Rationale: Air pollution exposure has been shown to potentiate plaque progression in humans and animals. Our previous studies have suggested a role for oxidized lipids in mediating adverse vascular effect of air pollution. However, the types of oxidized lipids formed in response to air pollutants and how this occurs and their relevance to atherosclerosis are not fully understood.

Objective: To investigate the mechanisms by which particulate matter <2.5 μm (PM_{2.5}) induces progression of atherosclerosis.

Methods and Results: Atherosclerosis-prone ApoE^{-/-} or LDLR^{-/-} mice were exposed to filtered air or concentrated ambient PM_{2.5} using a versatile aerosol concentrator enrichment system for 6 months. PM_{2.5} increased 7-ketocholesterol (7-KCh), an oxidatively modified form of cholesterol, in plasma intermediate density lipoprotein/low-density lipoprotein fraction and in aortic plaque concomitant with progression of atherosclerosis and increased CD36 expression in plaque macrophages from PM_{2.5}-exposed mice. Macrophages isolated from PM_{2.5}-exposed mice displayed increased uptake of oxidized lipids without alterations in their efflux capacity. Consistent with these findings, CD36-positive macrophages displayed a heightened capacity for oxidized lipid uptake. Deficiency of CD36 on hematopoietic cells diminished the effect of air pollution on 7-KCh accumulation, foam cell formation, and atherosclerosis.

Conclusions: Our results suggest a potential role for CD36-mediated abnormal accumulations of oxidized lipids, such as 7-KCh, in air pollution–induced atherosclerosis progression. (Circ Res. 2014;115:770-780.)

Key Words: 7-ketocholesterol ■ air pollution ■ atherosclerosis ■ foam cells ■ macrophages ■ particulate matter

Both epidemiological and empirical evidence suggest an important effect of inhaled particulate matter (<2.5 μm [PM_{2.5}]) in mediating cardiovascular events. The effect of air pollution on global cardiovascular disease and daily mortality is significant and substantial, given its pervasive presence and ubiquitous exposure, with recent data suggesting that >17% of global adult mortality is directly attributable to anthropogenic components of PM_{2.5}, primarily through ischemic heart disease events. In 2005, 89% of the world’s population lived in areas where the World Health Organization Air Quality Guideline of 10 μg/m^3 PM_{2.5} (annual average) was exceeded. We and others have previously demonstrated that controlled exposure to air pollution results in potentiation of atherosclerosis in genetically susceptible models of atherosclerosis, which have been corroborated in human studies using surrogate measures, such as coronary artery calcium and carotid intima-media thickness. We have also previously shown that chronic exposure to PM_{2.5} increases generation of derivatives of oxidized phospholipids with resultant mobilization of mononuclear cells from bone marrow to the circulation, lungs, and the vascular wall via a toll-like receptor 4 (TLR4) mechanism, providing a direct pathway by which air pollution may potentiate atherosclerosis. Although important questions remain, including the role of other oxidation such as oxidatively modified forms of cholesterol that are fundamental to atherosclerosis lesion progression.

In the present study, we found an increase of 7-ketocholesterol (7-KCh), a major component of oxysterol, in the intermediate density lipoproteins (IDL) and low-density lipoprotein...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>7-KCh</td>
<td>7-ketocholesterol</td>
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<tr>
<td>BMDM</td>
<td>bone marrow–derived macrophage</td>
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<tr>
<td>FA</td>
<td>filtered air</td>
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<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<tr>
<td>oxLDL</td>
<td>oxidized LDL</td>
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<tr>
<td>oxPAPC</td>
<td>oxidized 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphoryl-choline</td>
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<tr>
<td>PEM</td>
<td>peritoneal exudate macrophage</td>
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<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
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<td>WT</td>
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(LDL) fraction of plasma lipoproteins and within aortic plaque. In addition, air pollution–enhanced CD36-mediated uptake of 7-KCh by macrophages. Disruption of CD36 in hematopoietic cells abolished air pollution–induced 7-KCh accumulation in aortic wall and foam cell formation. Thus, our study provides new insights into adverse cardiovascular effects of air pollution exposure by linking air pollution–mediated lipoprotein oxidation with CD36-mediated abnormal deposition of oxidized lipids in atherosclerotic plaque and vascular dysfunction.

Methods

Animal Studies and Exposure Protocol

Eight-week-old apolipoprotein E<sup>−/−</sup> (ApoE<sup>−/−</sup>) or LDLR<sup>−/−</sup> male mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed at constant temperature (22±2°C) on a 12-hour light/dark cycle. The mice were maintained on regular chow (catalog #862; Harlan, Haslett, MI). Assignments to PM<sub>2.5</sub> group versus filtered air (FA) group were randomized. The animal exposure and the monitoring of the exposure environment and ambient aerosol were performed. Exposure to concentrated ambient PM<sub>2.5</sub> was performed at the Ohio Air Pollution Exposure System for Interrogation of (PM<sub>0.1</sub>) particles are not excluded. We have previously demonstrated that atherosclerosis progression enhanced in ApoE<sup>−/−</sup> mice fed high-fat chow and concomitantly exposed to PM<sub>2.5</sub> in Tuxedo, NY. However, because the biological effects of PM<sub>2.5</sub> may differ based on concomitant diet and composition and size of PM (which in turn is dependent on emission sources), we designed our experiments with a different exposure environment (Columbus, OH regional) and in the context of normal diet conditions. Throughout this article, concentrated ambient PM<sub>2.5</sub> exposure is referred to as PM<sub>2.5</sub> unless specified otherwise.

PM<sub>2.5</sub> Concentration and Composition Analysis in the Exposure Chamber

The overall mean ambient PM<sub>2.5</sub> level during the exposure periods was 9.1±7.3 μg/m<sup>3</sup>. Mean exposure enrichment over ambient levels in the chamber was ≈10-fold higher. Particles from the exposures were collected on Teflon filters (Gelman Teflo, 37 mm, 0.2-mm pore; Gelman Sciences, Ann Arbor, MI). Filters were weighed before and after collecting samples. Analysis of PM<sub>2.5</sub> composition was performed as previously described.

7-KCh and Cholesterol Levels

Liquid chromatography/mass spectrometry was used to determine the level of 7-KCh and cholesterol in the serum and tissues as described previously. Lipoproteins were isolated by fast protein liquid chromatography. 7-KCh and cholesterol level in lipoprotein fraction were determined by liquid chromatography/mass spectrometry.

Bone Marrow Transplantation

Bone marrow transplantation was performed as previously described. Briefly, LDLR<sup>−/−</sup> mice were irradiated at the dose of 950 Rad. Mice were intravenously injected with 15×10<sup>6</sup> bone marrow cells from C57BL/6 or CD36<sup>−/−</sup> mice 24 hour after irradiation.

Data Analyses

All the data in the article are expressed as mean±SEM unless otherwise specified (Online Table I). Difference between 2 groups was tested by Student t test. Differences among groups were tested by 2-way ANOVA and Bonferroni post hoc test using Graphpad Prizm 5.01 software. P values of <0.05 were considered statistically significant.

Results

PM<sub>2.5</sub> Concentrations During the Study Period

After exposure with FA or PM<sub>2.5</sub> for 6 months, ApoE<sup>−/−</sup> mice were euthanized, and atherosclerotic plaque burden was quantified at the level of the aortic sinus. Starting at the level of the aortic valve leaflet, serial 5-μm sections, covering a total length of 600 μm were stained with hematoxylin and cosin to evaluate the severity of atherosclerosis. PM<sub>2.5</sub> exposure significantly increased lesion area, lipid and collagen content of atherosclerotic plaque (Figure 1A–D). The plaque distribution and lipid content in the aorta are depicted from representative FA and PM<sub>2.5</sub> exposed animals stained with Oil-Red-O (Figure 1E).

Atherosclerosis Burden With PM<sub>2.5</sub>

After exposure with FA or PM<sub>2.5</sub> for 6 months, ApoE<sup>−/−</sup> mice 24 hour after irradiation. Mice were intravenously injected with 15×10<sup>6</sup> bone marrow derived macrophage

7-KCh Levels in Mice Exposed to PM<sub>2.5</sub>

A preponderance of lipids present in plaques is composed of oxidized cholesterol derivatives (oxysterols). 7-KCh, a major component of oxysterols in advanced plaque, has been postulated to play an important role in endothelial dysfunction and atherogenesis. Therefore, we evaluated levels of 7-KCh in the tissues of exposed mice. No significant difference of 7-KCh was detected in liver and plasma between PM<sub>2.5</sub>-exposed and FA-exposed group (Online Figure I). The level of 7-KCh was 7-fold higher in the aorta, whereas levels in the lung did not differ in response to PM<sub>2.5</sub> exposure (8.58±2.08 versus 1.25±0.45 μg/g tissue; P<0.05; n=5 in aorta and 4.55±0.28 versus 4.98±0.35 μg lung tissue in PM<sub>2.5</sub> versus FA; P>0.05; n=5 in lung; Figure 2A and 2B). We also assessed the amount of cholesterol in the same tissues. We failed to detect differences in cholesterol content between PM<sub>2.5</sub> and FA exposure group in either the aorta or the lung (Figure 2C and 2D). When adjusted for levels of cholesterol, the ratio of 7-KCh/cholesterol increased 10-fold in the aorta but not in the lung of PM<sub>2.5</sub> group (Figure 2E and 2F). Because 7-KCh has been demonstrated to accumulate in the plasma lipoproteins, with this representing a facile pathway of delivery to the...
arterial vessel wall, we quantified 7-KCh level in the lipoprotein fraction. As depicted in Figure 2G, plasma very LDL and IDL/LDL fractions contained the bulk of 7-KCh, with these levels being confined to the IDL/LDL fraction once the cholesterol content of the lipoprotein fractions were adjusted for. The 7-KCh/cholesterol in plasma LDL was ≈ 2-fold higher in PM2.5-exposed mice (Figure 2G and 2H).

**Effect of PM2.5 on 7-KCh and Cholesterol Uptake and Efflux**

The transport and uptake of oxysterols into macrophages and endothelial cells result in progressive accumulation of lipids in the arterial wall in atherosclerosis.21 To test the effect of PM2.5 exposure on macrophage-mediated sterol accumulation, we used peritoneal macrophages derived from PM2.5/FA-exposed mice. Previous studies have demonstrated that these cells are a reasonable surrogate for plaque macrophages and can be isolated in large enough quantities for our assays.22,23 As shown in Figure 3A, the uptake of 7-KCh by peritoneal exudate macrophages (PEM) derived from PM2.5-exposed mice was higher than that of FA-exposed mice (9.4±1.19% versus 8.18±0.76%; P<0.05; n=6; Figure 3A). To validate these results additionally, we also used bone marrow–derived macrophages (BMDM) to investigate the effect of collected PM2.5 particles on cholesterol/oxysterol transport. After overnight treatment with PM2.5, the ability of 7-KCh uptake by BMDM increased (Online Figure II). In contrast, no significant difference in 7-KCh uptake by cultured endothelial cells
was detected between PM$_{2.5}$-treated and vehicle-treated group (Online Figure IIIA). These results suggest that PM$_{2.5}$ exposure promotes uptake of 7-KCh selectively in macrophages but not in endothelial cells. Unmodified cholesterol uptake by PEM was not affected by PM$_{2.5}$ treatment (Figure 4B). Pretreatment with 7-KCh did not increase the cholesterol uptake by PEM (Figure 3B).

In addition to uptake, efflux is another process that affects the accumulation of oxysterols in the cell. PM$_{2.5}$ did not affect the efflux of 7-KCh in PEM derived from mice exposed to PM$_{2.5}$ (Figure 3C). Efflux of cholesterol in PEM was also not influenced by the treatment of PM$_{2.5}$ (Figure 3D). However, pretreatment of 7-KCh reduced the cholesterol efflux in PEM (Figure 3D).

**PM$_{2.5}$ Promotes CD36-Mediated 7-KCh Uptake by Macrophages**

Scavenger receptors, such as CD36, have been reported to mediate oxidized LDL (oxLDL) internalization in macrophages and have been implicated in the pathogenesis of atherosclerosis.\(^{24,25}\) To verify the involvement of CD36 in air pollution–induced 7-KCh accumulation, we evaluated the total expression of CD36 on circulating monocytes after PM$_{2.5}$ exposure for 12 weeks. PM$_{2.5}$ exposure doubled CD36 expression on CD11b+ monocytes (Figure 4A). To assess the expression of CD36 on plaque-infiltrating macrophage, aortic sinus sections of FA- and PM$_{2.5}$-exposed ApoE$^{-/-}$ mice were stained with F4/80 and CD36. Most F4/80$^+$ macrophages infiltrating the plaque expressed CD36, with an increased number of CD36-expressing macrophages observed in the plaque of PM$_{2.5}$-exposed mice (Figure 4B and 4C).

Because CD36 expression was increased in the monocytes and macrophages of PM$_{2.5}$-exposed mice, we investigated whether PM$_{2.5}$ facilitates 7-KCh uptake through a CD36-dependent mechanism. First, we tested the association between CD36 expression and 7-KCh uptake. BMDMs of wild-type (WT) mice were incubated with 7-KCh–loaded LDL (labeled with fluorescent dye, BODIPY) for 8 hours and subjected to flow cytometric analysis of CD36 expression and 7-KCh-LDL uptake after extensive washes. CD36-positive BMDMs were more efficient in taking up 7-KCh-LDL when compared with CD36-negative BMDMs (Figure 4D). Next, we investigated whether PM$_{2.5}$ could promote intracellular translocation of CD36. We detected cell surface and total CD36 expression on BMDMs after in vitro treatment with PM$_{2.5}$. Surface expression of CD36 on macrophages decreased after in vitro treatment with PM$_{2.5}$ (50 μg/mL) for 24 hours (Online Figure IVA). Furthermore, we detected total expression of CD36 by staining for CD36 after permeabilization. There was a small but statistically significant increase in total CD36 (Online Figure IVB), suggesting that the decrease of surface CD36 is caused by internalization rather than suppression of synthesis of CD36. To exclude the nonspecific effect of lipopolysaccharide that is usually found in PM particles, cells were incubated with lipopolysaccharide inhibitor polymyxin (25 μg/mL) and PM$_{2.5}$ particles (50 μg/mL) together. Blockade of lipopolysaccharide with polymyxin did not reverse the effect of PM particles on CD36 internalization (Online Figure V). In contrast, no difference in the expression of scavenger receptor class A (also known as CD204) was observed between PM$_{2.5}$- and vehicle-treated cells (Online Figure IV C and IVD). In line with this, nonspecific phagocytosis induced by microsphere (1 μm latex beads) did not affect 7-KCh uptake (Online Figure VI).

**Loss of CD36 Attenuates Air Pollution Effects on Atherosclerosis**

To demonstrate the role of CD36 in mediating the effects of PM$_{2.5}$ in vivo, LDLR$^{-/-}$ mice were transplanted with bone marrow cells isolated from WT or CD36$^{-/-}$ mice followed by 3-month exposure to PM$_{2.5}$. PM$_{2.5}$ exposure promoted atherosclerotic plaque development in LDLR$^{-/-}$ mice transplanted with WT bone marrows, whereas deficiency of CD36 on hematopoietic cells reduced plaque volume in response to PM$_{2.5}$ (Figure 5A and 5B). Lipid deposition in response to PM$_{2.5}$ exposure was reduced in mice transplanted with CD36$^{-/-}$ bone marrows in comparison with mice transplanted with WT marrow (Figure 5C and 5D).

Confocal analysis of aortic sinus sections suggested that PM$_{2.5}$ exposure elevated 7-KCh level in the plaque in
LDLR−/− mice transplanted with WT bone marrows when compared with FA, whereas 7-KCh level in the plaque of LDLR−/− mice transplanted with CD36−/− bone marrows was comparable with mice transplanted with WT marrow and exposed to FA (Figure 6A and 6B). Most macrophages were colocalized with 7-KCh, and there were also a lot of 7-KCh single positive areas surrounding macrophages (Figure 6A). To test whether the increased 7-KCh level was caused by increased plaque size, 7-KCh positive area was normalized by plaque area. As shown in Figure 6C, percentage of 7-KCh positive area increased in the plaque of LDLR−/− mice transplanted with WT bone marrows and exposed to PM2.5 when compared with FA, but not in LDLR−/− mice with CD36−/− bone marrows, suggesting that PM2.5 increased 7-KCh level independent of plaque lesion size and CD36 deficiency can blunt the effect of PM2.5. Similar results were obtained when normalized with macrophage area (F4/80 +), indicating increase of 7-KCh in aortic plaque is not because of the increase of macrophage (Figure 6D). To confirm the role of CD36 in oxysterol uptake further, PEMs isolated from FA- or PM2.5-exposed LDLR−/− mice with WT or CD36−/− bone marrows were incubated with DiI-labeled LDL, oxLDL, or 7-KCh–loaded LDL for 8 hours. Fluorescence intensity of the cells was measured by flow cytometry after extensive washes. As depicted in Figure 6E, similar levels of LDL uptake were observed in all four groups. However, the uptake of oxLDL or 7-KCh-LDL was enhanced by PM2.5 exposure in WT macrophages, and loss of CD36 reduced the uptake of oxLDL or 7-KCh-LDL by macrophages. The effect of air pollution on oxLDL or 7-KCh-LDL uptake was markedly diminished by CD36 deficiency (Figure 6E).

PM1.4 Increases Foam Cell Formation, Which Is Blunted by CD36 Deficiency

Foam cell formation from macrophages with subsequent fatty streaks plays an important role in the pathogenesis of atherosclerosis. Modified LDL, including oxidized LDL (oxLDL) or acetylated LDL, promotes foam cell formation, whereas LDL in its native form is not atherogenic.26,27 Likewise, there was a marked increase in foam cell formation when macrophages were incubated with 7-KCh-LDL when compared with LDL (Figure 7A and 7D). Dil-labeled 7-KCh-LDL was prepared by loading 7-KCh (2 mmol/L) to Dil-labeled LDL. Fluorescence intensity of macrophages incubated with Dil-labeled 7-KCh-LDL was significantly higher when compared with that incubated with Dil-labeled LDL, suggesting 7-KCh-LDL rather than native LDL accumulates in macrophages and promotes foam cell formation (Figure 7A and 7D). To determine the effect of PM1.4 and CD36 in the foam cell formation, PEMs isolated from FA- or PM2.5-exposed LDLR−/− mice with WT or CD36−/− bone marrows were incubated with DiI-labeled LDL, oxLDL, or 7-KCh–loaded LDL for 36 hours. PM2.5 exposure promoted foam cell formation as evidenced by Oil-Red-O staining and confocal visualization of Dil-labeled 7-KCh-LDL. There was no difference in foam cell formation between FA and PM2.5 in CD36-deficient macrophages, suggesting that this effect of PM1.4 in increasing 7-KCh uptake occurs via CD36 pathways (Figure 7B, 7C, 7E, and 7F).
Discussion

In this study, we provide evidence for a novel mechanism by which inhaled PM may promote atherosclerosis. 7-KCh, an oxysterol extensively implicated in atherosclerosis, increased with chronic exposure to PM$_{2.5}$ and preferentially accumulated in the aortic plaque in 2 different hypercholesterolemic rodent models. 7-KCh was almost exclusively present in the IDL/LDL fraction of plasma lipoproteins and selectively

![Figure 5](image_url)

**Figure 5.** Loss of CD36 diminished promotive effect of air pollution on atherosclerosis. A, LDLR$^{-/}$ mice transplanted with wild-type (WT) or CD36$^{-/}$ bone marrows were exposed to filtered air (FA) or particulate matter <2.5 μm (PM$_{2.5}$) for 3 months. Aortic sinus sections were used for the detection of plaque burden by hematoxylin and eosin (H&E) staining and lipid content detection by Oil-Red-O staining. A, Representative image of H&E staining. B, Statistical analysis of plaque volume. C, Representative images of Oil-Red-O staining. D, Statistical analysis of Oil-Red-O positive area. *P<0.05 when compared with FA; #P<0.05 when compared with WT.

![Figure 6](image_url)

**Figure 6.** Loss of CD36 reduces particulate matter <2.5 μm (PM$_{2.5}$)-mediated uptake of oxysterol. A–D, Aortic sinus sections from FA- or PM$_{2.5}$-exposed LDLR$^{-/}$ mice with wild-type (WT) or CD36$^{-/}$ bone marrows were used for the detection of 7-ketocholesterol (7-KCh; green) and macrophage (F4/80; red). Representative images (A) and statistical analysis of 7-KCh area (B), 7-KCh/plaque ratio (C), and 7-KCh/F4/80 ratio (D) were shown. E, PEMs isolated from FA- or PM$_{2.5}$-exposed LDLR$^{-/}$ mice with WT or CD36$^{-/}$ bone marrows were incubated with Dil-labeled low-density lipoprotein (LDL; 10 μg/mL), oxidized LDL (oxLDL; 10 μg/mL), or 7-KCh-LDL (10 μg/mL) for 8 hours. Cells were then harvested for the analysis of Dil intensity on LSRII flow cytometer. Flow histograms are shown. n=6. *P<0.05 when compared with FA; #P<0.05 when compared with WT.
accumulated in macrophages through a CD36-dependent process. Consistent with a role for CD36 in air pollution-mediated effects, hematopoietic deficiency of CD36 exerted a protective effect on atherosclerosis, inflammation, and lipid accumulation in response to air pollution. CD36+ macrophages derived from PM 2.5-exposed animals preferentially accumulated 7-KCh without demonstrating abnormalities in cholesterol efflux pathways. Our results provide new insights into mechanisms underlying air pollution–induced atherosclerosis progression by linking air pollution with vascular lipid dysregulation.

Multiple studies have confirmed an important proatherogenic effect of diesel exhaust particles, PM 2.5, and ultrafine components of air pollution. 5,6,10 Limited human cross-sectional studies have demonstrated higher carotid intima-media thickness and coronary artery calcium scores in patients exposed to higher levels of air pollution.28,29 Abnormalities in endothelial function have also been described in acute and chronic exposure studies (primarily in animal models).30,31 We and others have demonstrated increased lipid accumulation within plaques of animals exposed to air pollution together with macrophage accumulation.6,12-14 Increased expression of oxidative stress markers, such as iNOS, nitrotyrosine, and CD36, has been previously demonstrated in plaques after both PM2.5 and diesel exhaust exposure.34,35 Although the proatherogenic effects of PM 2.5 in animal models are consistent with the epidemiological evidence linking PM 2.5 with atherosclerotic events, such as acute myocardial infarction and stroke, the precise pathways that sustain progression of plaque with exposure resulting in unstable coronary syndromes are poorly understood.2 In atherosclerosis, a critical step is recognition and internalization of oxLDL by specific macrophage scavenger receptors (with CD36 accounting for 60%–70% of oxLDL uptake).36 CD36 has been shown to mediate oxLDL internalization in macrophages, with its expression correlating with exposure measures such as alveolar macrophages positive for inhaled particles.34 Therefore, we investigated this pathway and the mechanisms that may lead to CD36-mediated foam cell formation in response to PM 2.5 exposure. CD36 expression was increased in plaque macrophages from PM 2.5-exposed animals. In our studies, we found a marked increase in 7-KCh in the aorta of exposed hyperlipidemic animals with colocalization primarily in plaque macrophages. Interestingly, nonspecific phagocytosis mediated by in vitro exposure to microspheres did not enhance 7-KCh uptake. PM 2.5 exposure did enhance the uptake of 7-KCh. These results suggest that PM 2.5 promotes the accumulation of 7-KCh by enhancing CD36-mediated internalization rather than nonspecific phagocytosis. Consistent with our findings, other air pollutants have also been shown to increase CD36 expression. Bai et al14 reported a 1.5- to 2-fold increase of CD36 in plaque, accompanying with 1.5- to 3-fold increase of lipid content after exposure to diesel exhaust. CD36 expression was correlated positively with increased entry of monocytes into atherosclerotic plaques.34 Robertson et al have suggested that CD36 mediates the
vascular effect of circulating vasoactive factors released in response to ozone exposure. However, our study does not exclude other scavenger receptors, such as LOX-1, CD68, and SR-B1, that may also be involved in enhanced atherogenesis. Exposure to engine emissions increased lectin-like oxLDL receptor (LOX-1), plasma oxLDL, and vascular infiltration of monocyte/macrophage in ApoE−/− mice, which were attenuated by the blockade of LOX-1 using neutralizing anti-LOX-1 Ab. These findings raise the possibility that air pollutants, such as PM2.5, as well as gaseous components of vehicle emission, may also influence atherogenesis through similar mechanisms (scavenger receptor–mediated dysregulation of oxidized lipids).

We have previously demonstrated that chronic exposure to PM2.5 increased the generation of oxidized phospholipid derivatives of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (oxPAPC), such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine in the lungs. The generation of oxPAPC resulted in activation of nuclear factor-κB and proinflammatory genes, including chemokines such as chemokine (C-C motif) ligand 2 through a TLR4/NADPH oxidase-dependent pathway. Mice deficient in NADPH oxidase 2 (NOX2) and TLR4 demonstrated reduced vascular inflammation and improved vascular dysfunction seen with PM2.5 exposure. TLR4 deficiency also abolished circulating increase of Ly6C+ monocytes in response to long-term PM2.5 exposure, providing a cellular mechanism through which exposure may facilitate inflammation in atherosclerosis. We have postulated previously that because PAPC is abundantly present in tissues, such as the lung (dominant phospholipid in the surfactant fluid of lung), the generation of oxidized forms of PAPC could easily occur with air pollution exposure resulting in TLR4 activation and transcription of proinflammatory genes and chemokines. The role of oxidation products other than oxPAPC that may arise with air pollution exposure, such as oxidatively modified forms of cholesterol, has not been investigated to date. Because air pollution exposure serves as a potent redox trigger, facilitating the generation of a range of oxidatively modified products, it is entirely possible that other oxidation products of cholesterol are also generated. In atherosclerosis, lesion formation and progression are fundamentally dependent on the generation of oxidized forms of cholesterol, which are rapidly taken up by macrophages in lesions via scavenger receptor–dependent and scavenger receptor–independent pathways, resulting in lipid accumulation within the vessel wall. 7-KCh is a major oxidation product present in oxLDL and within atherosclerotic plaque. In current study, we detected elevated levels of 7-KCh in plasma lipoproteins (IDL/LDL) after chronic exposure to air pollution. 7-KCh but not cholesterol increased in the LDL/IDL fraction of plasma lipoproteins of PM2.5-exposed mice, raising the possibility that elevated 7-KCh in the aorta may result from increased accumulation, through delivery of ApoB lipoproteins enriched in 7-KCh. Consistent with these findings, deficiency of CD36 markedly decreased the internalization of oxidized LDL and 7-KCh–loaded LDL but had no effect on the uptake of native LDL. Macrophages with low expression of CD36 had a significantly lower ability to uptake oxidized and 7-KCh–loaded LDL when compared with macrophages with high expression of CD36. Furthermore, ex vivo macrophages uptake of 7-KCh–loaded LDL or oxLDL, as well as foam cell formation, were enhanced by PM2.5 exposure; results that were partially abolished by CD36 deficiency. We further demonstrated in carefully performed in vivo experiments that bone marrow deficiency of CD36 attenuates PM2.5–mediated effects on atherosclerotic plaque and lipid accumulation. F4/80+ macrophages in plaque colocalized with 7-KCh within the plaque with abundant 7-KCh surrounding macrophages, suggesting release of 7-KCh presumably from apoptotic macrophages containing the oxysterol. CD36 deficiency in bone marrow–derived cells also reduced atherosclerotic lesion in FA-exposed mice. This result is in accordance with previous reports. Although Moore et al41 reported that CD36 deficiency in ApoE−/− background did not affect atherosclerotic progression in their work; subsequent studies including those from Sheedy et al40,42,43 suggest CD36 deletion did reduce atherosclerotic plaque in ApoE−/− mice. The differences noted in these studies may be attributable to the use of 2 different mouse strains by the groups as pointed out previously.44 Our result that LDLR−/− mice with CD36-null bone marrow were protected from atherosclerosis is indeed consistent with another previous report that transplantation of CD36-null bone marrow reduces atherosclerotic lesion formation.49

The finding in the current investigation that CD36 mediates air pollution–induced 7-KCh accumulation and atherosclerosis progression provides complementary mechanisms to our previous studies linking TLR4 to abnormal vascular effects and cellular inflammation associated with air pollution exposure. Oxidized phospholipids, such as oxPAPC, may result in proinflammatory effects through nuclear factor-κB mechanisms that may synergistically interact with accumulation of lipids, such as 7-KCh in macrophages to accelerate atherogenesis. On the basis our findings of increased 7-KCh in lipoproteins, such as very LDL and LDL/IDL and lack of increase in lung-derived 7-KCh, it is likely that 7-KCh is continually formed in the vascular compartment during air pollution exposure particularly on the surface of cholesterol-loaded lipoproteins, such as LDL and IDL. We posit that the continual exposure of LDL to air pollution particularly at the alveolar–capillary membrane likely plays a role in this process. LDL/IDL-containing oxidized lipids, such as 7-KCh and oxPAPC in response to air pollution, may be delivered to the atherosclerotic aorta expressing scavenger receptors and CD36. A recent study suggested that CD36 may also activate TLR4 and induces sterile inflammation by forming a TLR4/TLR6 heterodimer, thus providing additional pathways by which TLR4 may interact with pathways involving CD36. In the current study, we did not measure oxPAPC in LDL fraction although previous studies have demonstrated increased oxPAPC in circulating lipoproteins, including LDL and high-density lipoprotein. Although oxPAPC may not be the major component of oxLDL or oxidized lipids within plaque, it certainly may represent an additional oxidation product that is delivered via scavenger receptors and may accumulate within atherosclerosis. In addition, the presence of oxPAPC may result in
proinflammatory effects within the vascular wall.

The relative contribution of 7-KCh versus other oxidized lipids in air pollution–mediated atherosclerosis progression will require additional studies.

Mass transfer of 7-KCh can also occur in the opposite direction from within the cell to a wide range of phospholipid-containing acceptors, including high-density lipoprotein. Recently, ATP-binding cassette subfamily G member 1 has been suggested to promote the export of 7-KCh and 7β-hydroxycholesterol to high-density lipoprotein, and to exert cytopenic effects.

In our study, efflux assays performed with both 7-KCh and cholesterol demonstrated that pretreatment with PM2.5 does not influence 7-KCh and cholesterol efflux in either macrophages or endothelial cells. Interestingly, we observed increased cholesterol uptake in endothelial cells and a decrease of cholesterol efflux in macrophages when cells were pretreated with 7-KCh. These findings suggest a feed-forward effect on lipid accumulation after 7-KCh exposure/accumulation. These results are consistent with those of Gelissen et al who demonstrated that the presence of 7-KCh suppresses the export of cholesterol from macrophage to apolipoprotein A-I acceptor. In addition, studies have suggested that 7-KCh suppresses incorporation of cholesterol to plasma membrane and may thus reduce cholesterol efflux to cholesterol acceptors.

Our study has several important limitations that must be acknowledged. Peritoneal macrophages from mice exposed to PM2.5 were used to assess 7-KCh uptake in plaque macrophage in light of the large amount of cells to perform the assays. Previous studies, however, have demonstrated that these cells are, however, reasonable surrogates for plaque macrophages. We think that despite this limitation, these studies help provide a framework of understanding of how exposure to PM2.5 may modulate uptake of 7-KCh. Accumulation of 7-KCh in plaque depends importantly on circulating levels of 7-KCh in lipoproteins, 7-KCh uptake, and retention of 7-KCh in the sites of lesion formation. In the present study, we have not provided insights on how 7-KCh accumulates selectively in lipoproteins, or have we provided information on the precise locus of 7-KCh formation in LDL. We postulate that plasma lipoproteins may undergo oxidation at the alveolar–capillary membrane interface in response to repetitive PM2.5 exposure. This may potentially explain lack of parenchymal lung accumulation of 7-KCh as was noted in the study.

In summary, our findings provide a novel mechanism by which air pollution may enhance the development of atherosclerosis via linking CD36-mediated abnormal accumulation of oxidized lipid in vasculature with air pollution–induced lipid oxidation. Additional studies are required to determine which components of PM2.5 are required for lipid oxidation, other receptors involved in the deposition of oxidized lipid in vascular wall, and the precise compartment where 7-KCh is generated.

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Disclosures

None.

References


What Is Known?

- Exposure to air pollution contributes to cardiovascular morbidity and mortality.
- Oxidized lipids promote atherosclerosis.
- Formation of oxidized lipid in the lung is increased in animals exposed to air pollution.

What New Information Does This Article Contribute?

- In mice, exposure to air pollution increases accumulation of oxidized lipids, such as 7-ketocholesterol in macrophages and the aortic wall.
- CD36 links air pollution and abnormal accumulation of oxidized lipid in macrophages and atherosclerotic lesion.
- Deficiency of CD36 reduces ability of macrophage to take up oxidized lipids and it diminishes the effect of air pollution on atherogenesis.

Both epidemiological and empirical evidence suggest an adverse effect of air pollution on cardiovascular disease.

However, the underlying mechanisms remain poorly understood. Previous studies have provided evidence of acceleration of atherosclerosis by air pollution in experimental models. Limited human studies using surrogate markers of subclinical cardiovascular disease, such as coronary artery calcium and carotid intima-media thickness, support these findings. We asked whether air pollution contributes to the formation of oxidized lipids in plasma lipoproteins and whether these are deposited in the arterial wall. We found that the levels of 7-ketocholesterol were increased in plasma lipoproteins in response to air pollution exposure in atherosclerosis-prone mice. These results suggest that air pollution increases CD36-mediated accumulation of oxidized lipids in macrophages and the arterial wall and thereby promotes atherogenesis. This study provides new insights into mechanisms underlying air pollution–induced atherosclerosis progression by linking air pollution with the formation of oxidized lipids.
CD36-Dependent 7-Ketocholesterol Accumulation in Macrophages Mediates Progression of Atherosclerosis in Response to Chronic Air Pollution Exposure

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Animals
Eight-week-old Apolipoprotein E−/− (apoE−/−) male mice from Jackson Laboratory (Bar Harbor, ME) were housed at constant temperature (22 ± 2°C) on a 12-h light/dark cycle. They were fed *ad libitum* on standard laboratory mouse chow and had free access to water. Assignments to PM$_{2.5}$ group vs. filtered air (FA) group were randomized. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and the Institutional Animal Care and Use Committee of the Ohio State University approved the study protocols.

Exposure to PM$_{2.5}$
The animal exposure and the monitoring of the exposure environment and ambient aerosol were performed as previously described.$^1$ Animals were randomly exposed to concentrated ambient PM$_{2.5}$ or filtered air (FA) for 6 hours/day, 5 days/week for a total duration of 6 months in Columbus, OH from December 23, 2011 to June 22, 2012. A versatile aerosol concentration enrichment system (VACES) was used to generate the concentrated PM$_{2.5}$ in the exposure chamber.$^2$ Inhalation exposures were conducted in OASIS (Ohio Air Pollution Exposure System for Interrogation of Systemic Effects) at the Ohio State University. OASIS is certified by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). In our study, OASIS was located at Polaris facility of the Ohio State University, which is 0.5 mile east of the I-71 highway.

PM$_{2.5}$ Concentration and Composition Analysis in the Exposure Chamber
Samples were collected on Teflon filters (Gelman Teflo, 37 mm, 0.2-mm pore, Gelman Sciences, Ann Arbor, MI). Before and after collecting samples, filters were weighed in a temperature- and humidity-controlled weighing room using an oscillating microbalance (Tapered-Element Oscillating Microbalance, model 1400, Rupprecht and Patashnick). Weight gains were used to calculate exposure concentrations of PM$_{2.5}$ in the exposure chambers. After that, analysis of PM$_{2.5}$ composition was performed by RTI following compendium Environmental Protection Agency method IO-3.3 [“Determination of metals in ambient particulate matter using X-ray fluorescence (XRF) spectroscopy” (http://www.epa.gov/ttnamti1/files/ambient/inorganic/mthd-3_3.pdf)] using the Thermo Quant’X XRF system (Thermo Fisher Scientific, Waltham, MA).$^1$

Plaque lesion analysis
To prepare sections for morphometric analysis,$^3$, hearts were perfusion fixed *in situ* with 10% neutral-buffered formalin. The heart with approximately 1 mm of proximal aorta attached and the aortic arch down toward the femoral branch were removed. The top half of the hearts containing the aortic root was then fixed for additional 8 hours and embedded. 20 µm sections were sequentially cut until the aortic valve leaflets appeared. From this point on, serial 5µm sections
were cut until a total of 600 µm sections were collected. To quantify lesion area of atherosclerotic plaques of the aortic roots, sections were stained routinely with hematoxylin and eosin (H&E) and analysis were performed using MetaMorph and ImageJ softwares.

For lipid staining, frozen sections of aortic sinus were stained with Oil-Red-O (0.24% in 62.5% isopropanol) and then counterstained with hematoxylin. For collagen staining, masson’s trichrome staining was performed with a standard procedure.

To determine the lesions on the aortic surface, we used the *en face* method. The aortic arch down toward the femoral branch was dissected under a dissecting microscopy to carefully remove the adventitia. The aortas were then soaked in 10% buffered formalin for 8 hours followed by Oil-Red-O staining.

### 7-ketocholesterol and cholesterol levels in the tissue

To determine the level of 7-ketocholesterol and cholesterol in the tissues, plasma and lipoprotein factions, liquid chromatography/mass spectrometry was used. Tissues were dried and weighted, then 1 ml of hexane-isopropanol 2: 1 (v/v) was added to each sample. For plasma, 0.5 ml of hexane-isopropanol 2: 1 (v/v) was added to 200µl plasma for each mouse. Lipoproteins were isolated by fast protein liquid chromatography. D7 7-ketocholesterol or D7 cholesterol was added as internal standard. The organic phase was collected by centrifuge and the collecting was repeated three times. The collected organic phase was evaporated dryness under nitrogen gas and further analyzed by liquid chromatography/mass spectrometry as described previously.

### Lipid uptake and efflux

Peritoneal exudate macrophages (PEM) isolated from mice and HUVECs were used in lipid uptake experiment. For the detection of 7-KCh uptake, PEM or HUVECs were treated with or without particles (50 µg/ml) overnight. Cells were then incubated with 

\[
\text{\textsuperscript{3}}\text{H}-\text{labled 7-KCh (1 \mu Ci/ml)}
\]

for 3 hours. After incubation, cells and media were collected separately for radioactivity measurement. For the detection of cholesterol uptake, PEM or HUVECs were treated with or without particles (50 µg/ml) collected from filters in exposure for overnight followed by 7-KCh (20 µg/ml) or vehicle incubation for 1 hour. After that, cells were incubated with \n
\[
\text{\textsuperscript{3}}\text{H}-\text{labled cholesterol (1 \mu Ci/ml)}
\]

for 3 hours. Cells and media were then collected separately for radioactivity measurement.

Peritoneal exudate macrophages (PEM) isolated from mice and HUVECs were used in efflux experiments. For 7-KCh efflux, PEM or HUVECs were treated with or without particles (50 µg/ml) overnigt, followed by incubation with \n
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\text{\textsuperscript{3}}\text{H}-\text{labled 7-KCh (1 \mu Ci/ml)}
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for 6 hours. After extensive washes with PBS, cells were incubated with HDL (50 mg/ml) for 16 hours. Cells and medium were then collected separately for radioactivity measurement. For cholesterol efflux, PEM or HUVECs with or without overnight treatment of PM2.5 were treated with 7-KCh (20 µg/ml) or vehicle for 1 hour. Cells were then incubated with \n
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\text{\textsuperscript{3}}\text{H}-\text{labled cholesterol (1 \mu Ci/ml)}
\]

for 6 hours. After extensive washes with PBS, cells were incubated with 50mg/ml HDL. After 16 hours of incubation, cells and medium were collected separately for radioactivity measurement.
**Immunofluorescence staining**

For the detection of CD36, F4/80, and 7-KCh in plaque, frozen sections of sinus were used for immunofluorescence staining. Briefly, 10-μm-thick tissue sections were incubated with indicated antibodies (rat anti-mouse F4/80, mouse anti-7-KCh [clone #35A], or American hamster anti-mouse CD36) at 4°C overnight. After extensive washes with PBS, the tissue sections were incubated with corresponding 2nd antibodies (FITC-labeled anti-rat IgG and rhodamine-labeled anti-Armenian hamster IgG, or FITC-labeled anti-mouse IgG and rhodamine-labeled anti-rat IgG) at room temperature for 1 hour. After washes with PBS, sections were mounted in Fluormount G. Images were captured by a Zeiss LSM510NLO confocal scan head mounted on a Zeiss Axiovert 200M on an inverted-based microscope with a 63x objective.

**Flow cytometry**

For the flow cytometric detection of surface CD36 and SR-A, peritoneal exudate macrophages were isolated from C57BL/6 or FVB/NJ mice as described earlier.7 Macrophages with indicated treatments were stained with FITC-labeled anti-mouse F4/80, PE-labeled anti-mouse CD36, and APC-labeled anti-mouse SR-A antibodies at 4°C for 30 min. After extensive washes, cells were suspended in flow buffer (2% FBS in 1x PBS) and analyzed on LSRII flow cytometer. For the detection of total CD36 and SR-A, macrophages with indicated treatments were incubated with FITC-labeled anti-mouse F4/80 at 4°C for 30 min. Cells were then fixed in a fixation buffer (4% paraformaldehyde in PBS) for 30 min and permeabilized in a permeabilization buffer (0.1% saponin in PBS) for 30 min. After permeabilization, cells were incubated with PE-labeled anti-mouse CD36, and APC-labeled anti-mouse SR-A antibodies at 4°C for 30 min. After extensive washes with permeabilization buffer, cells were suspended in permeabilization buffer and analyzed on LSRII flow cytometer.

**Detection of CD36-mediated internalization of 7-KCh-loaded LDL**

For the preparation of oxLDL, 200 μg/ml Bodipy-labeled LDL (Life Technologies) was incubated with 5 μM CuSO4 at 37°C overnight, followed by 24 hours of dialysis in PBS at 4°C. For the preparation of 7-KCh-loaded LDL, Bodipy-labeled LDL was incubated with 2 mM 7-KCh at 37°C for 5 hours and then dialyzed in PBS at 4°C for 24 hours. Macrophages were incubated with 25 μg/ml Bodipy-labeled 7-KCh-loaded LDL at 37°C for 8 hours. For flow cytometric detection, cells were collected and stained with APC-labeled anti-mouse F4/80 and PE-labeled anti-mouse CD36 antibodies, followed by analysis on a LSRII flow cytometer.

**Foam cell formation**

Dil-labeled LDL was prepared as previously described8 with minor modifications. LDL (density = 1.02 to 1.05) was obtained from human plasma by sequential density gradient ultracentrifugation. LDL was then labeled with 3,3’ –dioctadecylindocarbocyanine (Dil) probe as follows: 50μl of Dil (3 mg/ml) in dimethyl sulfoxide was added to 1 mg lipoprotein and incubated at 37°C for 15 hours. The density of the incubation mixture was raised to 1.063 g/ml using potassium bromide, followed by ultracentrifugation and dialysis with PBS. For the preparation of
7-KCh-loaded LDL, Dil-labeled LDL was incubated with 2 mM 7-KCh at 37°C for 5 hours. The mixture was ultracentrifuged, dialyzed in PBS at 4°C for 24 hours and sterilized before use. Macrophages were incubated with 5 μg/ml Dil-labeled LDL or 7-KCh-loaded Dil-labeled LDL at 37°C for 36 hours. After the incubation, cells were washed with PBS, fixed in 4% paraformaldehyde for 15 minutes followed by an incubation of 60% isopropanol for 5 minutes, and stained with Oil-Red-O for 10 minutes. Cells were then counterstained with hematoxylin for 5 dips. For Oil-Red-O staining, 20 images per group were captured under Nikon microscopy (40x objective). For Dil fluorescence, 15 images per group were captured by a Zeiss LSM510NLO confocal scan head mounted on a Zeiss Axiovert 200M on an inverted-based microscope with a 63x objective. Oil-Red-O positive area, fluorescence positive area and cell area were quantified by ImageJ or MetaMorph software.

Data analyses
All data are expressed as means ± SD unless otherwise specified. Difference between two groups was tested by student’s t test. Differences among groups were tested by two-way ANOVA and Boneferroni’s post hoc test using Graphpad Prizm software. P values of < 0.05 were considered statistically significant.
Supplemental Figure I: 7-KCh levels in liver and plasma are similar in FA- and PM-exposed mice. Lipids were extracted from liver and plasma of ApoE⁻/⁻ mice exposed to PM or FA and used for the detection of 7-KCh using liquid chromatography/mass spectrometry (LC/MS). A, 7-KCh levels in the liver of FA and PM-exposed mice. B, 7-KCh levels in the plasma of FA and PM-exposed mice.
Supplemental Figure II: 7-KCh uptake by macrophages increased after PM exposure. BMDMs isolated from mice were treated with PM particles or vehicles (Ctrl) overnight and then incubated with D7 7-KCh (20 μg/ml) for 3 hours. Cells and medium were then harvested for the detection of 7-KCh by liquid chromatography/mass spectrometry (LC/MS).
Supplemental Figure III: Effect of PM exposure on 7-KCh/cholesterol uptake and efflux in endothelial cells. A, 7-KCh uptake in EC: HUVECs were treated with PM$_{2.5}$ particles (50 ug/ml) or PBS (Ctrl) overnight and then incubated with $^3$H-labeled 7-KCh (1 μCi/ml) for 3 hours. Cells and medium were then harvested for the detection of radioactivity. B, Cholesterol uptake in EC: HUVECs were treated with PM$_{2.5}$ particles (50 ug/ml) or PBS (Ctrl) overnight and then incubated with H$^3$-labeled cholesterol (1 μCi/ml) for 3 hours with or without the presence of 7-ketocholesterol (20 ug/ml). Cells and medium were then harvested for the detection of radioactivity. C, 7-KCh efflux in EC: HUVECs were treated with PM$_{2.5}$ (50 ug/ml) or PBS overnight and then incubated with $^3$H-labeled 7-KCh (1 μCi/ml) for 6 hours. After washing out the remaining 7-KCh, cells were incubated with HDL (50 ug/ml) overnight. Cells and medium were then collected for the detection of 7-KCh. D, Cholesterol efflux in EC: HUVECs with or without overnight treatment of PM$_{2.5}$ were incubated with H$^3$-labeled cholesterol (1 μCi/ml) for 6 hours, followed by 1 hour incubation with 7-KCh or vehicle. After washes with fresh medium,
cells were incubated with 50 ug/ml HDL overnight. Cells and medium were then collected for the
detection of radioactivity.
Supplemental Figure IV. PM exposure enhances CD36-mediated uptake in macrophages

A, Surface expression of CD36 in PM$_{2.5}$-treated BMDMs: BMDMs were incubated with PM$_{2.5}$ (50 ug/ml) or vehicle overnight. Cells were then collected for the flow cytometric analysis of surface-expressing CD36. Representative image (left) and statistic bar graph (right) are shown. B, Total CD36 in PM$_{2.5}$-treated BMDMs: BMDMs with treatment as indicated above were fixed and permeabilized, followed by CD36 staining and flow cytometric analysis. Representative image (left) and statistic bar graph (right) are shown. C, Surface expression of SR-A in PM$_{2.5}$-treated BMDMs. Representative image (left) and statistic bar graph (right) are shown. D, Total SR-A expression in PM$_{2.5}$-treated BMDMs. Representative image (left) and statistic bar graph (right) are shown. *n=6. *, $P<0.05$. 
Supplemental Figure V: Blockade of lipopolysaccharide (LPS) with polymyxin B (PMB) did not reverse PM-induced internalization of surface CD36. Macrophages were treated with PBS (Ctrl), PM particles (50 ug/ml) or 50 ug/ml PM particles plus 25 ug/ml PMB for 24 hours. Cells were then harvested for the detection of surface expression of CD36.
**Supplemental Figure VI:** 7-KCh uptake by macrophages increased after PM but not Beads treatment. BMDMs isolated from WT mice were treated with PM particles, microspheres (latex beads with 1 μm in diameter) or vehicles (Ctrl) overnight and then incubated with D7 7-KCh (20 μg/ml) for 3 hours. Cells and medium were then harvested for the detection of 7-KCh by liquid chromatography/mass spectrometry (LC/MS).
### Supplemental Table I: Elemental composition of particles in ambient air and PM

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Supplemental References


