

Chronic exposure to air pollution particles increases the risk of obesity and metabolic syndrome: findings from a natural experiment in Beijing

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ABSTRACT: Epidemiologic evidence suggests that air pollution is a risk factor for childhood obesity. Limited experimental data have shown that early-life exposure to ambient particles either increases susceptibility to diet-induced weight gain in adulthood or increases insulin resistance, adiposity, and inflammation. However, no data have directly supported a link between air pollution and non-diet-induced weight increases. In a rodent model, we found that breathing Beijing's highly polluted air resulted in weight gain and cardiorespiratory and metabolic dysfunction. Compared to those exposed to filtered air, pregnant rats exposed to unfiltered Beijing air were significantly heavier at the end of pregnancy. At 8 wk old, the offspring prenatally and postnatally exposed to unfiltered air were significantly heavier than those exposed to filtered air. In both rat dams and their offspring, after continuous exposure to unfiltered air we observed pronounced histologic evidence for both perivascular and peribronchial inflammation in the lungs, increased tissue and systemic oxidative stress, dyslipidemia, and an enhanced proinflammatory status of epididymal fat. Results suggest that TLR2/4-dependent inflammatory activation and lipid oxidation in the lung can spill over systemically, leading to metabolic dysfunction and weight gain.—Wei, Y., Zhang, J., Li, Z., Gow, A., Chung, K. F., Hu, M., Sun, Z., Zeng, L., Zhu, T., Jia, G., Li, X., Duarte, M., Tang, X. Chronic exposure to air pollution particles increases the risk of obesity and metabolic syndrome: findings from a natural experiment in Beijing. *FASEB J.* 30, 000–000 (2016). www.fasebj.org

KEY WORDS: chronic inflammation · metabolic dysfunction · particulate matter

It is increasingly recognized that environmental factors such as air pollution may contribute to the global obesity epidemic. Epidemiologic studies have shown an association between traffic density (a proxy of air pollution

exposure) and body mass index levels at age 18 years in subjects living in Southern California (1) and an association between maternal exposure to polycyclic aromatic hydrocarbons in combustion particles during pregnancy with childhood obesity in children living in New York City (2). Limited animal experimental data support these epidemiologic associations. For example, in a mouse model, prenatal exposure to diesel exhaust particles predisposes the offspring to increased susceptibility to diet-induced weight gain and neuroinflammation in adulthood (3). However, this study did not establish a direct link between particle exposure and weight gain under normal dietary conditions. Similarly, another mouse study did not find a significant effect of early-life particle exposure on body weight in adulthood, although the exposure resulted in increased insulin resistance, adiposity, and inflammation (4, 5). A recent study using diesel exhaust exposure of pregnant

ABBREVIATIONS: CCL2, chemokine (C-C motif) ligand 2; CXCL5, C-X-C motif chemokine 5; GLP-1, glucagon-like peptide 1; GSH, glutathione; HEPA, high-efficiency particulate air; MDA, malondialdehyde; PM_{2.5}, particulate matter with a diameter of ≤ 2.5 μm ; RNA-Seq, second-generation RNA sequencing; qPCR, real-time PCR; TC, total cholesterol; TG, triglyceride

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doi: 10.1096/fj.201500142

This article includes supplemental data. Please visit <http://www.fasebj.org> to obtain this information.

mice showed increased placental inflammation and fetal resorption, along with increased weight in the surviving offspring at 10 wk of age (6). These experimental studies have focused on prenatal or early-life exposures and used an intermittent exposure protocol either with laboratory-resuspended particles or with artificially concentrated ambient particles (in the absence of gaseous copollutants). These exposure protocols may not represent real-life situations of continuous exposure to the air pollution mixture.

Ambient concentrations of PM_{2.5} (particulate matter with a diameter of $\leq 2.5 \mu\text{m}$) in Beijing have in recent years been high enough to reach comparable levels in an exposure chamber (5). Without the need for further artificial concentration, natural ambient air in Beijing was used in the present study to examine whether living naturally in such an environment can lead to weight gain, and if so, what pathophysiologic processes are involved at the organ, tissue, and molecular levels. Our control exposure was the same Beijing air with particulate matter partially removed and without changing gaseous pollutants, as we focused on examining the effects of PM_{2.5} in the context of the real-world air pollution mixture.

MATERIALS AND METHODS

Ethics

All animal experimentations were conducted in compliance with the guidelines of ethical animal research. The study protocol was approved by the Medicine Animal Care and Use Committee at Peking University Health Science Center before commencement of experiments.

Exposure protocol

Two identically sized chambers, each $\sim 1 \text{ m}^3$ in volume ($1.2 \times 0.8 \times 1.2 \text{ m}$), were used in the present study. Each chamber could house a maximum of 9 rat cages (4 on the upper rack and 5 on the lower rack). These chambers were placed side by side for whole body inhalation exposure of rats in an air-conditioned room in Beijing. The only difference between the 2 chambers was the presence or absence of a high-efficiency particulate air (HEPA) filter placed in the inlet duct. The room was about 2 km away from northwestern fourth Ring Road, a major artery of the city with 8 main lanes and 6 auxiliary lanes that carries nearly 220,000 vehicles per day. Particulate matter concentrations were measured using 2 sets of single particle soot photometer and scanning mobility particle sizer and aerodynamic particle size, one for the chamber airstream and the other for ambient atmosphere. We calculated the removal efficiency of every particulate size. The removal efficiency of the HEPA filter was $98.99\% \pm 0.86\%$ for particles larger than $2.5 \mu\text{m}$ in diameter and $70.61\% \pm 19.34\%$ for PM_{2.5}. PM_{2.5} concentrations inside the chamber equipped with the HEPA filter were estimated using outdoor PM_{2.5} concentrations and the filter removal efficiency for PM_{2.5}. The temperature inside these chambers was controlled at $24 \pm 1^\circ\text{C}$.

We started our experiments with pregnant Sprague Dawley rats. All the rats were kept in an animal care facility before the experiments commenced. We conducted the first set of experiments using 30 pregnant Sprague Dawley rats that were 12 wk old. On gestation d 4, rats were randomized into 2 groups: 18 entered the unfiltered chamber, and the remaining 12 entered the filtered chamber. In each chamber, no more than 2 rats were

housed per cage to avoid overcrowding. One group was placed inside a chamber with the inlet air directly coming from outdoors; the other group was housed inside a same-size chamber equipped with a HEPA filter at the air inlet. The chambers were placed side by side inside an air-conditioned room in Beijing. Rats lived in these chambers naturally under a 12 h light/12 h dark cycle and were fed a normal chow diet. The animals were weighed when entering the chamber (d 0) and then every 3 d until d 14, when a series of end points was analyzed. Over this 14-d period, (December 28, 2009, to January 10, 2010), PM_{2.5} concentrations were $73.5 \pm 61.3 \mu\text{g}/\text{m}^3$ in the unfiltered chamber and $19.8 \pm 9.0 \mu\text{g}/\text{m}^3$ in the filtered chamber (means \pm SD of hourly concentrations measured throughout the 14-d period). The SD values indicated large variability in the hourly data. We observed large diurnal and day-to-day fluctuations in ambient PM_{2.5} concentrations.

We found that 7 of 10 rats in the unfiltered air group and 4 of 6 in the filtered air group had produced pups. Among the rest of the pregnant rats not dissected, we found that 6 in the unfiltered group and 6 in the filtered group had delivered pups normally. The pups prenatally exposed to unfiltered air stayed in the unfiltered chamber, and the pups prenatally exposed to filtered air stayed in the filtered chamber continuously until they were analyzed either at 3 or 8 wk of age.

To allow for monitoring body weight for a longer period and for measuring more endpoints in pregnant rats, we conducted a second set of experiments using 16 pregnant Sprague Dawley rats at 12 wk old (10 in the unfiltered air group and 6 in the filtered air group). The experiments started at gestation d 1 and lasted for 19 d. Pups of these rats were not further studied. All the exposure experiments were carried out continuously 24 h/d and 7 d/wk under a 12 h light/12 h dark cycle throughout the 19 d period. During this set of the experiments (March 12 – 31, 2010), PM_{2.5} concentrations were $64.6 \pm 72.7 \mu\text{g}/\text{m}^3$ and $16.7 \pm 9.6 \mu\text{g}/\text{m}^3$ in the unfiltered and filtered chambers, respectively.

Blood and tissue biomarker assays

After euthanasia, the blood was collected in EDTA anticoagulant tubes, then centrifuged at 3000 rpm for 10 min at 4°C . The EDTA plasma was separated and collected. Plasma cholesterol, glucose, oxidative stress biomarkers, and inflammatory cytokines were measured using enzymatic colorimetric assays for malondialdehyde (MDA), glutathione (GSH), total cholesterol (TC), triglyceride (TG), LDL, HDL, glucagon-like peptide 1 (GLP-1), C-X-C motif chemokine 5 (CXCL5), chemokine (C-C motif) ligand 2 (CCL2), TNF- α , IL-10, and IL-6. We ground 30 g each of lung, liver, spleen, and brain in 1 ml PBS on ice and centrifuged the samples at 5000 rpm for 15 min. The clarifying middle layer was carefully aspirated for MDA and 8-isoprostane assay kit analyses.

Lung morphometric analysis

The completely cut-down left lungs were fixed in 10% formalin and embedded in paraffin for making hematoxylin and eosin-stained slides, performing morphometric analysis, and performing further immunohistochemistry using slides. These slides were analyzed by an optical microscope with a digital camera. The right lung tissue samples were immediately frozen in liquid nitrogen for further RNA extraction. Other organs were collected, weighed, and cryopreserved for further analysis.

Second-generation RNA sequencing for gene expression analysis

Second-generation RNA sequencing (RNA-Seq) was used to analyze the genome-wide gene expression profiling in the lungs.

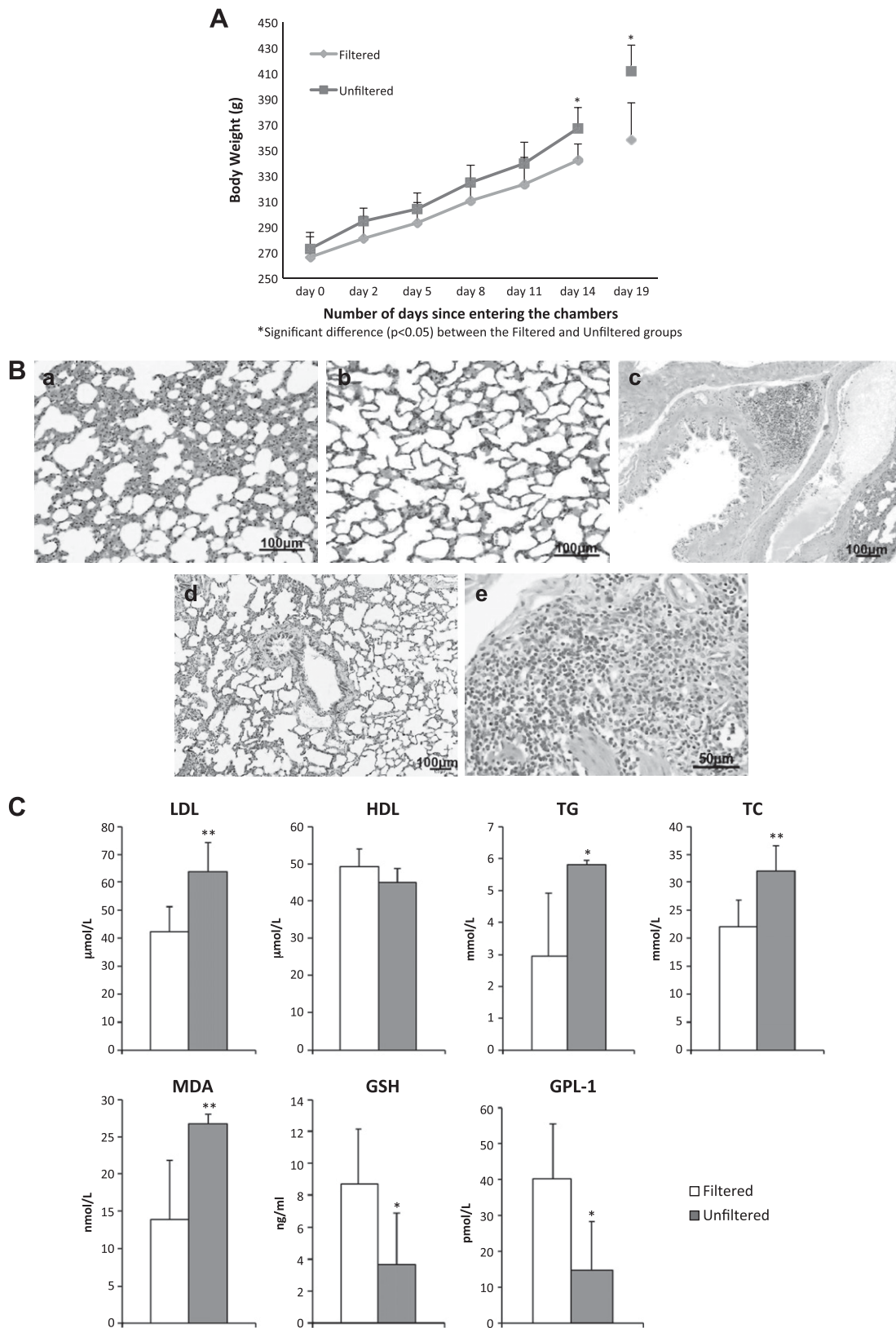


Figure 1. Pregnant rats exposed to unfiltered Beijing air *vs.* those exposed to filtered Beijing air. (A) Body mass measured from d 0, when rats went into chambers, to d 14 (in first set of experiments) and measured at d 19 (in second set of experiments). Error bars represent \pm SD. (B) Lung histology (hematoxylin and eosin stain) showing inflammation in alveoli of rat after 19 d exposure to unfiltered air (a), but no apparent inflammation in alveolus of rat after 19 d filtered air exposure (b). Similar histologic evidence for inflammation in bronchus of unfiltered air exposed rat (c) in reference to that of filtered air exposed rat (d). Higher-magnification micrograph indicates that inflammatory cells are mainly mononuclear cells (e). (C) Blood biomarkers measured in pregnant rats after 19 d exposure protocol. Significant difference between 2 groups denoted as * $P < 0.05$, ** $P < 0.01$.

TABLE 1. Body and organ mass of rats exposed prenatally and continuously for 8 wk to unfiltered or filtered Beijing air

Site	Male					Female				
	Filtered (n = 8)		Unfiltered (n = 6)		P	Filtered (n = 8)		Unfiltered (n = 6)		P
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Body	275	33.6	324	27.6	0.011	194	8.54	214	14.5	0.019
Liver	11.5	0.91	14.2	1.55	0.005	7.72	0.61	9.07	0.83	0.008
Spleen	0.79	0.05	0.88	0.06	0.008	0.49	0.06	0.65	0.10	0.009
Adrenal	0.04	0.00	0.06	0.01	0.002	0.05	0.01	0.06	0.01	0.092
Thymus	0.60	0.08	0.66	0.06	0.112	0.53	0.09	0.53	0.07	0.938
Heart	1.01	0.06	1.18	0.13	0.024	0.73	0.04	0.79	0.05	0.041
Brain	1.41	0.07	1.46	0.04	0.095	1.34	0.07	1.36	0.08	0.569
Lung	0.82	0.09	0.93	0.09	0.035	0.66	0.04	0.74	0.03	0.002
Epididymal fat	1.25	0.23	1.48	0.19	0.064					

RNA-Seq was analyzed by a commercial company (BGI, Beijing, China). Gene expression results are presented as the log₂ ratio of data from the unfiltered air group to the data from the filtered air group. Total RNA was extracted using a combination of Trizol reagent and ethanol precipitation according to manufacturer's instructions (Tiagen Biotech, Beijing, China). The integrity and quantity of RNA in the samples were determined using a spectrophotometer and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Real-time PCR (qPCR) for verification of selected genes

qPCR was used for verifying the significantly up- or down-regulated genes that were identified by RNA-Seq analysis. On the basis of the RNA-Seq results, we selected 30 genes for further qPCR analysis (Supplemental Table S1). The analysis was performed on an Opticon 2 qPCR machine (Bio-Rad, Hercules, CA, USA) using SYBR Green mix (Tiagen Biotech) with the following cycling program: 95°C for 10 min, 40 cycles of 95°C for 25 s, and 60°C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization, and each sample was run in triplicate. The aliquot of cDNA was amplified with a pair of primers for each.

Statistical analysis

SAS 9.1.3 for windows (SAS Institute, Cary, NC, USA) was used to perform data analysis. The data were expressed as means ± SD. Pairwise comparison was conducted by Student's *t* test (2-tailed) with equal or unequal variance depending on *F* test results. A value of *P* < 0.05 was considered statistically significant.

RESULTS

Effects in pregnant rats

At the beginning of the experiments, there was no difference in body weight between pregnant rats to be exposed to unfiltered air and those to be exposed to filtered air. The mean body weight of the unfiltered air group was consistently higher than that of the filtered air group throughout the experiments, reaching statistical significance on d 14 and 19 (Fig. 1A). The unfiltered

air group was 7% heavier on d 14 (*P* = 0.046) and 15% heavier on d 19 (*P* = 0.0041). On d 19, the lungs of the unfiltered group were heavier (by 25%; 1.47 ± 0.19 g vs. 1.18 ± 0.10; *P* = 0.0004) and displayed both perivascular and peribronchial inflammation (Fig. 1Ba–e). We observed thickened alveolar septa and the presence of mononuclear cell infiltration in the airway of the rats in the unfiltered group compared to the lung tissues of rats in the filtered group.

The rats in the unfiltered group also had heavier livers (by 16%; 15.62 ± 1.80 g vs. 13.42 ± 0.71 g; *P* = 0.011). The mass of other organs, including adrenal, thymus, brain, and epididymal fat, was not significantly different. As shown in Fig. 1C, after the 19 d exposure protocol, the unfiltered air group showed a significantly worsened plasma lipid profile compared to the filtered air group: LDL was 51% higher, TC was 97% higher, and TG was 46% higher. Moreover, GLP-1, an incretin hormone that reinforces glucose-dependent secretion of insulin, was significantly lower (by –64%) in the unfiltered air group. GLP-1 is regulated by its degradation, which is accelerated by systemic inflammation and has antiinflammatory properties within adipose tissue (7). This altered metabolic profile is consistent with a loss of insulin-dependent regulation of circulating glucose levels, which can lead to production of reactive oxygen species. There were accordingly a significant reduction (–62%) in plasma GSH (a major antioxidant) and a significant increase (+91%) in

TABLE 2. Biomarkers of male rats exposed prenatally and continuously for 8 wk to unfiltered or filtered Beijing air

Biomarker	Filtered (n = 10)		Unfiltered (n = 10)		P
	Mean	SD	Mean	SD	
LDL (μM)	50.7	8.02	63.0	9.18	0.005
HDL (μM)	58.8	7.84	46.5	3.49	0.001
TG (mM)	2.93	2.07	5.45	1.41	0.006
TC (mM)	23.4	5.39	27.2	2.15	0.058
MDA (nM)	13.4	6.84	25.5	6.05	0.001
GSH (ng/ml)	8.69	3.51	4.69	2.66	0.011
GLP-1 (pM)	30.9	14.0	17.4	12.0	0.033

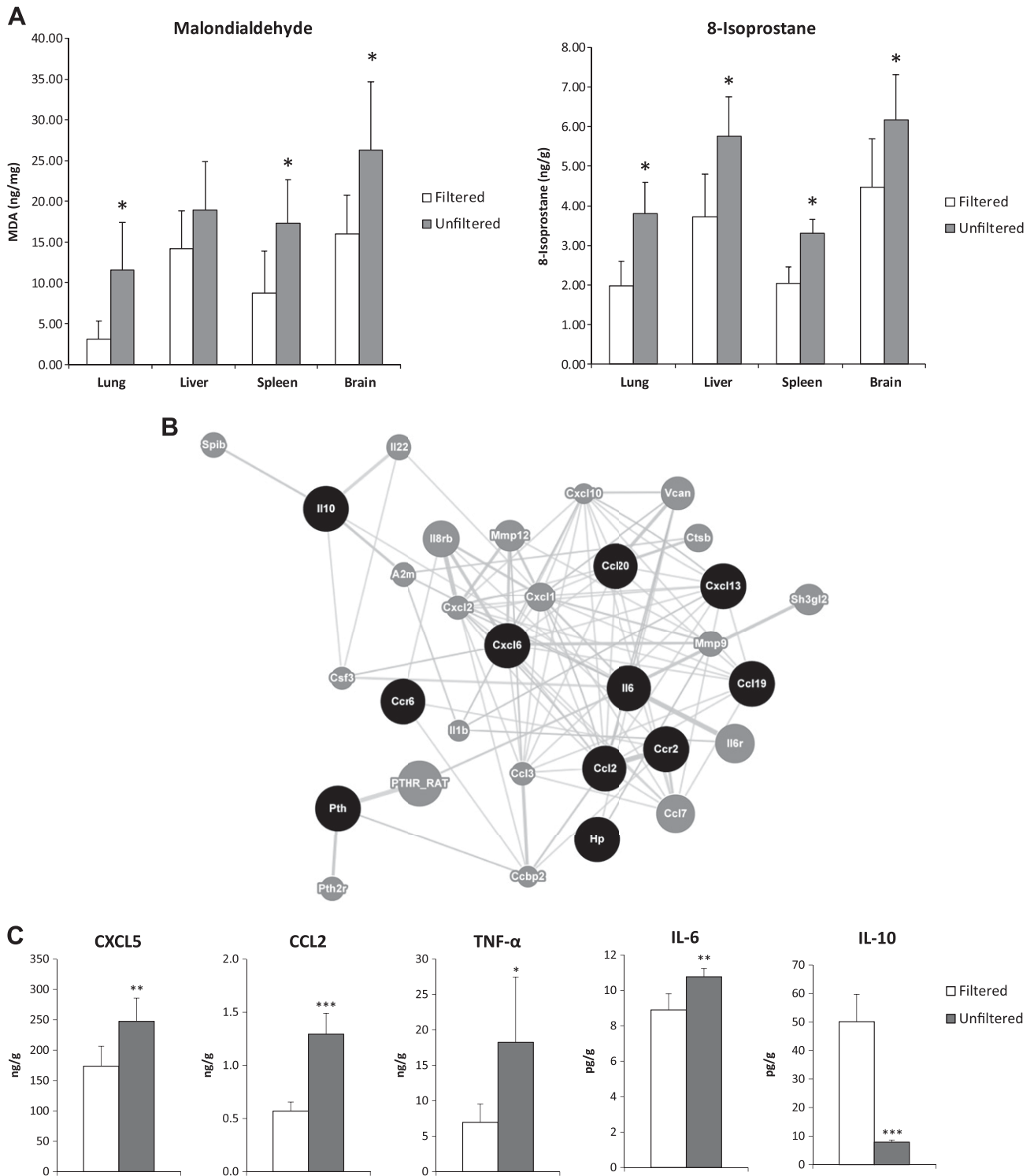


Figure 2. Rats exposed prenatally and continuously for 8 wk to unfiltered Beijing air *vs.* those exposed to filtered Beijing air. (A) Biomarkers of oxidative stress, MDA, and 8-isoprostane, in tissues of lung, liver, spleen, and brain of 8-wk-old male pups. (B) GeneMania network of up-regulated genes involved in cytokine signaling from lung tissue. Black circles indicate query genes identified as up-regulated between groups by RNA-Seq; gray circles, those connected *via* prediction or expression network analysis. (C) Cytokine/chemokine concentrations in epididymal fat tissue of male rats.

plasma MDA in the unfiltered air group. These indicate that unfiltered Beijing air induced a systemic oxidative stress resulting in an increase in oxidized lipids within the circulation, as MDA is a product of reactions between lipids (*e.g.*, n-6-polyunsaturated acids in cell membranes) and reactive oxygen species such as peroxides (8).

Effects in rat offspring

We continued our experiments with the offspring of the Sprague Dawley rats that had undergone the 14 d exposure protocol. The pups were placed into the same chambers where their mothers had lived and

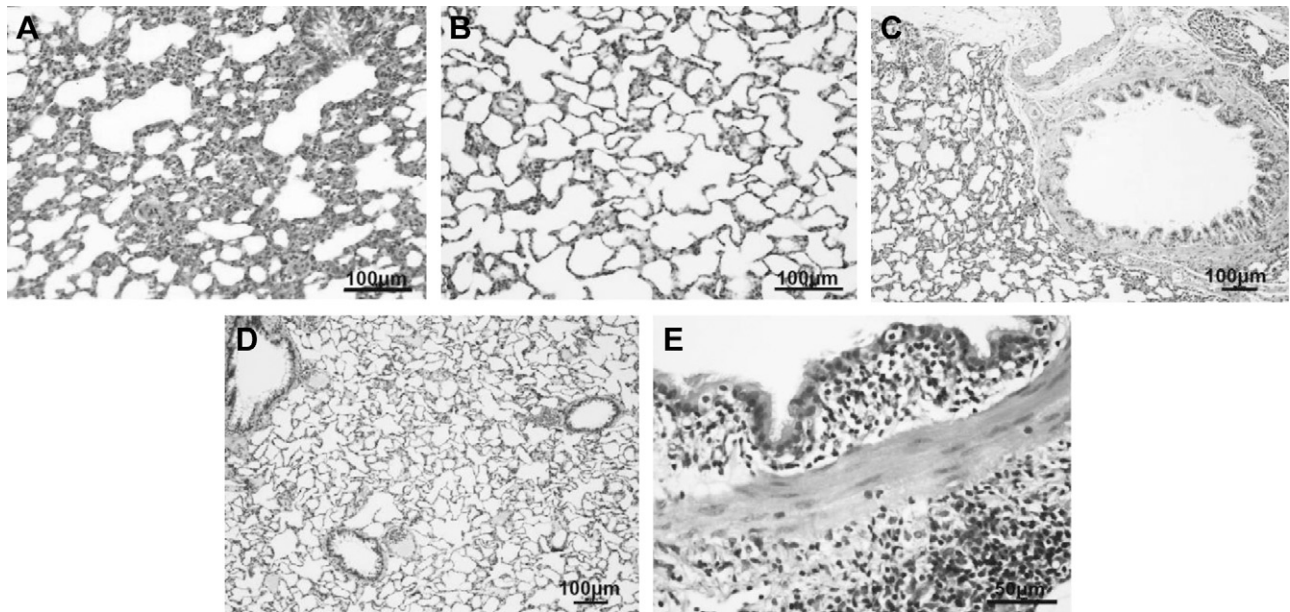


Figure 3. Lung histology (hematoxylin and eosin stain) of 8-wk-old rats, showing inflammation in alveoli of rat exposed to unfiltered air (A) compared to alveoli of rat exposed to filtered air exposure (B). Similar histologic evidence of inflammation in bronchus of unfiltered air exposed rats (C) in reference to that of filtered air exposed rats (D). Higher-magnification micrograph indicates that inflammatory cells are mainly mononuclear cells (E).

continuously lived there until humanely killed for analyses. At 8 wk old, both female and male rats in the unfiltered air group had significantly greater body mass (by 10 and 18%, respectively), lung mass (by 12 and 13%, respectively), and mass for other organs (liver, spleen, and heart) compared to the filtered air group (Table 1). Blood markers showed significant differences between the 2 groups, indicating that exposure to unfiltered air resulted in a worsened lipid profile (+24% LDL, -21% HDL, +86% TG, +16% TC), reduced incretin levels (-44% GLP-1), reduced antioxidant capacity (-46% GSH), and increased oxidative stress (+90% MDA) (Table 2). Furthermore, we observed significantly higher concentrations of MDA and 8-isoprostane in tissue samples of lung, liver, spleen, and brain in the unfiltered air group (Fig. 2A).

Similar to that for the rat dams, lung histology revealed both peribronchial and perivascular inflammation in these rat offspring (Fig. 3). When we conducted RNA-Seq analysis of lung tissue from both dams and pups comparing the unfiltered group to the filtered group, we found gene expression changes for over 100 genes, some of which were further confirmed for their expression changes using qPCR assays (Supplemental Table S1). Using the DAVID bioinformatic database revealed a focus of these up-regulated genes upon inflammatory processes and glucocorticoid response elements (9). A further analysis of the up-regulated genes, using the online network tool GeneMANIA (10), revealed a proinflammatory network focused on acute inflammatory signaling and classic macrophage activation, namely IL-6, CCL-2, CXCL-6, and CCL-19 (Fig. 2B). In this context, it is important to note that classic macrophage activation has been suggested to play a role in metabolic responses to inhaled pollutants (11).

In association with lung inflammation, we also found evidence of systemic inflammation in the epididymal fat, as indicated by the significantly raised concentrations of 4 proinflammatory chemokines and cytokines (CXCL5, CCL2, IL-6, and TNF- α) and significantly lower concentrations of the antiinflammatory cytokine (IL-10) in the unfiltered air group (Fig. 2C). We particularly observed a >6-fold reduction in IL-10 and >2-fold increases in TNF- α and CCL2, respectively. The presence of worsened metabolic profile and adipose inflammation led us to suspect that adipose tissue metabolism may be altered. We found that the mass of epididymal fat was 18% greater in the male rats exposed to unfiltered air than in those exposed to filtered air (Table 1). This indicates that the chronic exposure disrupted the balance between the proinflammatory and antiinflammatory functions, priming the adipose tissue toward inflammatory status that may be linked to metabolic dysfunction.

DISCUSSION

The explanation of how airborne particles (or an air pollution mixture) may cause metabolic dysfunction is important in understanding the role of air pollution in cardiometabolic diseases and underlying biologic mechanisms of particle health effects. In the present study, we examined whether and how inhaled particles could lead to non-diet-induced weight gain through one or more biologic pathways. We used a real-life air pollution exposure protocol, pathologic assessment, biochemical analysis, and gene expression assays. We also examined molecular processes involved in the pathophysiologic pathways by measuring RNA and protein biomarkers.

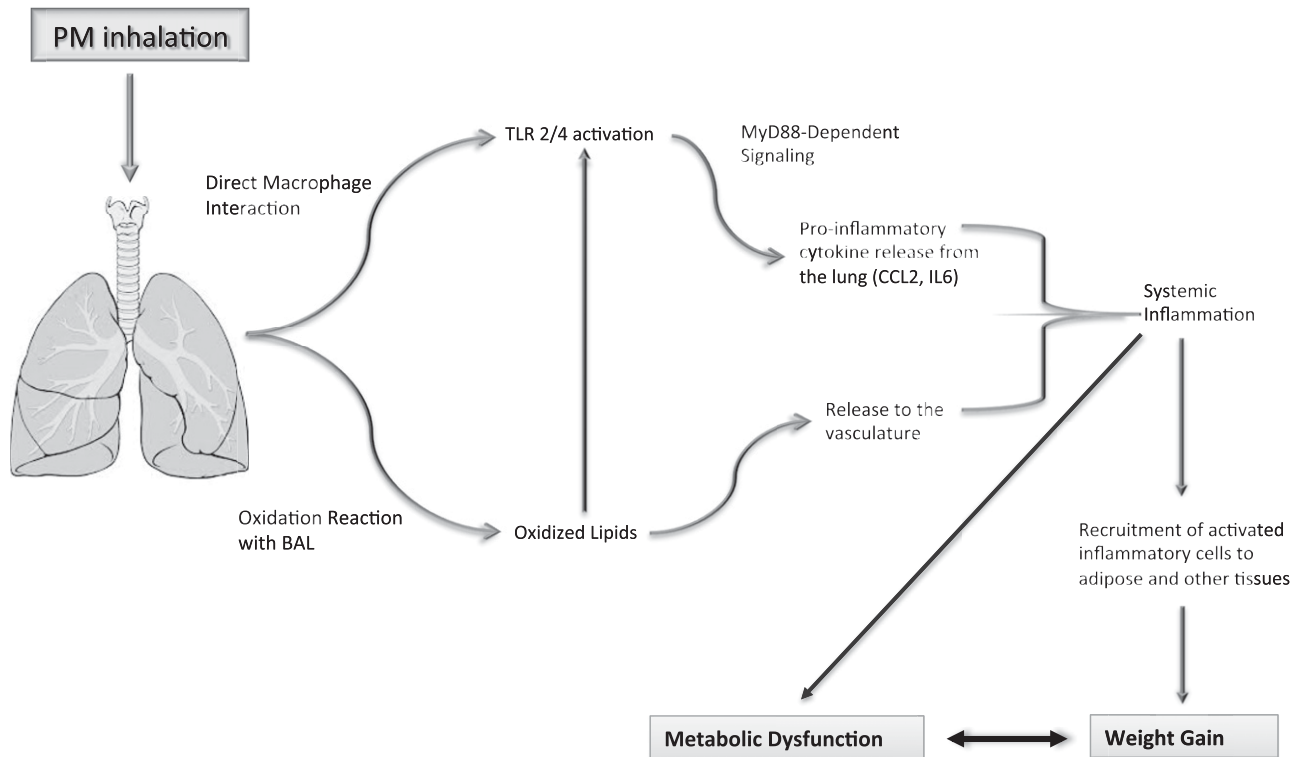


Figure 4. Mechanistic framework explaining how inhaled air pollutants disrupt metabolic state. Inhalation of air pollutants (especially particulate matter) can lead to direct activation of alveolar macrophages through TLR2/4-dependent mechanisms and generation of oxidized lipids within lung lining. These oxidized lipids can further activate inflammatory processes through TLR2/4 binding or be released to vasculature, where they will initiate systemic inflammation and oxidative stress responses. TLR2/4-dependent inflammatory activation, through activity of MyD88, will lead to release of proinflammatory cytokines, such as CCL-2 and IL-6, from lung, also generating systemic inflammation. These systemic inflammatory activation processes, along with loss of antiinflammatory functions from incretins such as GLP-1, will lead to increased recruitment of activated inflammatory cells to tissues and in particular adipose. Recruitment of such cells to adipose will worsen metabolic profile, leading to weight gain and metabolic disease state. Prenatal exposure may enhance cellular and functional responses shown here.

In both rat dams and their 8-wk-old offspring, we observed histologic evidence for perivascular and peribronchial inflammation in the lungs, increased concentration of MDA in the tissue, increased MDA and 8-isoprostane, and decreased concentration of GSH in the blood. The findings of the present study are consistent with previous studies reporting that $PM_{2.5}$ exposure induces oxidative stress and inflammation in organ tissues and the circulatory system, as summarized in a statement of American Heart Association (12).

The findings on epididymal fat mass and plasma incretin also augment to the emerging evidence that chronic exposure to $PM_{2.5}$ leads to increased lipid deposition in adipose tissue (13) and increased insulin resistance (14). Because chronic inflammation is increasingly recognized as part of the etiology of obesity, and because metabolic diseases and obesity are closely related (15, 16), our findings consistently provide evidence that chronic exposure to a real-world air pollution mixture containing high-level of $PM_{2.5}$ increases the risk for developing obesity.

Previous studies have shown that administration of $TNF-\alpha$ led to an increase in serum triglycerides and very-low-density lipoproteins in rats and humans (17) and inhibited insulin-stimulated glucose transport (18).

CXCL5 is produced concomitantly with IL-8 in response to stimulation with $TNF-\alpha$ and mediates the effects of $TNF-\alpha$ in insulin resistance (19, 20). Chronically elevated IL-6 levels have been associated with the development of insulin resistance (19). In contrast, levels of IL-10, an antiinflammatory cytokine that attenuates the inflammatory processes induced by $TNF-\alpha$, IL-6, and IL-1, have been adversely correlated with body mass index, fat mass, and fasting glucose levels (21, 22). Low levels of IL-10 in adipose tissues have been associated with both metabolic syndrome and type 2 diabetes (23).

In stark contrast to the above findings in the offspring after the 8 wk exposure protocol, we did not observe significant differences in body and organ mass and blood biomarkers (apart from HDL) between the 2 exposure groups in offspring that had only lived in the chamber for 3 wk (Supplemental Table S2). Lung histology results were also similar, showing no significant inflammation in both groups (Supplemental Fig. S1). This suggests that longer-term exposure may be needed to generate continuous inflammatory and metabolic changes that ultimately increase body weight.

Our exposure model used unfiltered and filtered Beijing air. The filtration we used in our study resulted in $PM_{2.5}$

concentrations comparable to ambient concentrations measured in typical urban/suburban areas in and below the United States. 24 h PM_{2.5} standard of 35 µg/m³. The filtration only removed the particulate component of the air pollution mixture. Therefore, the health effects we observed, more precisely speaking, reflect particulate effects in the presence of gaseous copollutants. Moreover, it is known that HEPA filters are more efficient in removing larger particles than smaller particles. In the present study, we observed a removal efficiency of 99% for PM larger than 2.5 µm and of 71% for PM_{2.5}. Within the PM_{2.5}, we did not measure ultrafine particles (PM_{0.1}), but we expect even lower removal efficiency for PM_{0.1}. Therefore, this study is limited in terms of further elucidating the role of specific role of ultrafine particles and PM_{2.5} within the context of the air pollution mixture.

Nevertheless, using the comprehensive data from our experiments in combination with previously postulated mechanisms (12), we developed a mechanistic framework, as shown in **Fig. 4**, for future hypothesis testing. In a rat model, we found that exposure to ambient air containing high concentrations of PM_{2.5} induced TLR2/4-dependent inflammatory activation and lipid oxidation in the lung, which then spilled over systemically, leading to metabolic dysfunction and weight gain. The study provides mechanistic evidence that chronic exposure to air pollution increases the risk for developing obesity and metabolic syndrome. If translated to and verified in humans, these findings will add support to the urgent need to reduce air pollution exposure, given the growing burden of obesity and related metabolic abnormalities in today's highly polluted world. FJ

This work was supported in part by grants from the National Natural Science Foundation of China (21190051, 21477119), the Open Fund of the State Key Joint Laboratory of Environment Simulation and Pollution Control (13K03ESPCP), and the China Postdoctoral Science Foundation (201003010).

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Received for publication December 15, 2015.
Accepted for publication January 31, 2016.