MATERIALS AND METHODS (Supplemental)

Immunofluorescent Staining

For immunofluorescent staining, the sections were incubated with primary antibodies against PKC-α, -βII, -δ, or -ε antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), and then with FITC-conjugated anti-rabbit IgG secondary antibody (1:200, Santa Cruz Biotechnology). For double staining, the sections were incubated with primary antibodies against PKC-βII (1:50, Santa Cruz Biotechnology) and IRS-1 (1:50, Cell Signaling Technology, Danvers, MA), and then with secondary antibodies (FITC-conjugated anti-goat IgG and Texas Red-conjugated anti-rabbit IgG, 1:200, Santa Cruz Biotechnology). Finally, 0.05% DAPI was used for nuclear staining. The sections were analyzed with a Zeiss LSM 510 confocal laser-scanning microscope and the fluorescent images of the representative areas of the aorta were photographed. A Zeiss C-Apochromat x63/1.2W high-power objective was used.

Magnetic Resonance Imaging (MRI)

In vivo MRI was performed in C57BL/6 mice when exposure ended. Briefly, a Bruker 11.7T NMR system with a 52 mm internal diameter vertical bore (Bruker Instruments, Billerica, MA) operating at a proton frequency of 500 MHz with a gradient strength of 300 gauss per centimeter was used. Mice were anesthetized continuously with inhaled isoflurane (1.5-2.0%) and placed in a 30 mm birdcage coil. Because the abdomen is relatively free from motion artifact, no respiratory or cardiac gating was necessary. A coronal spin-echo localizing

sequence was used to identify both kidneys. Twenty-five contiguous, 1-mm thick axial slices spanning from the superior pole of the uppermost kidney to the caudal aspect of the mouse were obtained using a spin-echo sequence with a 256 X 256 matrix size (pixel size, 117 $\times$ 117 $\times$ 1,000 μm^3). Repetition and echo times for the T1-weighted images were 1,000 and 13.0 ms, respectively. Usage of 4 signal averages provided the best tissue contrast. The imaging time was 17 minutes per scan. Data analysis was performed using the Image J software downloaded from the NIH website (Image J, http://rsb.info.nih.gov/ij/). Using the T1-weighted images, total abdominal volume, total adipose tissue, subcutaneous adipose tissue, and visceral adipose tissue were calculated as follows: the images were converted into two intensities, one corresponding to the adipose regions and the other corresponding to the remaining tissue. From these binary images, total adipose volume was equal to the volume of the intensity corresponding to the adipose region and total abdominal volume was equal to the volume of both intensities combined. Next, the visceral adipose volume was calculated. The subcutaneous adipose tissue was calculated by subtracting the visceral adipose volume from the total adipose volume. Due to varying anatomic lengths, all volumes were normalized using the formula: normalized volume = n $\times$ median number of images in a given population, where n is the number of slices measured in an individual T1-weighted image set.[1]

Myograph Experiments

At the end of the exposure period, mice were euthanized under isoflurane anesthesia. The aortic segments were removed, and the 2-mm thoracic aortic rings were suspended in individual organ chambers that were filled with physiological salt solution (PSS) using methods previously described.[2] The vessels were subjected to graded doses of vasoconstrictor phenylephrine (PE, 10^{-9} to 10^{-5} mol/L). After a stable contraction plateau was reached with PE, which was about 50% of peak tension generated with 120 mmol/L of KCl, the rings were exposed to graded doses of the endothelium-dependent agonist acetylcholine (Ach, 10^{-10} to 10^{-5} mol/L) or insulin (10^{-7} ~ 10^{-5} mol/L, Novolin®, Novo Nordisk Inc., Princeton, NJ). Basal NO bioavailability was assessed in preconstricted aortic rings by measuring the further increase in tension induced by the NOS inhibitor N_G-monomethyl-L-arginine (L-NMMA, 10^{-4} mol/L, Sigma-Aldrich, St. Louis, MO) for 30 minutes.

Adipose Stromal Vascular Fractionation and Macrophage Isolation

Epididymal fat pads from HFC fed C57BL/6 mice were excised, minced in PBS, and tissue suspensions were centrifuged, as detailed previously [9]. Stromal vascular fraction (SVF) pellet was harvested after the process with Collagenase II (Sigma-Aldrich) and incubated at 37°C for 30 minutes with shaking. The cell suspension was filtered through a 100-μm filter and then spun at 300 *g* for 5 minutes. Viable adipose tissue lymphocytes were isolated from the SVF using Lympholyte M (Cedarlane Laboratories Ltd, Birlington, NC). The lymphocyte layers were incubated with a biotinylated F4/80 antibody (Biolegend, San Diego,

CA) followed by anti-biotin superparamagnetic colloidal particles in MACS buffer (Miltenyi Biotec Inc., Auburn, CA). Cells were separated with MACS MS column/magnet according to the manufacturer's instruction. The purity of column separation was confirmed using flow cytometry (F4/80, CD11b positivity).

Immunochemical and Immunofluorescent Staining in Aortic Segments

Frozen aortic segments embedded in Tissue-Tek Optimal Cutting Temperature compound (OCT, Sakura Finetek USA Inc., Torrance, CA) were cut into 8-μm-thick sections, fixed with ice-cold methanol, and blocked with 1.5% horse serum. Immunohistochemical and immunocytochemical staining was performed with antibodies against 3-nitrotyrosine (1:200, Millipore, Billerica, MA) and inducible NOS (iNOS, 1:100, Santa Cruz Biotechnology). Quantification of 3-nitrotyrosine and iNOS staining were performed from 3 to 4 aortic sections per mouse in each group with software.

Confocal Microscopy of Fixed and Live Adipose Tissue

To image adipose tissue macrophages (ATMs) from C57BL/6 mice by immunofluorescence, epididymal fat pads were dissected and incubated in 1% paraformaldehyde (PFA) overnight. After 3 washes in PBS, portions from the fat pad tip were removed for imaging. Pieces were blocked in 5% BSA in PBS with 0.3% Triton X-100 (PBST) for 1 hour and the incubated with anti-F4/80 (Abcam Inc., Cambridge, MA) and anti-caveolin (BD Biosciences, Franklin Lakes, NJ) antibodies at 1:100 dilution with gentle rocking at room temperature for 2-3 hours.

After washing, fluorophor-conjugated secondary antibodies were added to the pads and incubated for 60 minutes at room temperature with gentle rocking. Fat pads were washed and placed on in a well of a chambered coverslip with 95% glycerol as a mounting media to hold the fat pad in place. To image live adipose tissue from c-*fms*^{YFP} mice, epididymal fat tissue was minced into small pieces, washed thoroughly with PBS, and incubated with antibody Isolectin GS-IB4 conjugated with Alexa Fluor (Molecular Probe, Carlsbad, CA). Nuclei were counterstained with Hoechst 33342 (Sigma-Aldrich). Tissue was imaged using an inverted confocal scanning microscope (Olympus).

Quantitative Real-time PCR

RNA was isolated using Trizol™ (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA quality and quantity were assessed by agarose gel electrophoresis and a Nanodrop™ spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA was reverse transcribed using 500 ng of total RNA according the manufacturer's instruction (Invitrogen Life Technologies – M-MLV reverse transcriptase) using random primers. PCR was performed using SYBR Green I master mix (Roche, Pleasanton, CA) on a Roche Lightcycler 480. All real-time reactions had the following profile conditions: 10 minute hot start at 95°C followed by 45 cycles of 94°C 10s, 60°C 20s, 72°C 20s. Reference and target gene dilution standards were run in triplicate for each primer set to calculate PCR efficiency using the above profile. The concentration ratios were determined after PCR efficiency correction by relative quantification analysis

using Lightcycler 480 software. All target genes were expressed as fold increase compared to the control. Melting/dissociation curves were run on each plate to assure the production of one amplicon of the same melting temperature for each primer set. Real time primers were designed to span genomic introns, thus avoiding amplification of genomic DNA possibly present in the RNA samples. "No template," cDNA negative controls were included for each gene set in all PCR reactions to detect contamination. Primers used were: *beta actin* For 5'-ctaggcaccagggtgtgatg 3'; *beta actin* Rev 5'-cttttcacggttggccttag-3'; *Hmg14* For 5′-gcagaaaatggagagacggaaaacc-3′; *Hmg14* Rev 5′-aagggaggcgggaccactgac-3′; *Nos2* For 5'-ccaagccctcacctacttcc-3'; *Nos2* Rev 5'-ctctgagggctgacacaagg-3'; *Tnf*α For 5'- caacggcatggatctcaaagac-3'; *Tnf*α Rev 5'- agatagcaaatcggctgacggt-3'; *Itgax* For 5'-acggaaccacagtcactctgtt-3'; *Itgax* Rev 5'-gtcacacatgaggtgcaggga-3'; *IL10* For 5'-tagagctgcggactgccttca-3'; *IL10* Rev 5'-atgctccttgatttctgggccat-3'; *Mgl1* For 5'-tggatgggaccgactttgagaa-3'; *Mgl1* Rev 5'-gggaccacctgtagtgatgtg-3; *Pparg* For 5'-tgaagacattccattcacaagagc-3'; *Pparg* Rev 5'-cacagactcggcactcaatgg-3'

Immunoblotting Analysis

Aortic tissue samples were homogenized and lyzed in Mammalian Protein Extraction Reagent (M-PER, Pirece, Rockford, IL) supplemented with protease inhibitor cocktail. The lysates were clarified at 12,000 *g* for 10 min at 4°C. Protein concentration of the lysates was measured by Bio-Rad protein assay reagents. Equal amounts (20 μg) of the lysates were prepared, subjected to

immunoblotting with antibodies against p-Akt(Ser473) (Cell Signaling Technology, Danvers, MA) and Akt (Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Figure Legends

Figure. Representative photomicrographs showing colocalization of PKC-βII and IRS-1 in aortic tissues by immunofluorescent staining in C57BL/6 mice fed with HFC. Colocalization of PKC-βII and IRS-1 in aortic tissues was detected via fluorescence by using FITC and Texas Red as the fluorochrome, respectively. Scale bar = 50 μm. DAPI was used for nuclear staining. Shown is the representative of each group providing similar results. n = 5-7.

Movie 1. Representative movie segment of rolling and adherent YFP cells in cremasteric circulation in c-*fms*^{YFP} mice fed with HFC treated by saline.

Movie 2. $PM_{2.5}$ exposure induces YFP cell adhesion in the cremasteric vasculature in c-*fms*^{YFP} mice fed with HFC. Representative movie segment of rolling and adherent YFP cells in cremasteric circulation in c-*fms*^{YFP} mice fed with HFC treated by $PM_{2.5}$.

REFERENCES

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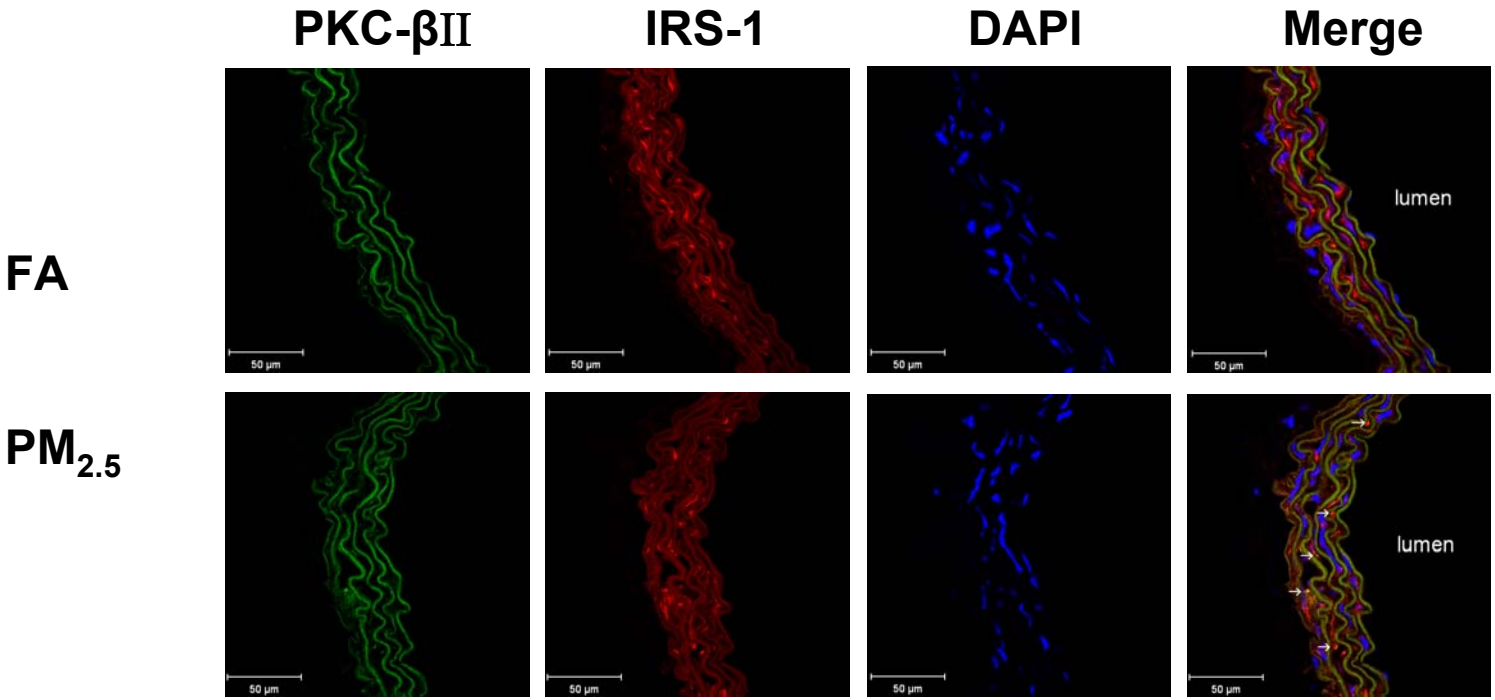
Table 1. Physical and biological data in C57BL/6 mice exposed to fine particulate matter ($PM_{2.5}$) or filtered air (FA)

	FA	$PM_{2.5}$
Weight, g	33.3 ± 1.2	35.0 ± 1.5
Total cholesterol, mg/dl	138.1 ± 6.5	123.4 ± 12.2
Triglycerides, mg/dl	121.5 ± 12.9	89.7 ± 9.8
HDL, mg/dl	98.2 ± 2.8	77.1 ± 5.6*****
LDL, mg/dl	16.4 ± 3.8	29.5 ± 4.5*****
TC/HDL	1.4 ± 0.1	1.6 ± 0.1*****
Fasting glucose, mg/dl	139.3 ± 5.6	161.2 ± 4.2*****
Fasting insulin, μU/ml	5.1 ± 0.4	15.8 ± 1.2*****
Insulin resistance (HOMA)	1.8 ± 0.7	6.2 ± 1.6*****

HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; HOMA, homeostatic model assessment. *, P <0.05, vs. FA.


PKC-βII
IRS-1
DAPI
Merge
FA
PM2.5
lumen
lumen
50 μm