Molecular Cardiology

Ambient Air Pollution Exaggerates Adipose Inflammation and Insulin Resistance in a Mouse Model of Diet-Induced Obesity

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Background—There is a strong link between urbanization and type 2 diabetes mellitus. Although a multitude of mechanisms have been proposed, there are no studies evaluating the impact of ambient air pollutants and the propensity to develop type 2 diabetes mellitus. We hypothesized that exposure to ambient fine particulate matter (PM2.5) exaggerates diet-induced insulin resistance, adipose inflammation, and visceral adiposity.

Methods and Results—Male C57BL/6 mice were fed high-fat chow for 10 weeks and randomly assigned to concentrated ambient PM2.5 or filtered air (n=14 per group) for 24 weeks. PM2.5-exposed C57BL/6 mice exhibited marked whole-body insulin resistance, systemic inflammation, and an increase in visceral adiposity. PM2.5 exposure induced signaling abnormalities characteristic of insulin resistance, including decreased Akt and endothelial nitric oxide synthase phosphorylation in the endothelium and increased protein kinase C expression. These abnormalities were associated with abnormalities in vascular relaxation to insulin and acetylcholine. PM2.5 increased adipose tissue macrophages (F4/80+ cells) in visceral fat expressing higher levels of tumor necrosis factor-α/interleukin-6 and lower interleukin-10/N-acetyl-galactosamine specific lectin 1. To test the impact of PM2.5 in eliciting direct monocyte infiltration into fat, we rendered FVBN mice expressing yellow fluorescent protein (YFP) under control of a monocyte-specific promoter (c-fms, c-fmsYFP) diabetic over 10 weeks and then exposed these mice to PM2.5 or saline intratracheally. PM2.5 induced YFP cell accumulation in visceral fat and potentiated YFP cell adhesion in the microcirculation.

Conclusion—PM2.5 exposure exaggerates insulin resistance and visceral inflammation/adiposity. These findings provide a new link between air pollution and type 2 diabetes mellitus. (Circulation. 2009;119:538-546.)

Key Words: air pollution ■ diabetes mellitus ■ macrophage ■ obesity

Substantial epidemiological evidence implicates air pollution as a major adverse risk factor with serious consequences on human health in both industrialized and developing countries.1,2 Recent data from large population cohorts have provided compelling associations between ambient PM2.5 pollution and increased cardiovascular morbidity and mortality.3,4 Substantial data exist implicating fine particulate matter (diameter <2.5 μm; PM2.5) as a major mediator of cardiovascular effects, including elevations in blood pressure and acute coronary syndromes.5,6 A major component of PM2.5 is pollutant particles generated as a consequence of urbanization, including automobile exhaust and particles generated from power plants. An increasing number of associative studies have suggested that exposure to low concentrations of inhaled environmental pollutants might be associated with a propensity to chronic diseases, including type 2 diabetes mellitus.7,8 Although a number of factors such as increased caloric intake and reduced physical activity undoubtedly play an important part in the genesis of these disorders, there is cumulative evidence that type 2 diabetes mellitus is a chronic inflammatory state aggravated by factors that promote inflammation at the level of vasculature and adipose tissue.9–11 Accordingly, we hypothesized that exposure to PM2.5 exaggerates insulin resistance (IR) and adipose inflammation and tested this hypothesis in a model of diet-induced obesity.
Methods

A detailed description of all materials and methods is provided in the online-only Data Supplement.

Animals and Animal Care

Six-week-old male C57BL/6 mice (The Jackson Laboratories, Bar Harbor, Me) were equilibrated for 2 weeks before being fed high-fat chow (HFC; 42% from fat-adjusted calorie diet, TD 88137, Harlan, Indianapolis, Ind; n=28) for 10 weeks and then randomized to exposure (see below). Ten-week-old transgenic mice expressing yellow fluorescent protein (YFP) under a monocyte-specific promoter (c-fms [CD115]; c-fmsYFP) were fed normal chow or were rendered diabetic by the use of a protocol similar to that stated above over 10 weeks (n=8) before being subjected to intratracheal PM2.5 exposure. The Committees on Use and Care of Animals of Ohio State University and New York University approved all experimental procedures.

Exposures to PM2.5

Ambient Whole-Body Inhalational Protocol

C57BL/6 mice were exposed in vivo from October 9, 2006, to March 26, 2007, for a total duration of 128 days, to concentrated PM2.5, composed of the northeastern regional background at the AJ Laney Laboratory in the Department of Environmental Medicine at New York University, which was characterized previously. The concentrated PM2.5 was generated by a versatile aerosol concentration enrichment system that was modified for longer-term exposures. The mice were exposed to PM2.5 (n=14) for 6 h/d for 5 d/wk. The filtered air (FA; n=14) control mice were exposed to an identical protocol except a high-efficiency particulate air filter (Pall Life Sciences, East Hills, NY) was positioned in the inlet valve to the exposure system to remove all of the particles from that airstream, as detailed previously.

Intratracheal Installation Protocol

c-fmsYFP mice were randomized to intratracheally delivered PM2.5 (1.6 mg/kg, collected from the filters after the exposure above) suspended in 25 μL sterile PBS or 25 μL sterile PBS (control) over a 5-minute period twice per week for 10 weeks, as detailed previously. PM2.5 in PBS was vortexed before instillation. Prior studies have demonstrated that delivery with this route results in similar levels of pulmonary distribution and bioavailability compared with ambient exposure.

Measures of Glucose Homeostasis

Mice were fasted overnight before the body weight was measured and lipid profile and intraperitoneal glucose tolerance tests were performed before and after PM2.5 whole-body exposure in C57BL/6 mice. The intraperitoneal glucose tolerance test also was performed with a T1-weighted gradient-echo sequence on all C57BL/6 mice when exposure ended. Magnetic resonance imaging was performed with a 11.7-T Bruker magnet.

Myograph Experiments

Aortic ring segments were subjected to graded doses of phenylephrine, acetylcholine, or insulin as described previously.

Adipose Stromal Vascular Fractionation and Macrophage Isolation

Stromal vascular fraction pellet was harvested from epididymal fat pads, and the lymphocyte layers were incubated with F4/80 antibody (Biolegend, San Diego, Calif) followed by anti-biotin superparamagnetic colloidal particles. Adipose tissue macrophages (ATMs) were separated with an MACS MS column/ magnet according to the manufacturer’s instruction (Miltenyi Biotec, Germany).

Confocal Microscopy Studies in Fixed and Live Adipose Tissue

To image ATMs in the C57BL/6 group by immunofluorescence, epididymal fat pads were dissected and incubated in 1% paraformaldehyde overnight and incubated with anti-F4/80 and anti-caveolin antibodies. Tissue was imaged with an inverted confocal scanning microscope. To image live adipose tissue from c-fmsYFP mice, epididymal fat tissue was minced into small pieces, washed thoroughly, and incubated with antibodies.

Intravital Microscopy in c-fmsYFP Mice

c-fmsYFP mice were anesthetized intraperitoneally by a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg). The mesenteric tissue and cremaster muscle were exteriorized.

On an optically coherent mount. The tissues were superfused with prewarmed Ringer’s lactate (37°C), and the number of adherent YFP cells in 5 to 10 movies was acquired with a 40×/0.80-W water-immersed objective using a Nikon Eclipse FN1 microscope (Nikon, Tokyo, Japan) and Metamorph software (version 7.1.2.0, Metamorph, Downingtown, Pa). The number of adherent cells in cremaster muscle was determined by counting stationary leukocytes in a 100-μm vessel length per 30 seconds; adherent cells in the mesenteric tissue were counted per image field (1.57×10 μm²).

Data Analyses

Data are expressed as mean+SEM unless otherwise indicated. For responses measured repeatedly at different time points or dose levels, a series of 2-sample independent Student’s t tests were used to detect the significant differences between 2 treatment groups at every time point and dose level with the Bonferroni correction for multiple comparison adjustment. Comparisons of other continuous variables were conducted with an independent 2-sample Student t test, with values of P<0.05 considered significant.

The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Whole-Body Exposure Data

Ambient mean daily PM2.5 concentration at the study site was 7.7 μg/m³ (SD, 5.0 μg/m³). Mean concentration of PM2.5 in the exposure chamber was 72.7 μg/m³ (=9-fold concentration from ambient levels). Because the mice were exposed for 6 hours a day, 5 days a week, the equivalent PM2.5 concentration to which the mice were exposed in the chamber normalized over the exposure period (128 days) was 13.0 μg/m³, which is well within the annual average PM2.5 National Ambient Air Quality Standard (NAAQS) of 15 μg/m³.
general exposure characteristics have previously been described.13

Metabolic Impairment by PM2.5

Before exposure began, there was no statistically significant difference between the 2 groups in body weight, chow consumption, lipid profile, and glucose tolerance response. Table I of the online Data Supplement summarizes the metabolic data in the FA and PM2.5 groups at the end of 24 weeks of exposure. C57BL/6 mice exposed to PM2.5 demonstrated identical chow consumption (2.2 ± 0.2 g/d in FA versus 2.2 ± 0.3 g/d in PM2.5) and weight gain over the duration of the entire experiment (Table I in the Data Supplement). PM2.5-exposed mice displayed abnormal indexes of glucose/insulin homeostasis as evidenced by elevated fasting glucose, insulin, homeostasis model assessment indexes, and abnormalities in lipid profile consistent with IR phenotype. Figure 1A displays responses to the intraperitoneal glucose tolerance test. PM2.5 mice demonstrated elevations in glucose levels at all time points after 20 minutes.

PM2.5 Impairs Vascular Endothelium-Dependent Responses

Because the vascular endothelium is a sensor for multiple stimuli, including inhaled particulates, and plays an important role in IR development, we investigated endothelial responses to acetylcholine and insulin (Figure 1B and 1C). PM2.5-exposed C57BL/6 mice demonstrated a decrease in peak relaxation and ED50 to acetylcholine and decreased peak relaxation to insulin compared with FA mice. Because these changes are generally consistent with a decrease in nitric oxide release in response to these agonists, we confirmed the effects of PM2.5 in reducing vascular nitric oxide bioavailability by the increment in tension in preconstricted aortic rings to N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 10\textsuperscript{-4} mol/L). PM2.5-exposed mice demonstrated reduced constriction to this agent (Figure 1D), consistent with lower levels of nitric oxide release.

PM2.5 Exposure Impairs Insulin Signaling

We examined the effect of PM2.5 exposure on insulin signaling in the aorta and liver. The phosphorylation of Akt in intact aorta but not denuded aortic segments was reduced in the PM2.5 group compared with the FA group (Figure 2A and 2B). These results suggest that the differential effect of PM2.5 on aorta was secondary to differences in endothelial Akt phosphorylation. We also investigated the time course of Akt phosphorylation in response to stimulation with insulin and found that Akt phosphorylation levels were decreased at 30 minutes in PM2.5 compared with FA (Figure 2C). To inves-
tigate whether protein kinase C (PKC) is involved in PM2.5-mediated IR and vascular dysfunction, we investigated the expression of multiple PKC isoforms by immunohistochemistry. PKC-βII but not PKC-α or PKC-δ was increased in PM2.5 compared with the FA groups (data not shown). The increased PKC-βII expression colocalized in aortic tissues, with anti-insulin receptor substrate-1 suggesting a role for this pathway in PM2.5-mediated IR (see the Data Supplement). Taken together, these data suggest that insulin signaling is abnormal in response to PM2.5 exposure.

PM2.5 Exposure Induces Adipose Inflammation and Visceral Adiposity

On the basis of the strong evidence that adipose tissue inflammation contributes to IR, we examined the effects of PM2.5 exposure on systemic and local adipose tissue inflammation. Figure 3 shows the plasma concentration of adipokines and inflammatory biomarkers in C57BL/6 mice fed HFC. A, TNF-α and IL-6. B, E-selectin, ICAM-1, and plasminogen activator inhibitor-1 (PAI-1). C, Resistin and leptin. n=7. ∗P<0.05 vs FA.

In addition, adipocyte size as measured by cross-sectional area was increased in PM2.5-exposed mice (FA, 883 μm²±21; PM2.5, 973 μm²±26; P<0.01) (Figure 5C and 5D). In view of the increased inflammation seen in adipose tissue, we investigated the expression of ATM-specific genes in the F4/80+ cells in the stromal vascular fraction. PM2.5 treatment resulted in significant increases in the expression of proinflammatory genes TNF-α and IL-6 (M1) with no change in nitric oxide synthase-2 expression. In contrast, IL-10 and the alternative (M2) macrophage activation marker galactose-N-acetylgalactosamine–specific lectin (Mgl1) were markedly downregulated (Figure 6B). These results indicated that PM2.5 down-
regulates genes associated with an anti-inflammatory "M2" phenotype while simultaneously inducing proinflammatory M1 phenotype genes.

PM2.5 Exposure Induces YFP Cell Adhesion and Infiltration

In view of the inflammatory macrophage phenotype in adipose tissue and an increase in adhesion molecules promoting leukocyte adhesion to endothelial cells (ICAM-1 and E-selectin), we tested the hypothesis that monocytes are recruited to adipose tissue in response to PM2.5 exposure, thus representing newly arrived monocyte-macrophages. To test this hypothesis, we used a transgenic animal model expressing YFP under the control of a c-fms promoter (c-fmsYFP). The mice were rendered diabetic (Figure 7A and 7B) and then exposed to FA or PM2.5 with an intratracheal protocol. Intravital microscopy was performed to determine the number of YFP cells in mesentery and adherent YFP cells in cremasteric vessels as representative of visceral and systemic microcirculation, respectively. Figure 7C indicates that more YFP cells in the adipose tissue were found in the PM2.5-exposed mice compared with the saline-exposed mice. Figure 8A and 8B are representative images of mesenteric tissue from c-fmsYFP mice exposed to either saline or PM2.5, respectively. Figure 8C shows a graphic of multiple experiments (n=7 per group). Exposure to PM2.5 significantly increased YFP cell infiltration in the mesenteric fat. Figure 8D is a representative image of cremasteric circulation from mice exposed to PM2.5. PM2.5 treatment resulted in increased YFP cell adhesion to endothelium compared with saline control in venules measuring 20 and 30 μm, respectively (Figure 8E).

Discussion

In the present study, ambient PM2.5 induced obesity to potentiate IR, visceral adiposity, and inflammation in a diet-induced murine model. Over the last few decades, a concerted scientific effort on risk factors such as glycemia, diet, and inactivity has resulted in fundamental insights into how they may increase propensity for type 2 diabetes mellitus. On the other hand, the link between chronic exposure to environmental factors in air/water and propensity to type 2 diabetes mellitus has gained only recent attention. PM2.5 exposure in conjunction with HFC feeding markedly worsened whole-body insulin glucose homeostasis and inflammation, providing evidence for an important interaction between environmental and dietary signals. Extensive evidence in animal models and humans implicates inflammation as a critical mechanism that is responsible, at least in part, for the

Figure 5. PM2.5 exposure increases ATM infiltration and adipocyte size in C57BL/6 mice fed HFC. A, Immunohistochemistry for macrophage-specific marker F4/80 in sections of epididymal fat pads from FA- and PM2.5-exposed mice. B, Quantification of ATMs in fat pads. F4/80° nuclei were counted and expressed as a percentage of the total nuclei per ×400 power field. Four sections were evaluated from each mouse in each group. C, Adipocyte-size histograms show increased adipocyte hypertrophy in PM2.5-exposed mice. Areas were calculated from 90 adipocytes from each of 5 mice in each group. D, Box plot of adipocyte size. The box represents the upper and lower quartiles. The whiskers or bars show the 5th or 95th percentiles. The line in the box represents the median. The + signs represent the mean. Raw numbers: FA: mean, 883±21 μm²; median, 839 μm²; PM2.5: mean, 973±26 μm²; median, 905 μm².

n=7. *P<0.05 vs FA.
pathophysiological abnormalities noted in response to high-fat diet and/or obesity. PM$_{2.5}$ exposure was associated with increases in systemic TNF-$\alpha$ and IL-6 levels in our study and is entirely consistent with short-term studies of both in vitro and inhalational exposure to PM 2.5 evoking a pronounced pulmonary and systemic inflammatory response. Corresponding human studies have demonstrated an effect of ambient and concentrated particles on inflammation.

A key defect in IR is abnormal insulin signaling through alterations in the insulin receptor substrate-1–phosphatidylinositol 3-kinase (PI3K)/Akt pathway. Reduced PI3K phosphorylation caused by abnormal anti-insulin receptor substrate-1 phosphorylation (increased serine phosphorylation or reduced tyrosine phosphorylation) and reduced phosphorylation of the downstream signaling mediator Akt is implicated as a key defect in IR. We have demonstrated a marked reduction in PI3K-mediated phosphorylation of basal and stimulated Akt in the aortic endothelium. The abnormalities in the PI3K/Akt pathway in the aorta were associated

Figure 6. Exposure to ambient PM$_{2.5}$ increases macrophage infiltration in adipose tissue and induces a shift in macrophage phenotype characteristics in C57BL/6 mice fed HFC. A, Immunofluorescence localization of ATMs (F4/80) in epididymal fat pads from mice exposed to FA or PM$_{2.5}$. Adipocytes identified by caveolin (Cav) staining and nuclei labeled with TOPRO3. Scale bar=100 $\mu$m. B, Real-time polymerase chain reaction measurement of macrophage M1/M2 gene expression. PM$_{2.5}$ treatment resulted in significant increases in the M1 phenotypic genes TNF-$\alpha$ and IL-6 in the F4/80+ cells of stromal vascular fraction (SVF). Nos2 indicates nitric oxide synthase-2; Itgax, integrin $\alpha$X, CD11c; and Ppar$\gamma$, peroxisome proliferator-activated receptor.

Figure 7. Glucose and insulin tolerance tests and YFP cell fluorescence staining of live adipose tissue in c-fms$^{YFP}$ mice fed HFC. A, Blood glucose levels during glucose tolerance test. B, Blood insulin levels during glucose tolerance test. C, Unfixed live adipose tissue from HFC-fed transgenic mice that express yellow fluorescent protein (c-fms$^{YFP}$, yellow) was stained with Hoechst 33342 (blue) and isolectin (red) by confocal microscopy. Isolectin is an endothelium-specific marker. PM$_{2.5}$ treatment resulted in increased YFP cell infiltration into the adipose tissue compared with the saline control. n=4. *$P<0.05$, normal chow vs HFC.

Figure 8. PM$_{2.5}$ exposure increases YFP cell infiltration in mesenteric tissue and YFP cell adhesion in the cremasteric vasculature in c-fms$^{YFP}$ mice fed HFC. A, B, Representative images of YFP cells in mesenteric tissue treated by either saline (A) or PM$_{2.5}$ (B) and the quantification of YFP cells (C). D, Representative image of YFP cells in cremasteric tissue treated by PM$_{2.5}$. The arrows depict YFP cells inside the vessel; closed arrowheads, YFP cells outside the vessel. E, Quantification of adherent YFP cells. The postcapillary venule boundaries are outlined in white lines. n=4. *$P<0.05$, saline vs PM$_{2.5}$. 

Sun et al Air Pollution Exaggerates Insulin Resistance
with markedly diminished dilation to classic endothelium-dependent agonists such as acetylcholine and insulin. This observation led us to hypothesize that the mechanism by which PM2.5 exacerbates IR is enhancement of inflammation in adipose tissue at the level of ATMs. ATMs are derived from circulating monocytes that are recruited across the endothelium in response to obesity and have been shown to be required for the development of IR in mouse models of diet-induced and genetic obesity. Consistent with this hypothesis, we observed that PM2.5 exposure increased systemic levels of inflammatory cytokines, increased adipokine levels, increased the numbers of F4/80+ ATMs, increased markers of classically activated (M1) ATMs, decreased markers of alternatively activated (M2) ATMs, and increased the adhesion of monocytes to adipose tissue vasculature.

In preliminary studies from our group, PM2.5 exposure in lean C57BL/6 mice, at levels and durations comparable to the exposure in this investigation, induced endothelial dysfunction and mild elevations in blood pressure but failed to induce alterations in lipid profile and insulin sensitivity. The metabolic changes in IR and obesity correspond strongly with low-grade inflammation or metaflammation characterized by increased abundance of ATMs. ATMs have been postulated to interfere with adipocyte function by secreting proinflammatory cytokines such as IL-1, IL-6, and TNF-α. We observed an increase in ATMs that was paralleled by an increase in adipose size and translated into a small but significant increase in overall visceral adipose tissue content in the PM2.5 group using highly sensitive magnetic resonance imaging methodologies. These changes raise the question of whether PM2.5 represents yet another factor in the complex relationship between urbanization and obesity and provide a basis for future studies in this area. As has been demonstrated in many epidemiological studies, visceral fat is the most predictive of postprandial glucose levels, indexes of IR, and finally cardiovascular disease in both men and women. Conversely, selective reduction in visceral adipose tissue reduces cardiovascular disease risk through improvements in hypertension, diabetes, IR, and other components of intermediary metabolism, implicating adipose tissue and adipose tissue–derived signals as key mediators in IR.

Our data demonstrate that PM2.5 alters the balance between M1 and M2 ATMs in adipose tissue. M1 macrophages accumulate in obesity and generate inflammatory signals that block insulin signaling in adipocytes. M2 ATMs are present primarily in lean animals and can block the negative effects of TNF-α on adipocytes. The importance of the balance between M1 and M2 ATMs has been emphasized by several recent studies. The M2 macrophage markers IL-10 and Mgl1 were found in our study to be dampened with PM2.5 exposure. In combination with the increase in inflammatory gene expression in adipose tissue, the attenuation of M2 gene expression may play a role in further amplifying inflammation. The balance between M1 and M2 macrophages in end organs appears to be an important aspect of and contributes to the inflammation seen in atherosclerosis and obesity. Thus, inflammatory diseases such as atherosclerosis that have been shown to be strongly associated with PM2.5 exposure may be caused not only by sustained proinflammatory effects but also by failure of antiinflammatory mechanisms. ATM content, adipocyte hypertrophy, and adipocyte death are closely linked, and our model cannot exclude the possibility that PM2.5 interacts directly with adipocytes, leading to secondary changes in ATMs.

Because the change in ATM numbers in visceral fat was paralleled by increases in circulating levels of ICAM and E-selectin, key integrins that play a role in the egress of monocytes from the vascular compartment to the adipose tissue, we investigated the effect of intratracheal PM2.5 on leukocyte trafficking in a novel mouse model expressing YFP under control of a monocyte specific c-fms promoter. In response to PM2.5, there was a marked increase in the number of adherent and rolling YFP cells and eventually infiltrating YFP cells. This further evidence that exposure to PM2.5 directly induces macrophage homing to the adipose tissue. The signals leading to their preferential homing and accumulation in response to environmental signals such as PM2.5 clearly need further investigation.

With the limited sample size in each group, we could not explore the longitudinal effect of exposure to PM2.5 on the health outcomes of interest. Instead, we focused on the differences at each individual time point. To adjust for the inflated type I error in multiple comparison, we used the Bonferroni correction, which is considered to be conservative. Nevertheless, our findings provide a potential biological basis for the link between particulate air pollution exposure and type 2 diabetes mellitus and further our understanding of the mechanisms involved in air pollution–induced cardiovascular diseases. Currently, the NAAQS standards mandate mean annual concentrations of <15.0 μg/m³ and daily concentrations of <35 μg/m³. PM2.5 annual levels seen in cities in Latin America, China, and India average 100 to 150 μg/m³, which are roughly 10- to 15-fold higher than concentrations in the United States and comparable to the levels accomplished in our study. The adjusted average concentration of PM2.5 after accounting for the duration of exposure per day (6 h/d, 5 d/wk) is well below the current NAAQS recommendations of 15 μg/m³ in the United States.

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Disclosures
None.

References
Sun et al.

Air Pollution Exaggerates Insulin Resistance

545


Obesity and type 2 diabetes represent chronic low-grade inflammatory states that have both genetic and environmental determinants. Although the role of diet and exercise as environmental determinants is defined, the interaction between particulate air pollution (PM2.5) and other metabolic determinants of obesity/insulin resistance has not yet been elucidated. In this experiment, we exposed C57BL/6 mice fed high-fat chow to concentrated ambient PM2.5 or filtered air for 24 weeks and demonstrated that PM2.5-exposed mice exhibited marked whole-body insulin resistance, increased visceral adiposity and inflammation, vascular relaxation abnormalities, and enhanced monocyte adhesion to microcirculatory beds. Our findings provide new insights into a potential role for PM2.5 exposure in the pathogenesis of diabetes/insulin resistance and further our understanding of how inhaled particulates may modulate inflammatory responses in distant target organs.
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SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

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 ($10^{-7}$ ~ $10^{-6}$ 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, Burlington, 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).

**Immunohistochemical 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 to 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'-ctaggcaccagggttgatg 3'; beta actin Rev 5'-ctttcacggttgctagt-3'; Hmg14 For 5'-gcagaaaatggagagacggaaacc-3'; Hmg14 Rev 5'-aaggagccggagccactgac-3'; Nos2 For 5'-ccaagccctcacctac-3'; Nos2 Rev 5'-ctctgagggctgacaccaagg-3'; Tnfα For 5'-caacggcatgtctcagac-3'; Tnfα Rev 5'-agatagcaaatcggtgacgg-3'; Itgax For 5'-acgggaaccacagtctgtt-3'; Itgax Rev 5'-gtcagagctggagatgagga-3'; IL10 For 5'-tagagctggagccattcct-3'; IL10 Rev 5'-agtgctctgtattttctgac-3'; Mgl1 For 5'-tggatggaccgctttgagaa-3'; Mgl1 Rev 5'-ggaccacgctgtgtgatgtg-3'; Pparg For 5'-tgaagacattcattcag-3'; Pparg Rev 5'-cacagactggacactgg-3'

Immunoblotting Analysis
Aortic tissue samples were homogenized and lysed 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 I.** Hypothetical schema illustrating potential interactions between particulate air pollution and over nutrition leading to the development of type 2 diabetes mellitus and insulin resistance. The asterisk indicates mechanisms for which evidence has been provided in this paper. M1=Classically activated macrophage phenotype; M2 = Alternatively activated macrophage phenotype.

**Figure II.** 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


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

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<td>Weight, g</td>
<td>$33.3 \pm 1.2$</td>
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<td>Total cholesterol, mg/dl</td>
<td>$138.1 \pm 6.5$</td>
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<td>Triglycerides, mg/dl</td>
<td>$121.5 \pm 12.9$</td>
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<td>HDL, mg/dl</td>
<td>$98.2 \pm 2.8$</td>
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<td>TC/HDL</td>
<td>$1.4 \pm 0.1$</td>
<td>$1.6 \pm 0.1^*$</td>
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<td>Fasting glucose, mg/dl</td>
<td>$139.3 \pm 5.6$</td>
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<td>Fasting insulin, μU/ml</td>
<td>$5.1 \pm 0.4$</td>
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<tr>
<td>Insulin resistance (HOMA)</td>
<td>$1.8 \pm 0.7$</td>
<td>$6.2 \pm 1.6^*$</td>
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HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; HOMA, homeostatic model assessment. *, P <0.05, vs. FA.
Figure II

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<th>PKC-β&lt;sub&gt;II&lt;/sub&gt;</th>
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