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Inhalation Exposure to Carbon Black Induces Inflammatory Response in Rats

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Background A link between exposure to fine particulate matter and cardiovascular events has been established. Inhaled nanoparticles are thought to pass through the lungs to reach other tissues via systemic circulation and to induce cell or tissue injuries. It was recently shown that long-term exposure to intra-tracheal dispersion of nano-sized carbon black (CB) exacerbates atherosclerotic lesions in low-density lipoprotein receptor-deficient mice. Because intra-tracheal dispersion of CB may be associated with aggregate formation and may not be an ideal method for CB exposure, whole-body inhalation exposure was used in the present study, the aim of which was to examine whether exposure of rats to nano-sized CB particles by inhalation leads to translocation of these particles into the circulation, exerting direct adverse effects on extrapulmonary tissues.

Methods and Results Sprague-Dawley rats were exposed to a high dose of CB or filtered air for 6 h/day, 5 days a week for a total of 4 weeks. Although the presence of CB was confirmed in pulmonary macrophages, electron microscopic survey did not detect CB in other tissues including liver, spleen and aorta. CB exposure raised blood pressure levels in an exposure-time dependent manner. Levels of circulating inflammatory marker proteins, including monocyte chemoattractant protein-1, interleukin-6, and C-reactive protein, were higher in the CB-treated group than in the controls.

Conclusion Evidence of translocation of inhaled CB was not obtained. It is likely that inhaled nano-sized CB particles form aggregations in the lung and do not exert direct adverse effects on extrapulmonary tissues. Air-pollution-mediated cardiovascular events appear to be induced by the low-grade inflammatory response to the accumulation of aggregated nano-sized particles in the lung. (*Circ J* 2008; **72:** 144 – 149)

Key Words: Atherosclerosis; Inflammation; Nanoparticles

Exposure to particulate matter air pollution has been reported to be associated with death and hospitalization from cardiovascular causes[1,2] The mechanism by which long-term exposure to fine particulate matter increases the risk of cardiovascular disease remains uncertain. Accelerated atherosclerosis and vulnerability to plaque rupture have been documented in experimental animal models exposed to particulate matter[3,4] and ambient pollution has been correlated with elevated blood pressure (BP)[5–8] and heart rate (HR)[9] in humans. Moreover, the duration of exhaust exposure in highway toll collectors has been shown to be associated with carotid intima – media thickening![10]

We recently showed that a 10-week intra-tracheal dispersion of carbon black (CB) induced atherosclerosis in low-density lipoprotein receptor knock-out (LDLR/KO) mice and the explanation appeared to be the inflammatory responses against deposited CB in the lungs![11] It has been postulated that inhalation of fine particulate matter might cause inflammation in the lung, and that this low-grade and prolonged inflammation might accelerate atherothrombotic diseases![12,13]

On the other hand, a more direct role of nanomaterials has been proposed, based on the study by Nemmar et al that showed rapid translocation of inhaled nano-sized carbon particles into the bloodstream of humans![14] Our in-vitro studies also suggested that nano-sized particulate matter might penetrate alveoli into circulating blood, and might directly damage endothelial cells, activate mononuclear cells, and aggregate platelets to accelerate the formation of atherothrombotic diseases![15–17] In our previous analysis of the effect of CB exposure in LDLR/KO mice![11] CB was not detected in tissues other than the lungs. Thus, it is unlikely that dispersed CB translocates into the circulating blood and directly damages target tissues or cells. However, the method of CB exposure in the previous study was intra-tracheal dispersion, which might not mimic the physiological responses elicited by the common route of exposure in humans. It is possible that CB particles administered by intra-tracheal dispersion may more easily aggregate and deposit in alveolar regions than CB particles dispersed in the respiratory air. With whole-body inhalation exposure to nano-sized CB particles, CB might translocate into circulating blood and reach target tissues. To assess this possibility, we examined whether exposure of rats to nano-sized CB particles by inhalation causes translocation of CB particles into the circulation and increases cardiovascular risk by exerting direct adverse effects on extrapulmonary tissues.

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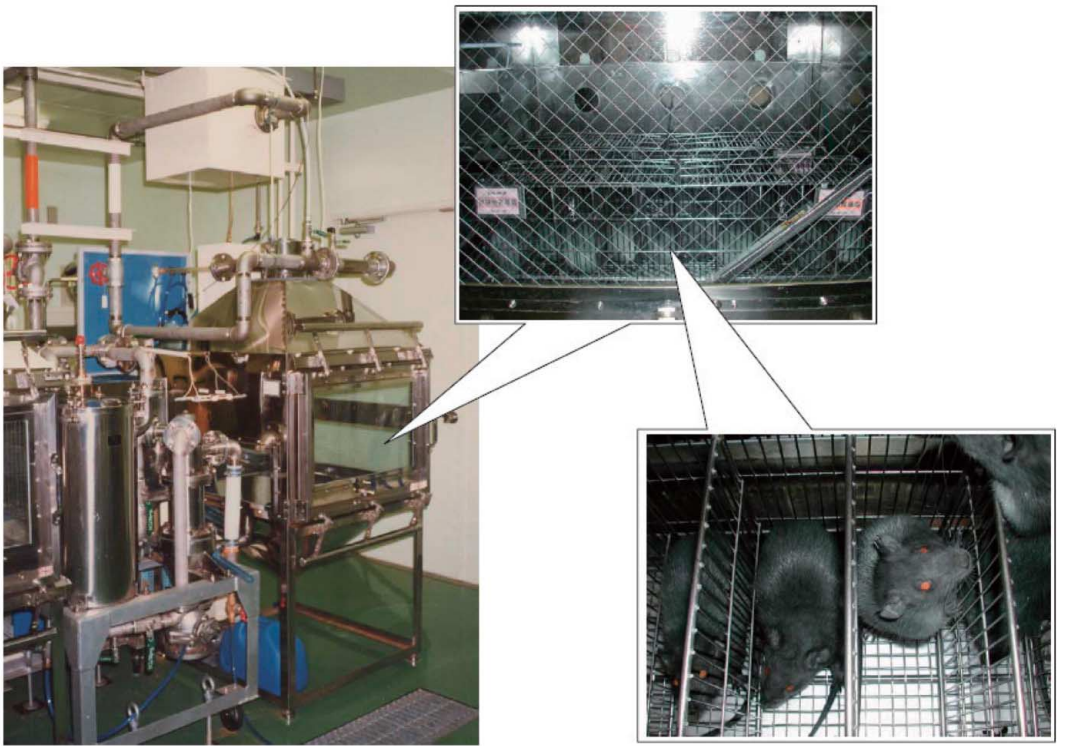

Fig 1. Facility and inhalation chamber used for whole-body inhalation exposure at the facility of the Chemicals Evaluation and Research Institute (CERI, Oita, Japan) using carbon black (CB, Association of Powder Process Industry and Engineering, Kyoto, Japan) generated by a versatile aerosol concentration enrichment system (Dust feeder, Model DF-5, Shibata Kagaku, Tokyo, Japan). Rats were exposed to CB in the inhalation chamber for 6 h/day, 5 days per week for a total of 4 weeks.

Methods

Animal Model

Sprague-Dawley (SD) rats aged 6 weeks (n=50) were obtained from Japan Charles River (Shiga, Japan) and randomly divided into the 2 groups: CB-treated group (n=25) or filtered air-exposed control group (n=25). Rats were housed individually in cages under controlled environment (23°C and 12-h light–dark cycle) with free access to normal chow and tap water. At 1, 7, 14, 28 and 30 days after exposure, 5 rats from each group were killed. They were anesthetized with a high dose of pentobarbital, and then the blood was collected directly from the abdominal aorta, immediately transferred to a tube containing EDTA-2Na or 3.8% sodium citrate and gently mixed. After centrifugation at 1,710 G for 5 min, the supernatant was transferred to a new tube and stored at –80°C until used. The liver, lungs, aorta and spleen were removed and weighed. BP and HR were measured by tail-cuff plethysmography (BP-98A, Softron, Tokyo, Japan) at 1, 14 and 28 days after CB exposure. Mean of 3 measurements was calculated for each rat. Blood samples collected on day 28 or day 30 were used for biochemical analysis. The study protocol was approved by the institutional ethics committee on animal research, and all animal experiments were performed in accordance with the institutional ethical guidelines for experiments with animals.

Exposure to CB

Fig 1 shows the facility and inhalation chamber at the Chemicals Evaluation and Research Institute (CERI, Oita, Japan) where inhalation exposure was carried out. CB (Association of Powder Process Industry and Engineering, Kyoto, Japan) was generated using a versatile aerosol concentration enrichment system (Dust feeder, Model DF-5, Shibata Kagaku, Tokyo, Japan). Rats were exposed to CB in the inhalation chamber at nominal concentrations of 15.6± 3.5 mg/m^3 (1.57±0.4×10^{10} particle number/m^3) for 6 h/day, 5 days per week for a total of 4 weeks. The control rats were exposed to clean, filtered air containing no CB for the same period. The concentrations of CB were monitored twice weekly by measuring the gross weight with a PTFE binder glass filter (TX40HI-20WW, Pall Corporation, NY, USA). Particle diameter of CB was measured by a Particle Size Analyzer (UPA-EX150, Nikkiso, Tokyo, Japan). Mean size (nm) ± SD determined at 1, 8, 15, 22 and 29 days after exposure was 118.1±2.4, 119.1±2.7, 122.2±2.0, 122.4±2.5, and 121.9±3.6, respectively. Concentration of CB particles below 100 nm was approximately 3% and 40% of the nominal concentrations of CB particles by weight and by number, respectively.

Biochemical Analysis

Levels of monocyte chemoattractant protein-1 (MCP-1; Pierce Biotechnology Inc, IL, USA), interleukin-6 (IL-6; Pierce Biotechnology Inc), C-reactive protein (CRP; Life diagnostics Inc, PA, USA), and 8-hydroxy-2'-deoxyguanosine (8-OHdG; Japan Institute for the Control of Aging Nikken SEIL Corporation, Shizuoka, Japan) were measured by ELISA according to the manufacturers' protocols. Blood samples collected on day 28 were analyzed for the number of blood cells (red blood cells, white blood cells and platelets) by an automated hematology analyzer (model KX-21NV, Sysmex Corporation, Kobe, Japan).

Table 1 Comparisons of the Hematological and Biochemical Parameters of the CB and Control Groups

	CB (n=5)	*Control (n=5)*	*p value*
Red blood cells (×10⁴/μl)	*727.6±12.7*	*713.6±38.6*	*NS*
White blood cells (×10²/μl)	*55.8±10.3*	*67.2±20.8*	*NS*
Platelets (×10⁴/μl)	*105.1±10.8*	*98.8±5.9*	*NS*
Creatine kinase (U/L)	*531.4±130.9*	*871.6±412.1*	*NS*
Creatinine (mg/dl)	*0.196±0.053*	*0.236±0.023*	*NS*
AST (IU/L)	*109.4±52.5*	*98.2±38.7*	*NS*
ALT (IU/L)	*30.4±7.2*	*35.6±9.9*	*NS*
Serum albumin (g/dl)	*3.7±0.5*	*3.7±0.3*	*NS*
8-OHdG (ng/ml)	*7.0±3.1*	*4.6±2.4*	*NS*
IL-6 (pg/ml)	*34.4±27.0*	*1.6±2.8*	*0.05*
CRP (μg/ml)	*88.4±21.2*	*28.9±6.0*	*0.0003*
MCP-1 (pg/ml)	*2,351.3±250.3*	*1,105.7±426.8*	*0.0024*

Blood samples collected on day 30 were used for the measurement of creatine kinase, creatinine, AST, ALT and albumin, whereas red blood cells, white blood cells, platelets, 8-OHdG, IL-6, CRP and MCP-1 were determined using blood samples collected on day 28. Differences between the groups were examined by unpaired Student's t-test.
CB, carbon black; AST, aspartate aminotransferase; ALT, alanine aminotransferase; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; IL-6, interleukin-6; CRP, C-reactive protein; MCP-1, monocyte chemoattractant protein-1.

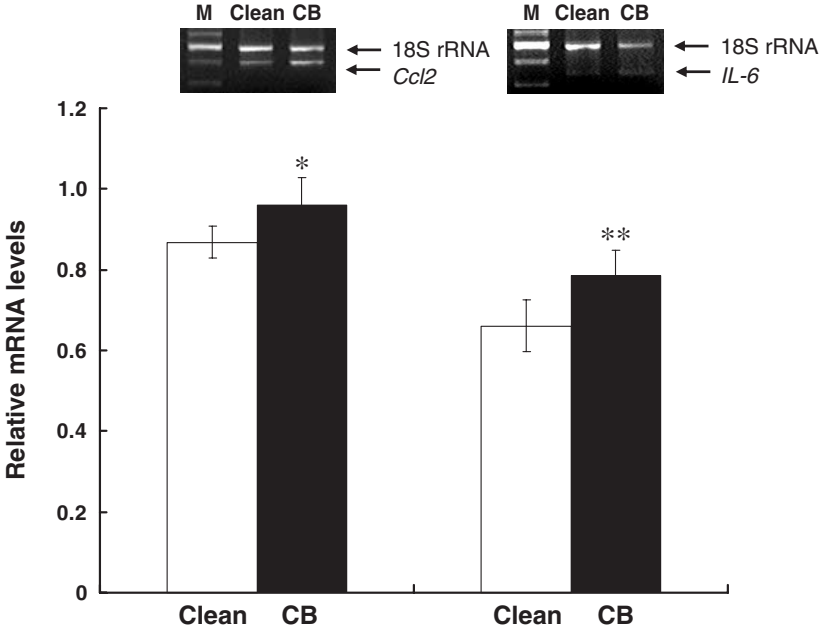

Fig 2. Expression levels of *Ccl2* and interleukin-6 (*IL-6*) mRNA in the lungs of carbon black (CB)-treated (n=4) and filtered-air treated (n=5) rats were compared by competitive RT-PCR with the use of 18S ribosomal RNA as an internal standard. The size of the PCR products for *Ccl2*, *IL-6* and 18S RNA was 381 bp, 346 bp, and 489 bp, respectively. Non-parametric tests revealed a statistically significant difference in the *Ccl2* and *IL-6* mRNA expression levels between the 2 groups; *p=0.05 and **p=0.0143. Representative PCR products are shown with a 100-bp DNA marker (M). Vertical bars indicate standard deviation.

Histology and Electron Microscopy

Lungs removed after 28 days of exposure were fixed in 4% paraformaldehyde-buffered solution (pH 7.4) overnight at 4°C and then placed in PBS-buffered solution (pH 7.4). The tissues were dissected from the left lobes between the lung bronchioles and alveolar lung, and the small tissues were embedded in paraffin. Sections of 1-μm thickness were stained with hematoxylin–eosin, Giemsa, and elastica van Gieson.

For electron microscopy, the lungs, liver, spleen and aorta from rats exposed for 14 days were used. After fixation for 1 h at 4°C in 0.1 mol/L sodium cacodylate buffer (pH 7.4) containing 2.0% glutaraldehyde, the tissues (lung, liver, spleen and aorta) were subjected to overnight post-fixation in 0.1 mol/L sodium cacodylate-buffer (pH7.4) with 1.0% osmium tetroxide at 4°C. After dehydration in an ethanol gradient (50–100% each for 10 min), samples were embedded in EPON812 at 60°C for 2 days. Ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate. The sections were examined with an electron microscope (JEM2000X, JEOL Ltd, Tokyo, Japan) at 100 kV.

Analysis of mRNA Expression Levels of IL-6 and Ccl2 by Semi-Quantitative Competitive RT-PCR

Total RNA was extracted from the lung tissues of CB-treated (n=4) and air-treated rats (n=5) using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instruction. Five micrograms of extracted total RNA were reverse transcribed using SuperScript II (Invitrogen, San Diego, CA, USA) and random primer (Takara-Bio, Shiga, Japan). No PCR product was detected when the reactions were carried out in the absence of reverse transcriptase. Levels of *IL-6* and *Ccl2* mRNA expression in the lung tissues were quantified using 18S ribosomal RNA as an internal standard (QuantumRNA 18S Internal Standards Kit, Ambion Inc, TX, USA) as previously described[18] The ratios of the 18S primers to 18S competimers were 1.5:8.5 for *Ccl2* and 1:9 for IL-6, respectively. Competitive PCR reactions were performed for each cDNA using the following sense and anti-sense primers; 5'-GAGTTCCGTTTCTACCTGGAGTTT-3' and 5'-CAGGATATATTTTCTGACCACAGTGAG-3' for *IL-6*, and 5'-CAGGTCTCTGTCACGCTTCTG-3' and 5'-GTGGAAAAGAGAGTGGATGCAT-3' for

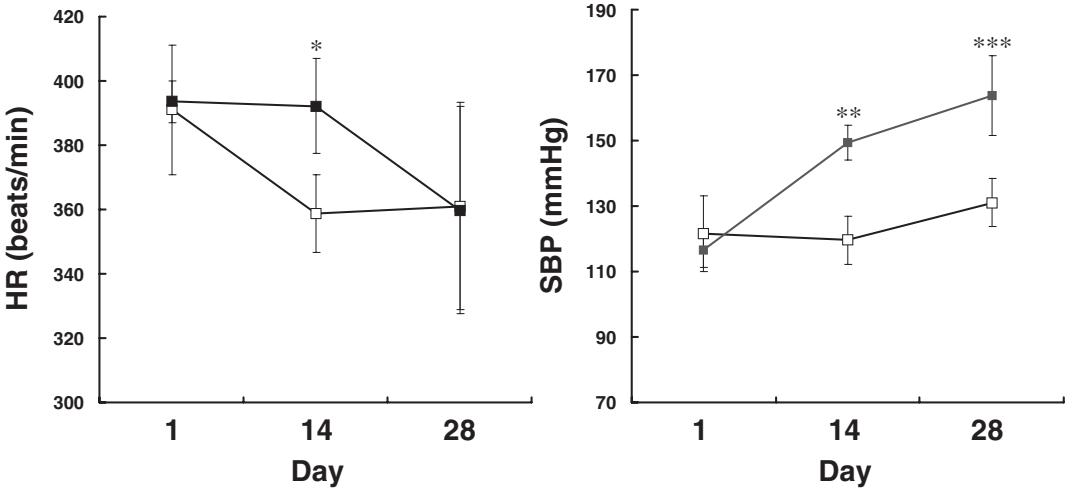

Fig 3. Effect of whole-body inhalation exposure of carbon black (CB) on heart rate (HR) and blood pressure (BP), which were measured by tail-cuff plethysmography after 1, 14, and 28 days of exposure. Each point represents a group of 3 rats. (Open squares) Control group (n=3), (filled squares) CB-treated group (n=3). Although HR measured on days 1 and 28 did not differ between the control and CB groups, HR on day 14 was significantly elevated in the CB group compared with the control group. In rats exposed to CB, levels of systolic BP (SBP) increased in an exposure-time dependent manner. Asterisks denote significant differences between the groups: *p=0.0383, **p=0.0046, and ***p=0.0164. Vertical bars indicate standard deviation.

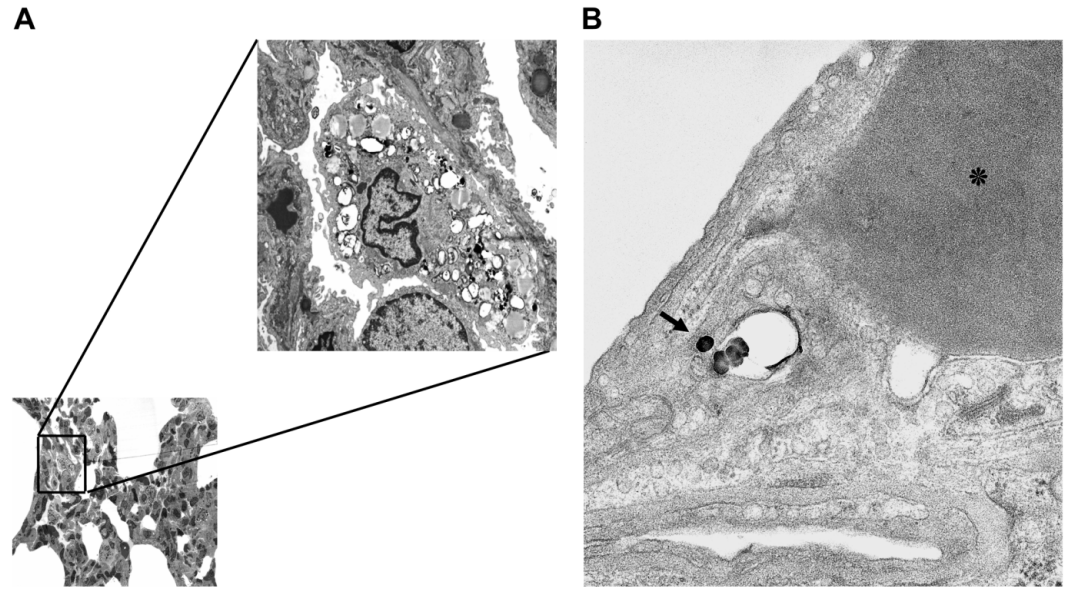

Fig 4. Representative electron micrograph of alveolar tissue in rats exposed to carbon black (CB) for 14 days shows CB in macrophage-like cells (A, ×2,500). CB is also seen in endothelial cells of a blood capillary (B, ×10,000). Arrow indicates a CB compartment in an autophagic-like structure in the endothelial cell. *Red blood cells.

Ccl2. The intensities of the PCR products from the *Ccl2* (381 bp) and *IL-6* (346 bp) mRNA relative to those from 18S RNA (489 bp) were assessed by densitometry. Results are expressed as arbitrary units.

Statistical Analysis

Results are expressed as mean ± SD. Differences in hematological and biochemical parameters between the CB-treated and filtered air-treated control groups were examined by 2-tailed unpaired Student's t-test. Differences in BP and HR measured at day 1, 14 and 28 (n=3 for each group at each point) were compared between the CB and control groups using a 2-tailed unpaired Student's t-test. Because of the non-normal distribution and semi-quantitative nature of RT-PCR, the non-parametric Wilcoxon/Kruskal-Wallis test was used for comparisons of the mRNA expression levels between the CB-treated and filtered air-treated control groups. Statistical analysis was performed using the JMP statistical package 6.0 (SAS Institute, Cary, NC, USA). P-values <0.05 were considered statistically significant.

Results

There were no differences between the CB and control groups in baseline body weight, and the time-course increase in body weight was not different between the 2 groups (data

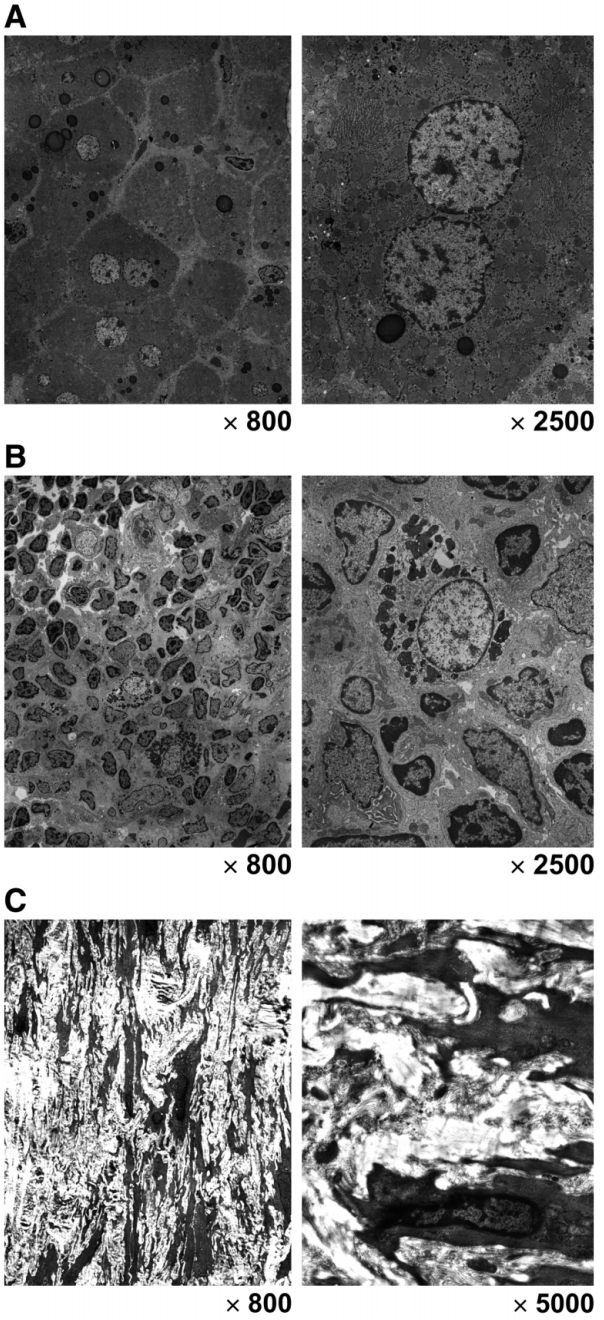

Fig 5. Representative electron micrographs of liver, spleen and aortic endothelial cells of carbon black (CB)-exposed rats. CB particles are not seen in (A) the liver sections (Left, ×800; Right, ×2,500), (B) the spleen (Left, ×800; Right, ×2,500) or (C) the endothelial cells (Left, ×800; Right, ×5,000).

not shown). Hematological and biochemical parameters measured on day 28 or 30 day were compared between the CB and control groups (Table 1). No significant differences were observed between the groups for the number of red blood cells, white blood cells or platelets. Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), often used as markers of liver injury, did not differ between the groups. There was no effect of CB inhalation on serum albumin, creatinine, creatine kinase, and 8-OHdG levels. Circulating levels of MCP-1 and CRP were significantly elevated in rats exposed to CB for 4 weeks ($p=0.0024$ and $p=0.0003$, respectively). IL-6 tended to be increased in the CB group compared with the control group ($p=0.05$). In accordance with this observation, higher expression levels of *IL-6* and *Ccl2* mRNA were observed in the lungs of rats exposed to CB for 28 days than in those of controls (Fig 2).

Although there was no difference between the control and CB groups in HR measured on days 1 and 28, HR was significantly elevated in the CB group compared with the control group after 14 days of inhalation of CB (Fig 3). CB exposure significantly raised systolic BP (SBP) levels, with the CB group having a significantly higher SBP on days 14 and 28 compared with controls (Fig 3).

Electron microscopic examinations revealed the presence of CB in macrophage-like cells and endothelial cells in blood capillaries deep in the lungs (Figs 4A,B). Most of the CB particles were located in phagosome-like structures in the cytoplasm of alveolar macrophages. CB was not detected in the liver, spleen or endothelial cells from the abdominal aorta (Fig 5). Examination of pulmonary tissues stained with hematoxylin–eosin, Giemsa, and elastica van Gieson showed no difference in the staining pattern between the CB and control groups, with no signs of alveolar destruction or fibrosis in the CB group (data not shown).

Discussion

One of the purposes of the present study was to clarify whether whole-body exposure of CB might result in translocation of the particles into the systemic circulation, eventually reaching the extrapulmonary tissues. Although it has been previously reported that nanoparticles are able to penetrate into the deep lung areas and pass through to reach the systemic circulation,[14] we did not observe any CB signals in extrapulmonary tissues, including the liver, spleen and aorta, by electron-microscopic examination. This is in agreement with our recent report showing the absence of CB particles in the liver, aorta, kidney and spleens of LDLR/KO mice exposed to CB for 10 weeks by intra-tracheal administration.[11]

In the present study, rats were exposed to CB for 6 h/day, 5 days a week for a total of 4 weeks at nominal concentrations of $15.6\pm3.5\ mg/m^3$, with a median particle diameter of 116.4 nm. According to the air-pollution data collected in the urban areas of China,[19] mean and maximum concentrations of particulate matter less than 2.5 μm in diameter have been reported to be 146.8 and 666.2 $\mu g/m^3$, respectively. The dosage used in the present analysis is approximately 20- to 100-fold higher than the ambient air pollution in China. Despite the higher dosage and different method of exposure used in our present analysis, we could not confirm the translocation of inhaled carbon nanoparticles into the bloodstream or other tissues. In line with these findings, 4-week exposure to CB did not have any significant effect on liver and renal functions as assessed by ALT, AST, creatinine and creatine kinase. Thus, nano-sized CB particles are likely to aggregate in the lung, and are unlikely to pass through the alveoli and exert direct toxic effects on other target tissues.

What is clear from our present study is that exposure to CB by inhalation induced incorporation of CB in pulmonary macrophages in SD rats. Electron microscopic examination revealed the existence of CB particles in autophagic-like cellular structures of pulmonary endothelial cells of the capillary vessels of CB-treated rats. Circulating levels of

MCP-1, IL-6 and CRP, known as inflammatory marker proteins, were markedly elevated in rats exposed to CB for 4 weeks compared with the controls. Although a severe form of alveolar inflammation or fibrosis was not observed in rats exposed to CB, it is certain that CB exposure induced a mild inflammatory response in the lung. Exposure to diesel exhaust particles has been reported to induce the release of proinflammatory cytokines, such as IL-1β and IL-8, in human bronchial epithelial cells[20,21] A study examining the effect of silica exposure on the production of inflammatory mediators in the lung has shown upregulation of *IL-6* and *MCP-1* in alveolar macrophages and fibroblasts[22] Therefore, in the present study the increased levels of *Ccl2* and *IL-6* mRNA expression in the lungs of CB-treated rats may account for the differences in the circulating levels of IL-6 and MCP-1 between the 2 groups. Cytokines associated with inflammation are known to trigger the production of acute-phase proteins, with IL-6 being the major stimulator of CRP synthesis in the liver[23] Local production of IL-6 may be responsible for a rise in CRP. Our results may support the hypothesis that a mild inflammatory response elicited in the lung by deposition of inhaled particulate matter leads to atherothrombotic diseases[12]

It is noteworthy that exposure to CB raised SBP in an exposure-time dependent manner. Although there was no difference between the CB and control groups in HR measured at the end of 4-week exposure, rats exposed to CB had a significantly higher HR at day 14 after exposure. Associations between ambient air pollution and elevated BP have been documented in humans[5–8] Although the retrospective analysis of the Augsburg MONICA surveys (1984–1985/1987–1988) did not have detailed data on the concentrations of nano-sized particles, an effect of total suspended particulates on SBP was demonstrated, with greater effects being observed among the subgroups with high plasma viscosity and elevated HR[5] The exact mechanisms of how CB exposure leads to higher BP await further investigation, but may involve activation of the sympathetic nervous system by the inflammatory response and/or respiratory distress because of the accumulation of CB particles in the lungs.

Although the method of CB exposure was improved from the intra-tracheal dispersion used in our previous report to whole-body inhalation in the present study, we found no evidence of the translocation of inhaled nano-sized particles to the circulation or extrapulmonary tissues. Thus, the possibility of a direct, deleterious effect of nano-sized particles on endothelial cells or extrapulmonary tissues can be considered minimal. Although the question of whether a trace amount of CB particles penetrated into the systemic circulation, but was not detected in ultrathin sections and caused toxic effects remains unanswered, we have confirmed that relatively high concentrations of CB particles are necessary to damage cells in vitro[16] Thus, the association between cardiovascular risk and air pollution is more likely to be explained by the inflammatory response induced by the accumulation of inhaled nano-sized particles in the deep lung. Determination of safety levels of particulate matter in the air from the viewpoint of health hazard is warranted.

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