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RESEARCH ARTICLE

Exposure to diesel exhaust upregulates COX-2 expression in ApoE knockout mice

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Abstract

Introduction: We have shown that diesel exhaust (DE) inhalation caused progression of atherosclerosis; however, the mechanisms are not fully understood. We hypothesize that exposure to DE upregulates cyclooxygenase (COX) expression and activity, which could play a role in DE-induced atherosclerosis.

Methods: ApoE knockout mice (30-week old) fed with regular chow 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). The protein and mRNA expression of COX-1 and COX-2 were evaluated by immunohistochemistry analysis and quantitative real-time PCR, respectively. To examine COX activity, thoracic aortae were mounted in a wire myograph, and phenylephrine (PE)-stimulated vasoconstriction was measured with and without the presence of COX antagonists (indomethacin). COX-2 activity was further assessed by urine 2,3-dinor-6-keto $PGF_{1\alpha}$ level, a major metabolite of prostacyclin I_2 (PGI_2).

Results: Immunohistochemistry analysis demonstrates that DE exposure enhanced COX-2 expression in both thoracic aorta ($p < 0.01$) and aortic root ($p < 0.03$), with no modification of COX-1 expression. The increased COX-2 expression was positively correlated with smooth muscle cell content in aortic lesions ($R^2 = 0.4081$, $p < 0.008$). The fractional changes of maximal vasoconstriction in the presence of indomethacin was attenuated by 3-fold after DE exposure ($p < 0.02$). Urine 2,3-dinor-6-keto $PGF_{1\alpha}$ level was 15-fold higher in DE group than the control ($p < 0.007$). The mRNA expression of COX-2 ($p < 0.006$) and PGI synthase ($p < 0.02$), but not COX-1, was significantly augmented after DE exposure.

Conclusion: We show that DE inhalation enhanced COX-2 expression, which is also associated with phenotypic changes of aortic lesion.

Keywords: Diesel exhaust, atherosclerosis, cyclooxygenase, ApoE knockout mouse

Introduction

Epidemiological studies have shown that exposure to fine ambient particulate matter air pollution ($PM_{2.5}$) can be an independent risk factor for cardiovascular morbidity and mortality (Dockery et al., 1993; Brook et al., 2010). The mechanisms underlying $PM_{2.5}$ induced vascular disease have not been fully investigated. Evidence from both human and animal studies has indicated that deposition of particles in the lung provokes a low-grade lung inflammation with a secondary systemic inflammatory response characterized by increased circulating pro-inflammatory mediators, such as interleukin-1 (IL-1), and IL-6 (Ishii et al., 2004; Tamagawa et al., 2008). The systemic responses are thought to cause the downstream

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Abbreviations

COX: cyclooxygenase
DE: diesel exhaust
HPRT1: hypoxanthine phosphoribosyltransferase-1
HSP: Heat shock protein
IL-1: interleukin-1
NSAIDs: nonsteroidal anti-inflammatory drugs
PE: phenylephrine
PM_{10}: particulate matter air pollution with diameter less than 10 μm
PGH_2: prostaglandin (PG) H2
PGI_2: prostacyclin I_2
PGIS: PGI synthase
PSS: physiological salt solution
RT-PCR: Real-time reverse transcription polymerase chain reaction
TXA2: thromboxane A2

cardiovascular diseases, especially in individuals who have pre-existing cardiovascular dysfunction (Seaton et al., 1995; Bai et al., 2007).

Prostanoids play an important role in cardiovascular function, such as regulate vascular tone, modulate vascular inflammatory response, and control leukocyte-endothelial cell adhesion and platelet aggregation (Kawabe et al., 2010a). Cyclooxygenase (COX) is the key regulatory enzyme responsible for the formation of prostanoids. COX catalyzes the conversion of arachidonic acid to prostaglandin (PG) H_2, which is subsequently converted to a variety of eicosanoids, including PGE_2, PGD_2, $PGF_{2\alpha}$, prostacyclin I_2 (PGI_2), and thromboxane A2 (TXA2) (Dubois et al., 1998). The array of PGs produced varies depending on the downstream enzymes present in different types of cells. For example, endothelial cells primarily produce PGI_2, whereas platelets mainly produce TXA2. Two COX-isozymes, COX-1 and COX-2, have been characterized. COX-1 is the enzyme responsible for basal, constitutive prostaglandin synthesis, whereas upregulation of COX-2 is implicated in inflammatory settings (Cipollone et al., 2004). PGI_2 and TXA2 are important components of prostaglandin metabolism that regulate cardiovascular homeostasis. Under physiological conditions, PGI_2 is released by endothelial cells to mediate several protective effects on the vascular wall, including balance excessive vascular contraction, and inhibit platelet aggregation and adhesion (Egan et al., 2004; Kawabe et al., 2010b). TXA2, a potent vasoconstrictor, is the predominant mediator having opposite effects of PGI_2. Under pathological conditions, such as inflammation or atherosclerosis, PGI_2 production decreases, and the release of TXA2 becomes dominant (Warner & Mitchell, 2004). Increased TXA2 production is implicated in endothelial dysfunction, hypertension, and atherothrombosis.

Samet and colleagues have shown that exposure to PM_{10} caused overexpression of COX-2 in human airway epithelial cells, which could result in lung injury (Samet et al., 2000). It was reported that exposure to diesel exhaust particles increased COX-2 protein and mRNA expression in human macrophage cells (U937) that were associated with a subsequent increase of cholesterol accumulation and foam cell formation (Vogel et al., 2005). Studies from our laboratory have previously shown that exposure to PM_{10} caused progression of atherosclerosis in rabbits (Suwa et al., 2002).

Diesel exhaust (DE) is a major component of urban $PM_{2.5}$, and could account for up to 90% of the fine particulate mass in ambient air of many major cities (Brook et al., 2010). DE is a mixture of fine particles and gases, and represents a useful model of traffic-related air pollutants. The particulate from DE consists of a central carbon core nucleus onto which an estimated 18,000 combustion products are absorbed, including organic chemicals, such as polycyclic aromatic hydrocarbons, and transition metals (Schuetzle et al., 1981).

Recent studies have shown an association between progression of atherosclerosis and people living near major roads, and traffic-related air pollution exposure and acute coronary events (Hoek et al., 2002). We have also demonstrated that exposure ApoE mice to DE for 7 weeks induced progression of atherosclerosis (Bai et al., 2011b); however, the underlying mechanisms for these changes in plaques are not fully understood. We hypothesize that exposure to DE upregulates COX-2 activity and expression in blood vessels and heart that may play a role on these DE exposure-induced changes in atherosclerotic plaques.

Material and methods

Exposure system, experimental animals and exposure protocol

Characteristics of the exposure system have been described previously (Gould et al., 2008). DE was derived from a 2002 model turbocharged direct-injection 5.9-L Cummins B-series engine (6BT5.9G6; 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 the exposures, DE concentrations were continuously measured. Multistage samples collected on a micro-orifice uniform deposition impactor (MOUDI; MSP, Shoreview, MN) indicated a mass median diameter of 0.104 μm. Elemental carbon and organic carbon were determined by thermal optical transmittance analysis of quartz filter samples,

while metals concentrations were determined by X-ray fluorescence analysis of Teflon filter samples. Gaseous pollutants including carbon monoxide and oxides of nitrogen (NOx) have been characterized and the exposure conditions have been found to be both stable and reproducible. The most recent National Ambient Air Quality Standards issued by the U.S EPA in 2006 for $PM_{2.5}$ levels have been strengthened to 15 μg/m³ (annual) and 35 μg/m³ (24 h) based on vast evidence showing a strong association between fine particles and cardiovascular diseases (Donaldson et al., 2001). The exposure level (200 μg/m³) in this study was determined to reflect the ambient PM level of a heavy traffic condition. The average calculated exposure throughout a 24-h period in our study was less than 35 μg/m³, which is within the range of the National Ambient Air Quality Standard and is environmentally relevant.

Male ApoE knockout mice were housed in a temperature- and humidity-controlled environment, which was on a 12-h light/dark cycle. These mice had free access to water and standard rodent chow. At the age of 30-week old, mice were randomly chosen (10 mice/group) and 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 periods. We exposed ApoE knockout mice to DE for 7 weeks (5 days/week, 6 h/day) at the concentration of 200 μg/m³ particulate matter. Exposing mice to filtered air was the control. Animal procedures were approved by the Animal Care and Use Committee of the University of Washington.

Sample collection

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, blood was collected from inferior vena cava into EDTA tubes. Plasma was obtained after the centrifugation of blood and stored at –80°C until assay. Thoracic aorta, aortic root, and heart were carefully dissected from the connective tissues and kept in appropriate solution until assay. Spot urine samples were collected from the bladder after euthanasia and kept at –80°C.

Measurement of vascular tone

Thoracic aortae that were free of atherosclerotic lesions were carefully cleaned off the 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 610M; 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 also studied by the addition of cumulative concentrations of phenylephrine (PE, 1 nM–10 μM). To access the effect of DE exposure on cyclooxygenase and TXA2 production, indomethacin (1 μM) and SQ29548 (10 μM) (selective TXA2 receptor antagonist) were administered, respectively.

Immunohistochemical staining of COX-1, COX-2 and macrophage

The thoracic aortae adjacent to the segments used for functional studies, and aortic root sections that contained three complete valve leaflets were embedded and cut into 5μm. After deparaffinization, hydration, and antigen retrieval using citrate buffer (Invitrogen), aorta and root sections were incubated with 10% goat serum at room temperature for 30 min to block non-specific binding proteins, followed by incubation with specific primary antibodies: rabbit anti mouse antibody for COX-1 (1:150, Cayman Chemical), rabbit anti mouse antibody for COX-2 (1:100, Santa Cruz), and rat anti mouse antibody for F4/80 (1:50, AbD Serotec) 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. Five tissue sections were randomly chosen for each targeted tissue, and stained for each marker. Images for these sections were captured by a spot digital camera (Microspot, Nikon, Tokyo, Japan), coded and examined without knowledge of the experimental groups. Using Image Pro Plus software, positive red staining was recognized, quantified by color segmentation, normalized respectively to the thickness of vascular wall or the area of atherosclerotic lesions and expressed as volume fraction (V/v%) as previously described in detail (Bai et al., 2011b).

Measurement of urine 2,3-dinor-6-keto $PGF_{1\alpha}$

PGI_2 level was assessed by measuring urine 2,3-dinor-6-keto prostaglandin $F_{1\alpha}$, the major urinary metabolite converted from PGI_2, using an EIA kit according to the manufacturer's instruction (Cayman Chemical). Creatinine levels were measured by a creatinine assay kit (Cayman Chemical).

Real-time reverse transcription polymerase chain reaction analysis of the mRNA expression of COX-1, COX-2 and PGI synthase

Due to limited vessel samples, total RNA was extracted from the heart tissue using RNeasy Fibrous Tissue Mini Kit (Qiagen). RNA concentration was measured using Nanodrop (Thermo Scientific). Reverse transcription was performed using an RT kit (Invitrogen). The same amount of RNA from different samples was loaded in triplicates for each assay, and reverse transcription polymerase chain reaction (RT-PCR) was performed

using TaqMan Universal PCR master mix and Taqman gene expression assay system (Applied Biosystems) according to the manufacturer's instructions. COX-1, COX-2, PGI synthase (PGIS), β-actin and hypoxanthine phosphoribosyltransferase-1 (HPRT1) mRNA expression were measured by RT-PCR (TaqMan) using the ABI Prism 7900 HT sequence detection system (Applied Biosystems). Gene expression values were calculated based on the comparative threshold cycle (Ct) 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

Results are reported as mean ± SEM. The statistical significance was evaluated using the unpaired Student's t test for simple comparison between two values. The concentration-response curves of the different groups were compared by ANOVA for repeated measurements followed by Bonferroni's correction. $p < 0.05$ was considered to be significant. In all experiments, n equals the number of mice from which samples were obtained.

Results

Immunohistochemistry analysis of COX-1 and COX-2 expression in thoracic aorta and aortic root

In thoracic aorta, COX-1 was expressed by endothelial cells, smooth muscle cells and macrophages. COX-2 was expressed by smooth muscle cells and macrophages (Supplementary Figure 1). In aortic root, COX-1 and COX-2 were predominantly expressed by macrophages (Supplementary Figure 2).

DE exposure did not change COX-1 expression in thoracic aorta and aortic root, as compared to filtered air (Figures 1A–1C and 2A–2C). However, we observed a significant enhancement of COX-2 expression in both thoracic aorta (1.1 ± 0.1% vs 1.6 ± 0.1%, Filtered air vs DE; $p < 0.007$) (Figure 1D–1F) and aortic root (0.81 ± 0.06% vs 0.99 ± 0.17%, Filtered air vs DE; $p < 0.02$) (Figure 2D–2F) in DE exposure group. In addition, this increased COX-2 expression in aortic root was positively correlated with V/v of smooth muscle cells in aortic plaques (Bai et al., 2011b) (Supplementary Figure 3).

Vascular constriction and COX activity

PE-induced constriction was significantly suppressed in DE exposure group, as compared to filter air (Figure 3). The EC50 values were not affected (7.16 ± 0.09 vs 7.27 ± 0.12, Filtered air vs DE, $p > 0.05$). In the presence of COX blocker, indomethacin, PE-elicited constriction was reduced only in control but not DE exposure group (Figure 4A and 4B). The fractional changes of maximal constriction was significantly reduced in DE group (31.7 ± 4.6% vs 12.6 ± 4.2%, Filtered air vs DE, $p < 0.02$) (Figure 4C). The EC50 (or –LogEC50) values were not affected (7.16 ± 0.09 vs 7.15 ± 0.15; 7.27 ± 0.12 vs 7.08 ± 0.14, Filtered air vs Filtered air + Indo; DE vs DE+indo, $p > 0.05$). The presence of selective TXA2 receptor antagonist (SQ29548) did not alter PE-stimulated constriction after DE exposure (Supplementary Figure 4).

Urine 6-keto $PGF_{1\alpha}$ production

Urinary excretion of 6-keto $PGF_{1\alpha}$ is the major urinary metabolite of PGI_2. DE inhalation increased 6-keto $PGF_{1\alpha}$ concentration by 15-fold compared with the control (22.68 ± 5.5 vs 344.6 ± 117.1 pg/mL per Cr (mg/dL), Filtered air vs DE, $p < 0.007$) (Figure 5).

Real-time RT-PCR of the mRNA expression of COX-1, COX-2 and PGIS in the heart

To examine whether the mRNA expression of COX-1, COX-2 and PGIS was affected by DE exposure, we extracted RNA from the heart and performed quantitative real-time PCR using Taqman assays. We found no significant change in COX-1 mRNA expression after exposure to DE (Supplementary Figure 5A). However, the mRNA expression of COX-2 was significantly increased after DE exposure (38.0 ± 1.8% vs 50.4 ± 3.6%, Filtered air vs DE; $p < 0.006$) (Supplementary Figure 5B). The mRNA expression of PGIS in the heart was also significantly augmented in DE exposure group (71.4 ± 1.1% vs 78.4 ± 2.5%, Filtered air vs DE; $p < 0.02$) (Supplementary Figure 5C).

Discussion

Numerous epidemiological studies have shown that exposure to fine particulate matter air pollution, including DE, is association with cardiovascular disease, such as atherosclerosis (Dockery et al., 1993; Brook et al., 2010). Other (Campen et al., 2010) and our laboratory have reported that exposure to DE promotes atherosclerotic lesions to a more vulnerable status, indicated by an increase of macrophage accumulation and a variety of other markers for atherosclerosis progression (Bai et al., 2011b). COX-1 or/and COX-2 has been implicated in atherogenesis (Schönbeck et al., 1999; Praticò et al., 2001). The aim of this study was to examine potential contributions of COX to DE-induced atherosclerosis. We found that exposure to DE upregulated the expression of COX-2, but not COX-1, in both blood vessels (Figure 1) and aortic lesions (Figure 2). The production of PGI_2, a downstream metabolite of COX-2, was also increased (Figure 5), suggesting DE enhanced COX-2 activity. That augmented COX-2 expression in aortic lesions is correlated with aortic smooth muscle cell content (Supplementary Figure 3), implying a role of COX-2 and phenotypic changes in atherosclerotic lesions.

Prostanoid biosynthesis is significantly increased in response to inflammatory stimuli. Non-selective COX-1/COX-2 inhibitors, or nonsteroidal anti-inflammatory

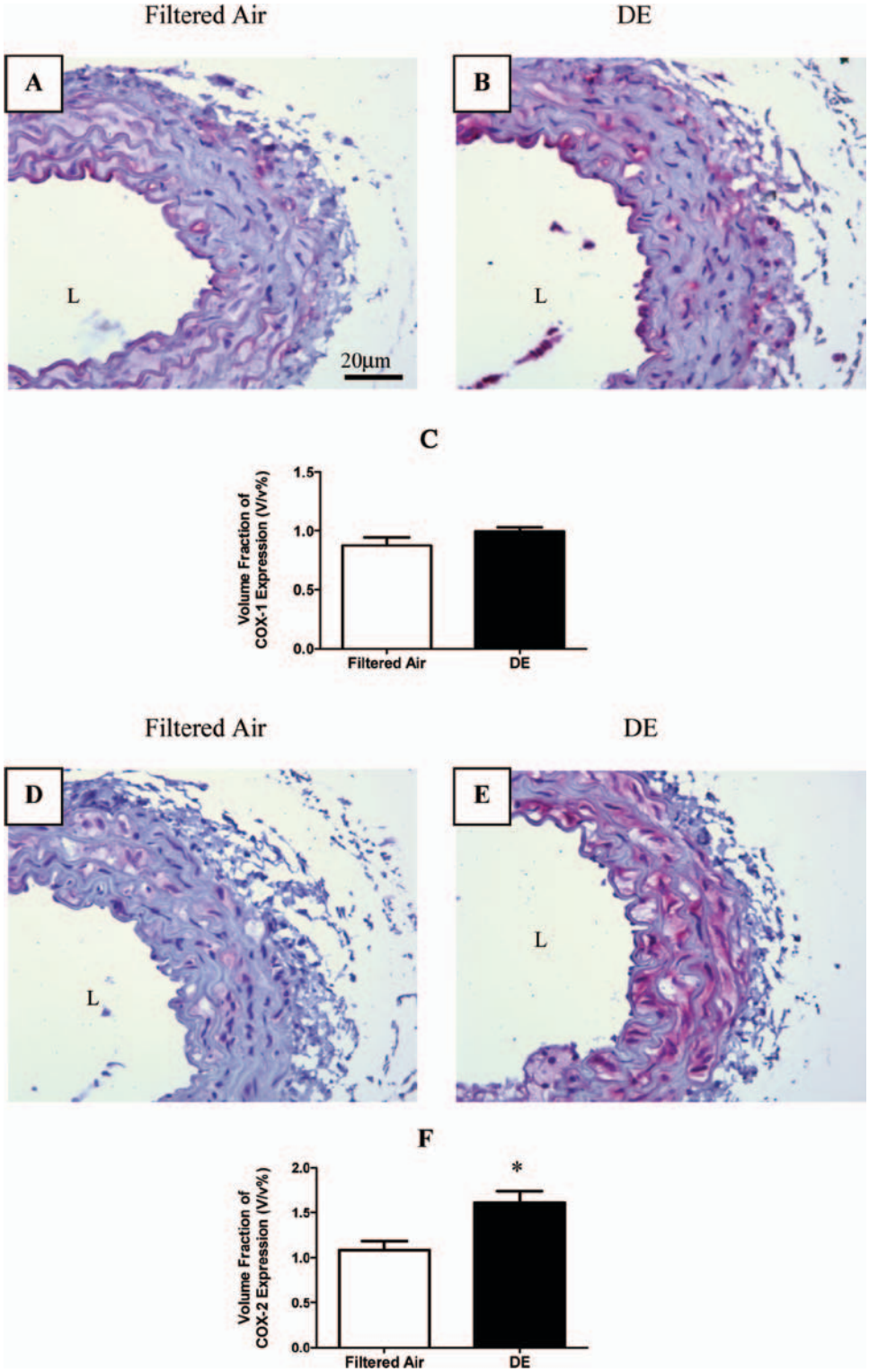

Figure 1. Immunohistochemical analysis of COX-1 and COX-2 expression in the thoracic aorta. Representative photomicrographs of the thoracic aorta stained for COX-1 (A, B) and COX-2 (D, E); C) COX-1 expression was not altered after DE exposure, $n = 8$, $p = 0.2$; F) COX-2 expression was significantly increased in DE exposure group, compared with the control, $n = 8$, $^*p < 0.007$. L: lumen. Magnification: original ×400. Values are mean ± SEM.

drugs (NSAIDs), have been widely used as anti-inflammatory agents, highlighting the pro-inflammatory role of these prostanoids. It is believed that COX-1 functions as a housekeeping enzyme that is constitutively expressed in endothelial cells as well as vascular smooth muscle cells, and responsible for the production of physiological prostanoids to maintain cardiovascular homeostasis. Nevertheless, overexpression of COX-1 is also implicated in a number of cardiovascular diseases, including hypertension (Virdis et al., 2007) and

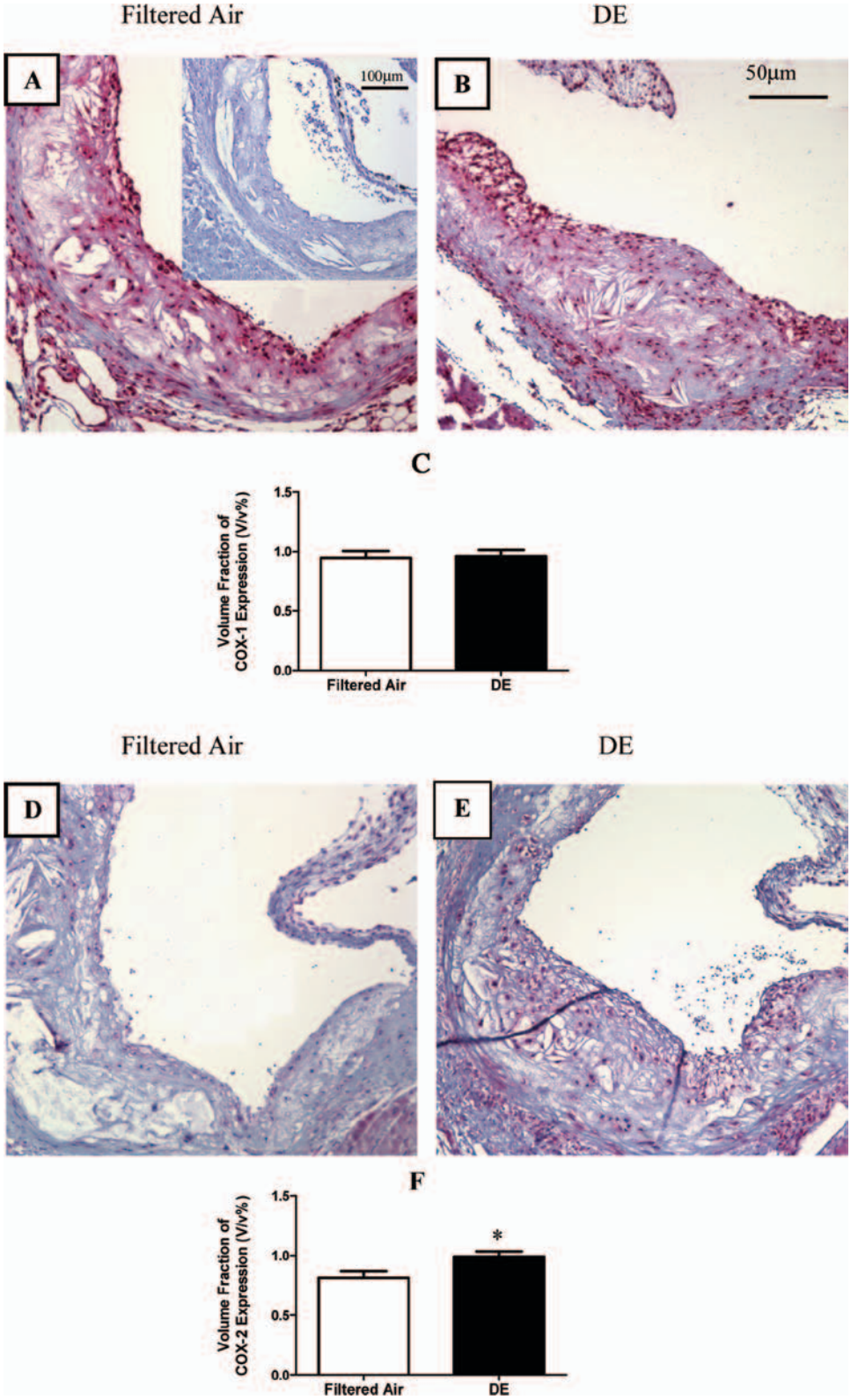

Figure 2. Immunohistochemical analysis of COX-1 and COX-2 expression in aortic root. Representative photomicrographs of aortic roots stained for COX-1(A, B) and COX-2 (D, E); C) COX-1 expression was not altered after DE exposure, $n = 8$, $p = 0.3$; F) COX-2 expression was increased in DE exposure group, $n = 8$, $^*p < 0.02$. Magnification: original ×200. Inset in A is the negative control. Values are mean ± SEM.

atherosclerosis (Praticò et al., 2001). These deleterious effects are mainly resulted from COX-1-derived TXA2, a potent vasoconstrictor and platelet activator. TXA2 causes endothelial dysfunction and promotes vascular smooth muscle cell adhesion, migration and proliferation (McClelland et al., 2009). In this study, we

examined COX-1 protein and mRNA expression in blood vessels and the heart, respectively. We noted that COX-1 was expressed by endothelial cells, smooth muscle cells and macrophages (Supplementary Figure 1). COX-1 protein and mRNA expressions were not altered by DE exposure (Figures 1C and 2C, Supplementary 5A). Vascular functional study showed that the presence of selective TXA2 receptor antagonist (SQ29548) did not modify vasoconstriction in DE exposed group (Supplementary Figure 4), suggesting that DE did not affect TXA2 activity, which is predominantly regulated by COX-1. It was reported that the upregulation of COX-1 expression mainly occurred in the early or acute stages of cardiovascular dysfunction (Belton et al., 2003). This notion may explain why we did not observe an increase in our 7-week exposure model.

COX-2 is usually not detectable under normal physiological conditions, but can be upregulated by inflammatory or other pathological stimuli. We noted that COX-2 was expressed in smooth muscle cells and macrophages in mouse aorta (Supplementary Figures 1 and 2), and the expression of COX-2 was augmented at both protein and mRNA levels (Figures 1F and 2F, Supplementary 5B). Elevated concentrations of a major metabolite of PGI_2, 2,3-dinor-6-keto $PGF_{1\alpha}$, in urine

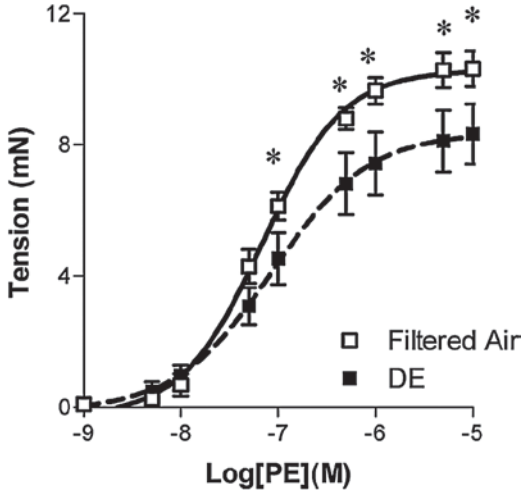

Figure 3. PE-stimulated vasoconstriction in the thoracic aorta. Exposure to DE significantly attenuated PE-stimulated vasoconstriction, $n = 9$, $^*p < 0.05$.

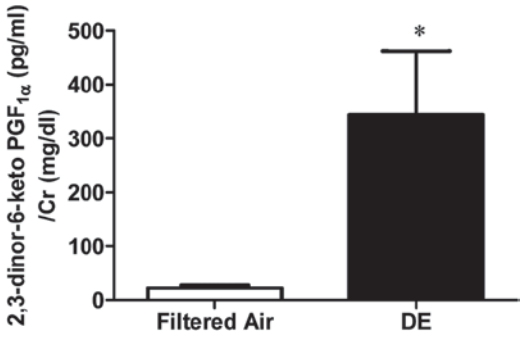

Figure 5. Increased urine 2,3-dinor-6-keto $PGF_{1\alpha}$ concentration. Exposure to DE significantly increased urine 2,3-dinor-6-keto $PGF_{1\alpha}$ concentration, $n = 10$, $^*p < 0.007$. Values are mean ± SEM.

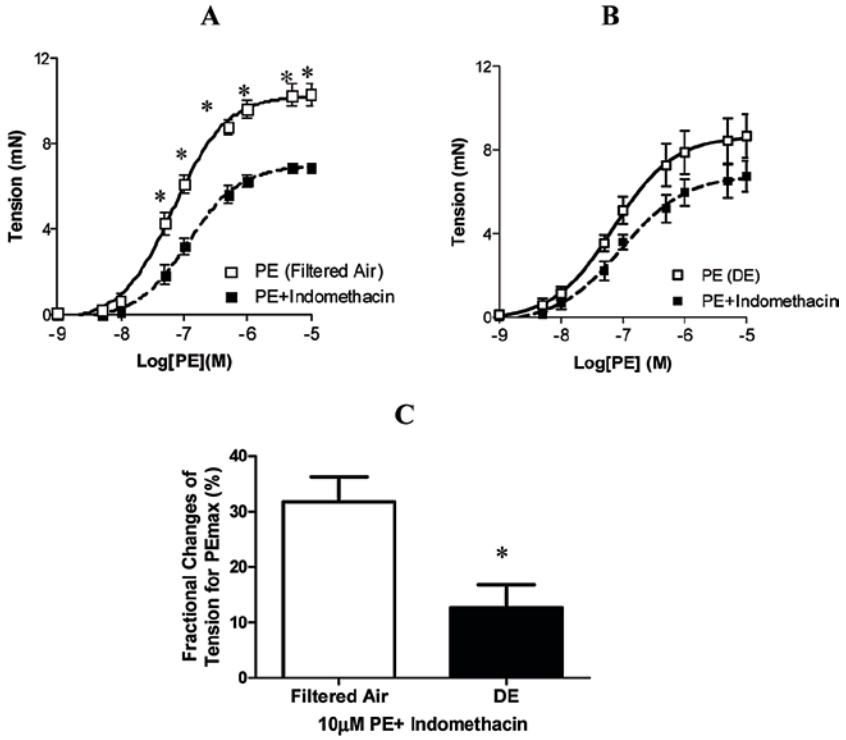

Figure 4. The effect of DE exposure on COX activity. Concentration-response curves of PE-stimulated vasoconstriction show that COX inhibitor caused significant reduction of constriction only in filtered air exposed mice (A) ($n = 9$, $^*p < 0.001$), but not in DE group (B), $n = 9$, $p = 0.17$; C) The fractional changes of maximal vasoconstriction in the presence of indomethacin were significantly lower in DE exposure group than the control, $n = 9$, $^*p < 0.02$.

(Figure 5), and increased PGIS mRNA expression in the heart (Supplementary Figure 5C) indicate that COX-2 activity was also upregulated. Consistent with our results, it was reported that DE increased COX-2 protein and mRNA expression in human macrophages (Hofer et al., 2004). COX-2 expression was observed in cardiovascular tissue of ApoE mice exposed to cigarette smoke for 2 weeks (Barbieri & Weksler, 2007). In addition, PM-induced overexpression of COX-2 in primary human bronchial epithelial cells (Zhao et al., 2009).

Both human and animal studies have demonstrated that COX-2 is implicated in the progression of atherosclerosis (Schönbeck et al., 1999; Rott et al., 2003; Burleigh et al., 2005), potentially by causing reactive oxidative species production (Racz et al., 2010), endothelial dysfunction (Hermann et al., 2003) and/or smooth muscle activation and migration (Li et al., 2006). We recently reported that exposure DE to ApoE mice caused progression of atherosclerosis (Bai et al., 2011b). The positive correlation between increased aortic COX-2 expression and aortic smooth muscle cell content (Supplementary Figure 3) implies a potential mechanism underlying the association between COX-2 and atherosclerosis. We found that DE increased oxidative stress (Bai et al., 2011b), but no association was noted between COX-2 and ROS. In addition, ACh-induced relaxation was not affected in this exposure model (unpublished results).

In contrast to studies showing increased vasoconstriction (Sun et al., 2005), PE-stimulated vasoconstriction was significantly attenuated in DE exposure group, as compared to the controls (Bai et al., 2011a). We postulate that DE exposure caused an imbalance between PGI_2 and TXA2 production, favoring more COX-2 mediated PGI_2 production (Figure 4). This notion is supported by data showing elevated concentrations of a major metabolite of PGI_2, 2,3-dinor-6-keto $PGF_{1\alpha}$, in urine (Figure 5), and increased PGIS mRNA expression in the heart (Supplementary Figure 5C). PGI_2 is a potent vasodilator and a pro-inflammatory prostanoid that enhances vascular permeability, whereby promoting leukocyte trafficking. This notion further supports COX-2-mediated increase of smooth muscle cells in aortic lesions.

It is believed that PM-induced cardiovascular effect results from lung and systemic inflammation following exposure (Dockery et al., 1993; Seaton et al., 1995; Bai et al., 2007; Brook et al., 2010). Using the same exposure model, we have demonstrated that DE exposure caused lung inflammation, which was indicated by increased alveolar macrophages (Bai et al., 2011b) as well as the expression of heat shock protein 70 (HSP70) in the lung (Kido et al., 2011). In addition, we showed increased band cell counts (Bai et al., 2011b) and systemic HSP70 concentration in DE exposed ApoE mice (Kido et al., 2011), suggesting systemic inflammation. We correlated COX-2 with both the magnitude of DE exposure (indicated by total alveolar macrophages in the lung and the alveolar macrophages positive for particles) and systemic response (indicated by band cells) to determine the relationship between COX-2, the lung effect and the systemic response, but no significant association was found (data not shown). We speculate that COX-2 expression in atherosclerosis plaque following DE exposure was mediated locally in vascular walls. We recently demonstrated that DE exposure increased iNOS expression (Bai et al., 2011a). iNOS has been reported to cause upregulation of COX-2, especially under pathological conditions (Kim et al., 2005; Ye et al., 2008) . We noted that the expression of COX-2 and iNOS by macrophages and smooth muscle cells are co-localized in blood vessels (unpublished data). We speculate that iNOS contributes to the upregulation of COX-2 locally. The pathway(s) leading to COX activation by NO/iNOS are unclear, but may involve an interaction at the iron-heme center of COX molecule where NO interacts with iron-containing enzymes leading to either a stimulation (such as via soluble guanylate cyclase) or inhibition (such as via aconitase) of the enzymatic activity (Kalyanaraman et al., 1982; Karthein et al., 1987). This interaction between iNOS and COX activation may represent an important mechanism by which the initial inflammatory response is amplified. Further investigations are warranted to clarify relationships between iNOS and COX-2, using iNOS and COX-2 knockout mice.

Conclusion

In summary, we show that DE inhalation causes upregulation of COX-2 (but not COX-1) activity and expression in both blood vessels and aortic lesions. We previously have shown that exposure to DE caused progression of atherosclerosis in ApoE knockout mice (Bai et al., 2011b), and we postulate that this upregulation of COX-2 may play a role in DE-induced atherosclerosis.

Declaration of interest

This work was supported by the NIH grants R01ES13434 (MER), K24ES013195 (JDK), P50ES015915 (MER, SVE, JDK), the Heart and Stroke Foundation of Canada (SVE, NB), and Michael Smith Foundation for Health Research (NB). SVE is a Senior Scholar with the Michael Smith Foundation for Health Research and CIHR/GSK professor in Chronic Obstructive Pulmonary Disease.

References

Bai N, Khazaei M, van Eeden SF, Laher I. 2007. The pharmacology of particulate matter air pollution-induced cardiovascular dysfunction. Pharmacol Ther 113:16–29.

Bai N, Kido T, Kavanagh TJ, Kaufman JD, Rosenfeld ME, van Breemen C, van Eeden SF. 2011a. Exposure to diesel exhaust up-regulates iNOS expression in ApoE knockout mice. Toxicol Appl Pharmacol 255:184–192.

Bai N, Kido T, Suzuki H, Yang G, Kavanagh TJ, Kaufman JD, Rosenfeld ME, van Breemen C, Eeden SF. 2011b. Changes in atherosclerotic

plaques induced by inhalation of diesel exhaust. Atherosclerosis 216:299–306.

Barbieri SS, Weksler BB. 2007. Tobacco smoke cooperates with interleukin-1β to alter β-catenin trafficking in vascular endothelium resulting in increased permeability and induction of cyclooxygenase-2 expression *in vitro* and in vivo. FASEB J 21:1831–1843.

Belton OA, Duffy A, Toomey S, Fitzgerald DJ. 2003. Cyclooxygenase isoforms and platelet vessel wall interactions in the apolipoprotein E knockout mouse model of atherosclerosis. Circulation 108:3017–3023.

Brook RD, Rajagopalan S, Pope CA 3rd, Brook JR, Bhatnagar A, Diez-Roux AV, Holguin F, Hong Y, Luepker RV, Mittleman MA, Peters A, Siscovick D, Smith SC Jr, Whitsel L, Kaufman JD; American Heart Association Council on Epidemiology and Prevention, Council on the Kidney in Cardiovascular Disease, and Council on Nutrition, Physical Activity and Metabolism. 2010. Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association. Circulation 121:2331–2378.

Burleigh ME, Babaev VR, Yancey PG, Major AS, McCaleb JL, Oates JA, Morrow JD, Fazio S, Linton MF. 2005. Cyclooxygenase-2 promotes early atherosclerotic lesion formation in ApoE-deficient and C57BL/6 mice. J Mol Cell Cardiol 39:443–452.

Campen MJ, Lund AK, Knuckles TL, Conklin DJ, Bishop B, Young D, Seilkop S, Seagrave J, Reed MD, McDonald JD. 2010. Inhaled diesel emissions alter atherosclerotic plaque composition in ApoE(-/-) mice. Toxicol Appl Pharmacol 242:310–317.

Cipollone F, Rocca B, Patrono C. 2004. Cyclooxygenase-2 expression and inhibition in atherothrombosis. Arterioscler Thromb Vasc Biol 24:246–255.

Dockery DW, Pope CA 3rd, Xu X, Spengler JD, Ware JH, Fay ME, Ferris BG Jr, Speizer FE. 1993. An association between air pollution and mortality in six U.S. cities. N Engl J Med 329:1753–1759.

Donaldson K, Stone V, Clouter A, Renwick L, MacNee W. 2001. Ultrafine particles. Occup Environ Med 58:211–6, 199.

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE. 1998. Cyclooxygenase in biology and disease. FASEB J 12:1063–1073.

Egan KM, Lawson JA, Fries S, Koller B, Rader DJ, Smyth EM, Fitzgerald GA. 2004. COX-2-derived prostacyclin confers atheroprotection on female mice. Science 306:1954–1957.

Gould T, Larson T, Stewart J, Kaufman JD, Slater D, McEwen N. 2008. A controlled inhalation diesel exhaust exposure facility with dynamic feedback control of PM concentration. Inhal Toxicol 20:49–52.

Hermann M, Camici G, Fratton A, Hurlimann D, Tanner FC, Hellermann JP, Fiedler M, Thiery J, Neidhart M, Gay RE, Gay S, Lüscher TF, Ruschitzka F. 2003. Differential effects of selective cyclooxygenase-2 inhibitors on endothelial function in salt-induced hypertension. Circulation 108:2308–2311.

Hofer TP, Bitterle E, Beck-Speier I, Maier KL, Frankenberger M, Heyder J, Ziegler-Heitbrock L. 2004. Diesel exhaust particles increase LPS-stimulated COX-2 expression and PGE2 production in human monocytes. J Leukoc Biol 75:856–864.

Hoek G, Brunekreef B, Goldbohm S, Fischer P, van den Brandt PA. 2002. Association between mortality and indicators of traffic-related air pollution in the Netherlands: A cohort study. Lancet 360:1203–1209.

Ishii H, Fujii T, Hogg JC, Hayashi S, Mukae H, Vincent R, van Eeden SF. 2004. Contribution of IL-1 β and TNF-α to the initiation of the peripheral lung response to atmospheric particulates (PM10). Am J Physiol Lung Cell Mol Physiol 287:L176–L183.

Kalyanaraman B, Mason RP, Tainer B, Eling TE. 1982. The free radical formed during the hydroperoxide-mediated deactivation of ram seminal vesicles is hemoprotein-derived. J Biol Chem 257:4764–4768.

Karthein R, Nastainczyk W, Ruf HH. 1987. EPR study of ferric native prostaglandin H synthase and its ferrous NO derivative. Eur J Biochem 166:173–180.

Kawabe J, Ushikubi F, Hasebe N. 2010a. Prostacyclin in vascular diseases. Recent insights and future perspectives. Circ J 74:836–843.

Kawabe J, Yuhki K, Okada M, Kanno T, Yamauchi A, Tashiro N, Sasaki T, Okumura S, Nakagawa N, Aburakawa Y, Takehara N, Fujino T, Hasebe N, Narumiya S, Ushikubi F. 2010a. Prostaglandin I2 promotes recruitment of endothelial progenitor cells and limits vascular remodeling. Arterioscler Thromb Vasc Biol 30: 464–470.

Kido T, Bai N, Yatera K, Suzuki H, Meredith A, Mukae H, Rosenfeld ME, van Eeden SF. 2011. Diesel exhaust inhalation induces heat shock protein 70 expression in vivo. Inhal Toxicol 23: 593–601.

Kim SF, Huri DA, Snyder SH. 2005. Inducible nitric oxide synthase binds, S-nitrosylates, and activates cyclooxygenase-2. Science 310:1966–1970.

Li SL, Reddy MA, Cai Q, Meng L, Yuan H, Lanting L, Natarajan R. 2006. Enhanced proatherogenic responses in macrophages and vascular smooth muscle cells derived from diabetic db/db mice. Diabetes 55:2611–2619.

McClelland S, Gawaz M, Kennerknecht E, Konrad CS, Sauer S, Schuerzinger K, Massberg S, Fitzgerald DJ, Belton O. 2009. Contribution of cyclooxygenase-1 to thromboxane formation, platelet-vessel wall interactions and atherosclerosis in the ApoE null mouse. Atherosclerosis 202:84–91.

Praticò D, Tillmann C, Zhang ZB, Li H, FitzGerald GA. 2001. Acceleration of atherogenesis by COX-1-dependent prostanoid formation in low density lipoprotein receptor knockout mice. Proc Natl Acad Sci USA 98:3358–3363.

Racz A, Veresh Z, Lotz G, Bagi Z, Koller A. 2010. Cyclooxygenase-2 derived thromboxane A(2) and reactive oxygen species mediate flow-induced constrictions of venules in hyperhomocysteinemia. Atherosclerosis 208:43–49.

Rott D, Zhu J, Burnett MS, Zhou YF, Zalles-Ganley A, Ogunmakinwa J, Epstein SE. 2003. Effects of MF-tricyclic, a selective cyclooxygenase-2 inhibitor, on atherosclerosis progression and susceptibility to cytomegalovirus replication in apolipoprotein-E knockout mice. J Am Coll Cardiol 41:1812–1819.

Samet JM, Ghio AJ, Costa DL, Madden MC. 2000. Increased expression of cyclooxygenase 2 mediates oil fly ash-induced lung injury. Exp Lung Res 26:57–69.

Schönbeck U, Sukhova GK, Graber P, Coulter S, Libby P. 1999. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. Am J Pathol 155:1281–1291.

Schuetzle D, Lee FS, Prater TJ. 1981. The identification of polynuclear aromatic hydrocarbon (PAH) derivatives in mutagenic fractions of diesel particulate extracts. Int J Environ Anal Chem 9: 93–144.

Seaton A, MacNee W, Donaldson K, Godden D. 1995. Particulate air pollution and acute health effects. Lancet 345:176–178.

Sun Q, Wang A, Jin X, Natanzon A, Duquaine D, Brook RD, Aguinaldo JG, Fayad ZA, Fuster V, Lippmann M, Chen LC, Rajagopalan S. 2005. Long-term air pollution exposure and acceleration of atherosclerosis and vascular inflammation in an animal model. JAMA 294:3003–3010.

Suwa T, Hogg JC, Quinlan KB, Ohgami A, Vincent R, van Eeden SF. 2002. Particulate air pollution induces progression of atherosclerosis. J Am Coll Cardiol 39:935–942.

Tamagawa E, Bai N, Morimoto K, Gray C, Mui T, Yatera K, Zhang X, Xing L, Li Y, Laher I, Sin DD, Man SF, van Eeden SF. 2008. Particulate matter exposure induces persistent lung inflammation and endothelial dysfunction. Am J Physiol Lung Cell Mol Physiol 295:L79–L85.

Virdis A, Colucci R, Fornai M, Duranti E, Giannarelli C, Bernardini N, Segnani C, Ippolito C, Antonioli L, Blandizzi C, Taddei S, Salvetti A, Del Tacca M. 2007. Cyclooxygenase-1 is involved in endothelial dysfunction of mesenteric small arteries from angiotensin II-infused mice. Hypertension 49:679–686.

Vogel CF, Sciullo E, Wong P, Kuzmicky P, Kado N, Matsumura F. 2005. Induction of proinflammatory cytokines and C-reactive protein

in human macrophage cell line U937 exposed to air pollution particulates. Environ Health Perspect 113:1536–1541.

Warner TD, Mitchell JA. 2004. Cyclooxygenases: New forms, new inhibitors, and lessons from the clinic. FASEB J 18: 790–804.

Ye Y, Martinez JD, Perez-Polo RJ, Lin Y, Uretsky BF, Birnbaum Y. 2008. The role of eNOS, iNOS, and NF-κB in upregulation and activation of cyclooxygenase-2 and infarct size reduction by atorvastatin. Am J Physiol Heart Circ Physiol 295:H343–H351.

Zhao Y, Usatyuk PV, Gorshkova IA, He D, Wang T, Moreno-Vinasco L, Geyh AS, Breysse PN, Samet JM, Spannhake EW, Garcia JG, Natarajan V. 2009. Regulation of COX-2 expression and IL-6 release by particulate matter in airway epithelial cells. Am J Respir Cell Mol Biol 40:19–30.