1. Material and methods

1.1 Exposure protocol and experimental animals

1.1.1 DE exposure system

DE was derived from a 2002 model turbocharged direct-injection 5.9-L Cummins B-series engine (6BT5.9G6; Cummins, Inc.) 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 15W-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.

1.1.2 Animals and exposure protocol

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-week old, these mice were moved in 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. ApoE knockout mice (12/group) were randomly chosen and exposed for 7 weeks (5days/week, 6hrs/day) to DE at the concentration of 200 $\mu g/m^3$. Mice exposed to filtered air were the control. Animal procedures were approved by the Animal Care and Use Committee of the University of Washington.

1.2 Sample collection

After exposure, sodium pentobarbital (100mg/kg, Abbott Laboratories) and heparin sulfate (500U/kg) were administered intraperitoneally. Upon the loss of all reflexes, blood was collected from inferior vena cava and put into EDTA tubes. Plasma was obtained after centrifugation and stored in –80°C until assay. Thoracic aorta, aortic root

and lung were carefully dissected from their connective tissue and kept in appropriate conditions until assay. Urine was also collected and stored in –80°C until assay.

1.3 Sample processing and image acquisition

Samples were fixed with 10% neutral formalin for 24 hours, then embedded in paraffin. Before staining, sections were cut into 5μm, deparaffinized in xylene and hydrated by passing through a series of graded alcohol. After staining for specific targets as explained in details in the following sections, images were captured by a spot digital camera (Microspot, Nikon, Tokyo, Japan), coded and examined by a researcher without knowledge of the experimental groups. The size of an atherosclerotic plaque and its compositions were determined by using quantitative morphologic analysis.

1.4 Lung tissue analysis

Lungs were inflated and fixed with 10% neutral formalin for 24 hours, cut into 4 pieces longitudinal slices, embedded in paraffin blocks, sectioned at 10μm and stained with hematoxylin and eosin (H&E). The images were captured, coded, and analyzed using point counting method. Briefly, a grid of points superimposed onto the captured images by Image Pro Plus software. The density of the grid and the number of fields were selected to maintain the coefficient of error of the estimate of the volume below 0.1. The points that had contacted with alveolar macrophages were counted. The volume fraction (V/v %) of alveolar macrophages was obtained by normalizing the total points for alveolar macrophages to the total points in the area of alveoli. In addition, 100 alveolar macrophages were randomly selected in these lung sections and the number of alveolar macrophages positive for particles was counted.

1.5 Histochemical analysis of atherosclerotic plaque

1.5.1 Morphometric evaluation of the size of atheroslcerotic lesions, cellularity and foam cells of atheroslcerotic lesions in aortic roots

Sections that contain three complete valve leaflets were stained for Movat pentachrome to quantify atherosclerotic lesion area, total cell counts and foam cells. To identify foam cells, we also stained aortic root sections with rat anti-mouse antibody for F4/80 (1:50,

AbD Serotec); a specific marker for mature mouse macrophages. However, we observed that some of the foam cells were not stained or identified, on the other hand, some of these stained cells did not contain lipid in their cytoplasm. Therefore, we decided to quantify foam cells using Movat pentachrome staining, by which foam cells or macrophages contain lipid in their cytoplasm can be identified easily. The images were captured, coded, and analyzed using a grid of points superimposed onto the captured images by Image Pro Plus software. The volume fraction (V/v %) of atherosclerotic lesions was obtained by counting the total points falling in the atherosclerotic lesions divided by the total points in the valve leaflets area. Using the same method, total points for foam cells in atherosclerotic lesions were counted and normalized to the total points in the lesion area to obtain the volume fraction of foam cells. The nuclei of cells in each lesion were manually counted and normalized to the size of atherosclerotic lesion to obtain the cellularity.

1.5.2 Lipid content of plaque

Frozen aortic roots embedded in OCT (Sakura Finetek) were cut (5μm). Sections that contain three complete valve leaflets were fixed in 4% paraformaldehyde at room temperature for 30 min, and stained for Oil-Red-O. Using Image Pro Plus software, the area of positive staining was recognized, analysis by colour segmentation, and normalized to the area of atherosclerotic lesion to obtain the volume fraction (V/v %) of positive staining for Oil-Red-O, representing the lipid content.

1.5.3 Collagen content

Aortic root sections that contain three complete valve leaflets were stained in picro-sirius red (Sigma) for one hour, washed in two changes of acidified water, dehydrated in three changes of 100% ethanol. Using Image Pro Plus software, the area of red staining was recognized, quantified by colour segmentation, and normalized to the area of the atherosclerotic lesion to obtain the volume fraction (V/v %) of positive staining for picro-sirius red, representing collagen synthesis.

1.5.4 Immunohistochemical analysis of macrophage, iNOS, CD36, nitrotyrosine and α-actin expression

Aortic root sections that contain three complete valve leaflets were used. After unmasking the antigenic sites by antigen retrieval using citrate buffer (Invitrogen), sections were incubated with 10% goat serum at room temperature for 30min to block nonspecific binding proteins, followed by incubation with specific primary antibodies: rat anti-mouse antibody for F4/80, a marker for mature macrophages (1:50, AbD Serotec); rabbit anti-mouse antibody for inducible nitric oxide synthase (iNOS) (1:100, Santa Cruz); rabbit anti-mouse antibody for CD36 (1:50, Santa Cruz); rabbit anti-mouse antibody for nitrotyrosine (NT) (1:400, Upstate Biotechnology), and rabbit anti-mouse antibody for α-actin (1:600, Abcam) 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. Using Image Pro Plus software, the area of positive staining was recognized, quantified by colour segmentation, and normalized to the area of the atherosclerotic lesion to obtain the volume fraction (V/v %) of expression for iNOS, CD36 and NT, respectively.

1.6 Systemic oxidative stress

It has been reported that isoprostanes are stable end products of arachidonic acid peroxidation[35]. Of the variety of isoprostanes detected, 15-F_{2t}-isoprostane (15-F_{2t}-IsoP) has been found to be a specific, reliable marker of oxidative stress. We measured 15-F_{2t}-IsoP in urine using 8-Isoprostane EIA kit according to the manufacturer's instruction (Cayman Chemical). Urine samples were purified using 8-isoprostane affinity sorbent, and creatinine levels were measured by creatinine assay kit (Cayman Chemical). The results were expressed in pg of 15-F_{2t}-Iso per mmol creatinine.

8-hydroxy-2-deoxy guanosine (8-OH-dG) is produced by oxidative damage of DNA by reactive oxygen and nitrogen species, and 8-OH-dG serves as an established marker for oxidative stress. Because of the complexity of plasma samples, urine is more suitable for

measuring free 8-OH-dG. The urine creatinine levels were measured by creatinine asssy kit (Cayman Chemical). 8-OH-dG concentration was measured using 8-OH-dG kit (Cayman Chemical), and the results were expressed in pg of 8-OH-dG per mmol creatinine.

2. Results

2.1 No changes in plasma cholesterol and triglyceride

To examine whether increased lipid content in atherosclerotic plaque was due to higher levels of plasma lipid, we measured plasma cholesterol and triglyceride, and found that exposure to DE did not affect plasma lipid levels (Table 1).

Table 1. Plasma cholesterol and triglyceride (data are the mean±SE)

	Filtered air	DE	
Cholesterol (mol/L)	26.2 ± 1.0	28.0 ± 1.8	n=12, P=0.4
Triglyceride (mg/dl)	135.8 ± 11.8	140.6 ± 12.9	n=12, P=0.8

2.2 Systematic oxidative stress

To determinate the effect of DE inhalation on systemic oxidative stress, we measured lipid and DNA oxidation markers in urine using EIA assays. We found that both15-F_{2t}-isoprostane (Fig 1A), and 8- hydroxy -2-deoxy guanosine (8-OH-dG) (Fig 1B) production were significantly higher in DE exposure group than filtered air exposure. The plasma MPO levels were similar between DE and filtered air exposure groups (n=12, p=0.5; Fig 2).

3. Figure legend

Fig. 1. Analysis of systemic oxidative stress. A) Increased protein oxidation supported by enhanced urine 8-isoprostane production, n=10, *P<0.02; B) Increased DNA oxidation demonstrated by augmented urine 8-OH-dG production, n=11, *P<0.02. Values are mean±SE

Fig. 2. No changes in plasma MPO levels after exposure to DE.