. The promoter of the murine gene coding for iNOS contains the binding sites for NF-κB, which are sensitive to oxidative stress stimulation (Xie et al., 1993). It was reported that exposure to ambient particles caused oxidative stress-induced NF-κB activation in epithelial cells (Shukla et al., 2000).

Exposure to diesel exhaust particles was found to cause ROS generation in epithelial cells, alveolar macrophages, blood vessels, endothelial cells, and cardiac myocytes (Bai et al., 2011; Ikeda et al., 1995; Ito et al., 2000; Okayama et al., 2006; Shukla et al., 2000). We recently demonstrated that DE inhalation increased systemic and tissue (aortic root) oxidative stress (Bai et al., 2011) In the present study, we show that CD36 and nitrotyrosine are concomitantly up-regulated with iNOS after DE inhalation (Fig. 3), suggesting that ROS could play a key role in the activation of NF-κB, whereby up-regulates iNOS expression and activation. ROS generation may arise directly from the surface of ambient particles (Brown et al., 2000), soluble compounds (e.g., transition metals) (Donaldson and MacNee, 2001; Li et al., 2002), or organic compounds (Baulig et al., 2003; Hirano et al., 2003; Sagai et al., 1993). In addition, direct interactions between DE, airway epithelial cells and alveolar macrophages may also contribute to ROS generation (Bai et al., 2007; Rudell et al., 1999).

### *iNOS and atherosclerosis*

The notion that overexpression of iNOS has causal effect on the development of atherosclerosis has previously been established by showing that atherosclerotic lesions were attenuated when iNOS was knocked out in ApoE mice (Detmers et al., 2000; Kuhlencordt et al., 2001). iNOS is capable of generating superoxide (Xia et al., 1998; Xia and Zweier, 1997), which can rapidly deplete endothelium-derived NO, attenuate eNOS activity and expression, leading to endothelial dysfunction and development of atherosclerosis. It is well known that oxidative modification of low-density lipoprotein (LDL) accelerates lipoprotein-uptake by macrophages and the formation of foam cells (Krieger et al., 1993), which is the characteristic component of atherosclerotic lesions and landmark of development of atherosclerosis. CD36 is one of the major macrophage scavenger receptor that uptakes oxidized LDL (oxLDL), and is also known as oxLDL receptor (Endemann et al., 1993). We recently reported that DE inhalation increased CD36 expression (Bai et al., 2011). CD36 is up-regulated by proatherogenic cytokines and oxidized lipid. In this study we show that the expression of iNOS and CD36 was co-localized in atherosclerotic lesions (Fig. 3). The positive correlation between iNOS and CD36 (Fig. 3G) suggests that iNOS, as a free radical generator or a surrogated marker for ROS, may contribute to the augmented CD36 expression leading to the foam formation and progression of atherosclerosis. In addition, up-regulation of iNOS also contributes to the generation of peroxynitrite, which is a potent oxidant and implicated in the pathogenesis of several cardiovascular diseases, including atherosclerosis, myocardial infarction, and heart failure (Graham et al., 1993).

## Conclusions

In summary, we show that DE inhalation causes significant up-regulation of iNOS expression and activity in blood vessels and heart. Our data indicate that the up-regulation of iNOS was mediated by the activation of NF-κB. This study provides some novel insights into the mechanisms underlying DE inhalation-induced progression of atherosclerosis, including increased lipid deposition, increased macrophages and smooth muscle cells in the lesion (Bai et al., 2011). iNOS may be a potential target to decrease the impact of air pollution on cardiovascular morbidity and mortality.

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## Conflict of interest statement

Nothing to declare.

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