Advanced Oxidation Protein Products Exacerbates Lipid Accumulation and Atherosclerosis Through Downregulation of ATP-Binding Cassette Transporter A1 and G1 Expression in Apolipoprotein E Knockout Mice

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Background: Both clinical data and basic science studies suggest that advanced oxidation protein products (AOPPs) may contribute to the progression of atherosclerosis. The aim of this study was to investigate the effects of AOPPs on ATP-binding cassette transporter (ABC) A1 and ABCG1 expression, lipid accumulation and atherosclerotic lesions in apolipoprotein E knockout (apoE-KO) mice.

Methods and Results: Male 8-week-old apoE-KO mice were fed a high-fat/high-cholesterol diet. Mice received intraperitoneal injections of AOPPs (5 mg/kg) and/or Janus Kinase (JAK) inhibitor AG-490 (5 mg/kg) once every other day for 8 weeks. As shown in our data, AOPPs increased lipid levels of plasma, and promoted advanced lesions in the aortic regions in apoE-KO mice. The ABCA1, ABCG1 and liver X receptor alpha (LXRα) expression were downregulated in apoE-KO mice treated with AOPPs, whereas the lesions in the aortas were decreased, and the ABCA1, ABCG1 and LXRα expression were upregulated in mice treated with AOPPs plus AG-490, compared to the mice treated with AOPPs only. The ABCA1 and LXRα expressions of aortas, liver and intestine were downregulated in the AOPPs group, while the expressions were upregulated in the AOPPs-plus-AG-490 group when compared to the AOPPs group. The same results can be also observed in peritoneal macrophages.

Conclusions: AOPPs increase accumulation of lipids and exacerbate atherosclerosis through downregulation of ABCA1 and ABCG1 expression, and the JAK-LXRα signaling pathway in apoE-KO mice. (Circ J 2014; 78: 2760–2770)

Key Words: Advanced oxidation protein products; Atherosclerosis; ATP-binding cassette transporter A1; ATP-binding cassette transporter G1; Lipid metabolism

Atherosclerosis is a chronic disease process that takes place in the intima-media layer of the arterial wall. A variety of cellular elements are involved in this process including endothelial cells, macrophages and platelets, as well as a host of compounds produced by them, such as cytokines, adhesion molecules, oxidative and glycation modifications of lipoproteins and other proteins.1–3 The particular importance in this context may be the recent observation made by...
Liu et al\(^4\) that advanced oxidation protein products (AOPPs) significantly accelerated atherosclerosis through promoting oxidative stress and inflammation.

Advanced oxidation protein products can be formed in vitro by exposure of serum albumin to hypochlorous acid (HOCl). In vivo, plasma AOPPs are mainly carried by albumin, and their concentrations are closely correlated with the levels of dityrosine. AOPPs result from the effects of free radicals on proteins, which might act as inflammatory mediators triggering the oxidative “ignition” of neutrophils, monocytes and T-lymphocytes, thus leading to upregulation and excessive stimulation of dendritic cells.\(^5\) These processes might account for immune disorders in atherosclerosis. More interestingly, it was found that AOPPs are likely to be related to atherosclerotic cardiovascular events.\(^6\) Increased levels of AOPPs were also found in diabetic and non-uremic subjects with coronary artery disease, suggesting that AOPPs might be relevant in atherosclerosis.\(^7\) Although the observational studies suggest a close relationship between AOPPs and atherosclerosis, there is little evidence to show that AOPPs contribute to the occurrence and/or progression of atherosclerosis.

ATP-binding cassette transporter A1 (ABCA1) is an ATP-binding cassette protein that promotes efflux of cholesterol and phospholipids from intracellular compartments to extracellular cholesterol acceptors. ABCA1 is regulated by the liver X receptor (LXR). Our previous studies demonstrated that oleate and interferon (IFN)-\(\gamma\) reduce the level of ABCA1 and impair ABCA1-dependent cholesterol efflux in THP-1 cells. A LXR agonist promoted ABCA1 expression in apolipoprotein E knockout (apoE-KO) mice.\(^8\) More importantly, our studies indicated that AOPPs might first downregulate the expression of LXR\(\alpha\) and ABCA1 through activation of the Janus kinase/signal transducers and activators of the transcription (JAK/STAT) signaling pathway and then inhibit cholesterol efflux in the THP-1 macrophage.\(^9\) However, the effects and mechanism of AOPPs remain to be further investigated in vivo.

The present study was designed to investigate the effects of AOPPs on ABCA1, ABCG1 expression and cholesterol efflux, and further determine the possible mechanism in vivo. In the present study, apoE-KO mice were fed a high-fat/high-cholesterol diet (HHFD), and received intraperitoneal injections of AOPPs and/or the JAK inhibitor, AG-490, once every other day; the control animals received intraperitoneal injections of the same volume of normal saline. All mice were killed after 8 weeks for evaluation of the atherosclerotic lesions, with the tissues collected for further analysis. All animal experiments were done in accordance with the Institutional Animal Ethics Committee and the University of South China Animal Care Guidelines for Use of Experimental Animals. All protocols and procedures were approved by the Institutional Animal Care and Use Committee.

### Methods

#### Preparation of Advanced Oxidation Protein Products-Bovine Serum Albumin (AOPPs-BSA)

AOPPs-BSA was prepared in vitro as described previously.\(^12,13\) Briefly, fatty acid-free BSA was exposed to 200 mmol/L HOCl for 30 min and then dialyzed overnight against phosphate-buffered saline (PBS) to remove any free HOCl. AOPP concentrations were measured by a spectrophotometric assay, as described previously.\(^12,14\) The content of AOPPs was 62.2±13.5 nmol/mg protein in prepared AOPPs-BSA.

#### Animal Model

Male 8-week-old apoE-KO mice (purchased from the Laboratory Animal Center of Peking University, Beijing, China) were randomly divided into 4 groups (n=10). All of the mice were fed a HFHC diet (15% fat wt/wt, 0.25% cholesterol wt/wt). The AOPP group received intraperitoneal injections of AOPPs (5 mg/kg) once every other day; the AG-490 group received intraperitoneal injections of AG-490 (5 mg/kg) once every other day; the AOPP and AG-490 group received the same doses of AOPPs and AG-490 once every other day; the control animals received intraperitoneal injections of the same volume of normal saline. All mice were killed after 8 weeks for evaluation of the atherosclerotic lesions, with the tissues collected for further analysis. All animal experiments were done in accordance with the Institutional Animal Ethics Committee and the University of South China Animal Care Guidelines for Use of Experimental Animals. All protocols and procedures were approved by the Institutional Animal Care and Use Committee.

#### Biochemical Variables in Terminal Plasma Analysis

Mice were fasted overnight and euthanized, and blood samples were obtained from the retro-orbital plexus. Triglyceride (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) were determined by commercial enzymatic methods (Test kits, Shanghai Rongsheng Biotech Inc, Shanghai, China). Plasma levels of AOPPs were determined by spectrophotometry as previously described.\(^15\) The assay was calibrated using chloraamine-T. The absorbance was read at 340 nm and AOPP concentrations were expressed as \(\mu\)mol/L of chloraamine-T equivalents. The levels of tumor necrosis factor-alpha (TNF-\(\alpha\)) and interleukin-1beta (IL-1\(\beta\)) in plasma were measured using ELISA kits strictly according to the manufacturer’s instructions.

#### Histology and Morphometric Analyses

Right after removal of the upper portion of the heart and proximal aorta, the pieces of aorta and liver tissues were immersed in Optimal Cutting Temperature solution on dry ice and stored at \(-80^\circ\)C. Serial 10-\(\mu\)m-thick cryosections of aorta starting from the aortic root were collected at a distance of 400\(\mu\)m. The aorta sections were stained by Oil red O, Hematoxylin-Eosin (H&E) and Masson’s Trichrome (MT) as described previously.\(^16,17\) The liver sections were stained only by Oil red O and H&E. Collagen content was assessed by MT staining of consecutive slides from serial sections. Image-Pro Plus image analysis software (Media Cybernetics, Shanghai, China) was used for all quantifications. The macrophage accumulation in atherosclerotic lesions was evaluated by CD68 immunohistochemistry staining. The lipid accumulation of the liver was quantified using the hepatic lipid accumulation assay kit (Abcam Trading (Shanghai) Company, Shanghai, China) according the kit instruction. The dye was eluted by adding 100% isopropanol and the extracts were determined by measuring the absorbance at 490 nm. Protein oxidation was assayed using a nitrotyrosine ELISA kit (Abcam) according to the manufacturer’s instructions, as described previously.\(^18\)

#### Macrophage-Specific Reverse Cholesterol Transport (RCT) In Vivo

To assay macrophage-specific reverse cholesterol transport in vivo, the radiolabeled cholesterol from prepared macrophages were used as described previously.\(^19,20\) J774 macrophages were cholesterol-loaded with oxidized low-density lipoprotein (ox-LDL) to become foam cells and labeled with \(^{3}\text{H}\)-cholesterol. Then, the macrophages were injected intraperitoneally into apoE-KO mice, which were treated by AOPPs (5 mg/kg) and/or AG-490 (5 mg/kg). At 48 h, the blood, collected via the retro-orbital plexus from anesthetized mice, was centrifuged for plasma supernatant. The liver and gall bladder were isolated. Feces were collected continuously over 48 h. \(^{3}\text{H}\)-cholesterol levels were measured by liquid scintillation counting.
The data are expressed as mean ± SD, n = 10. *P < 0.01 vs. the control group; #P < 0.05 vs. the AOPP group.

**Results**

Regulation of Plasma Lipids and Inflammatory Factors by AOPPs and AG490 in apoE-KO Mice

We determined the efficacy of AOPP and AG-490 treatments by measuring circulating lipid and inflammatory response levels in apoE-KO mice fed a HFHC diet. All animals survived through the course of the experiment. We examined the terminal levels of plasma lipids and inflammatory factors in all experimental mice. As shown in Table 1, AOPPs increased the beled counts in the plasma, liver, bile and feces were expressed as a percentage of total [3H]-cholesterol injected.

**Peritoneal Macrophage (PM) Isolation and Culture**

Elicited PMs were collected 4 days after injection of 1 ml of 10% thioglycolate into the peritoneal cavity of 9–15-week-old mice as described previously.21 PMs were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium and plated on tissue culture plates. Two hours later, non-adherent cells were removed by washing with PBS, and adherent macrophages were used for the studies.

**Cellular Cholesterol Content Detection and Cholesterol Efflux Experiments**

Cellular cholesterol content was detected by using high performance liquid chromatography (HPLC) as described previously.21 Cells were labeled with [3H]-cholesterol (0.2 μCi/ml) for the experiments of cellular cholesterol efflux. Medium and cell-associated [3H]-cholesterol was then measured by liquid scintillation counting. Percent efflux was calculated by using the following equation: (total media counts/(total cellular counts+total media counts))×100.

**Measurement of Cellular Phospholipids and TGs**

Ceramides (CM), sphingomyelins (SM) and phosphatidylcholines (PC) were measured in lipid extracts as described previously.22,23 In brief, cells were labeled with [3H]-cholesterol (0.2 μCi/ml) and phospholipid subclasses were analyzed by thin-layer chromatography (TLC). The spots were scraped, and their radioactivity was quantitated in a liquid scintillation counter. The phospholipids content was normalized by cell protein (dpm/mg cell protein). TG levels were measured by Glyceral Phosphate Oxidase-Peroxidase method using a commercial assay kit (BioSino Biotechnology & Science Inc, Beijing, China) The TG content was normalized by cell protein (mg/g cell protein).

**Western Immunoblotting**

Protein (20 μg from lysates) was loaded on a 8% SDS-polyacrylamide electrophoresis gel and was electrophoresed for 2 h at 100 V in buffer, and transferred to polyvinylidene fluoride (PVDF) membranes. Immunoreactivity was detected by using the enhanced chemiluminescence (ECL) test. Protein content was calculated by densitometry using Labworks analysis software (Shenteng, Shanghai, China).

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction Analysis (qPCR)

Total RNA was extracted using TRizol reagent. qPCR using SYBR Green detection chemistry, was performed on a Roche light Cycler Run 5.32 Real-Time PCR System (Roche, Mannheim, Germany). The sequences of the real-time PCR primers are as follows: ABCA1, forward primer 5'-GATTGGCTTCAGGATGTCCATGTTGGAA-3'; reverse primer 5'-GTATTTTGTCAAGGCTACCAGTTACATTGACCA-3'; LXRα, forward primer 5'-AGGGCCATCAGAGGACAGACGTTTCGAC-3'; reverse primer 5'-GGGACAGAACAGTATTCG-3', ABCG1, forward primer 5'-GATTTGCTTCAGGATGTCCATGTTGGAA-3'; reverse primer 5'-GTATTTTGTCAAGGCTACCAGTTACATTGACCA-3'; and the expression of β-actin was used as the internal control.

**Statistical Analysis**

All data are presented as mean ± SD. Results were analyzed by using one-way ANOVA and Student’s t-test using SPSS 16.0 software. A probability value < 0.05 was regarded as significant.

**Small Interference RNA (siRNA) Transfection**

Predesigned siRNA targeting JAK and control non-silencing siRNA were synthesized by Biology Engineering Corporation (Shanghai, China). Cells were transfected with siRNA for JAK or control siRNA using Lipofectamine 2000 (Invitrogen, CA, USA) and the efficacy of gene silencing was evaluated by Western blotting.

**Table 1.** Biochemical Variables in Terminal Plasma of apoE-KO Mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>AOPPs</th>
<th>AG-490</th>
<th>AOPPs and AG-490</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.90±3.32</td>
<td>28.6±2.69</td>
<td>27.5±2.96</td>
<td>29.2±3.04</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.96±0.56</td>
<td>2.49±0.65</td>
<td>2.01±0.49</td>
<td>2.25±0.55</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>15.20±3.43</td>
<td>19.63±4.11</td>
<td>15.31±3.7</td>
<td>18.33±3.98</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>3.36±1.02</td>
<td>4.25±0.89</td>
<td>3.50±0.81</td>
<td>4.90±0.77</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>11.62±2.06</td>
<td>15.20±2.90</td>
<td>12.12±2.19</td>
<td>13.23±2.56</td>
</tr>
<tr>
<td>AOPPs (μmol/L)</td>
<td>36.38±4.51</td>
<td>73.86±9.55</td>
<td>39.27±3.33</td>
<td>51.67±8.25</td>
</tr>
<tr>
<td>TNFa (pg/ml)</td>
<td>10.22±1.19</td>
<td>27.56±3.75</td>
<td>12.06±2.03</td>
<td>20.54±3.51</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>6.58±1.03</td>
<td>15.24±2.09</td>
<td>7.37±1.35</td>
<td>12.59±2.05</td>
</tr>
</tbody>
</table>

apoE-KO, apolipoprotein E knockout; AOPPs, advanced oxidation protein products; HDL-C, high-density lipoprotein cholesterol; IL-1β, interleukin-1β; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride; TC, total cholesterol; TNF-α, tumor necrosis factor.

All mice were fed a high-fat/high-cholesterol diet (15% fat wt/wt, 0.25% cholesterol wt/wt). The AOPP group received intraperitoneal injection of AOPPs (5 mg/kg) once every other day. The AG-490 group received intraperitoneal injection of AG-490 (5 mg/kg) once every other day. The AOPP and AG-490 group received the same volume of AOPPs and AG-490 once every other day. The control animals received an intraperitoneal injection of the volumes of normal saline.
Figure 1. Evaluation of atherosclerotic lesions at the aortic root region of apolipoprotein E knockout (apoE-KO) mice. The apoE-KO mice were fed a high-fat/high-cholesterol diet and treated with advanced oxidation protein products (AOPPs) and/or AG-490, as described in the Methods section. (A–D) Representative micrographs of histological analysis of cross-sections from the aortic sinus stained with Oil red O (A, ×40), Hematoxylin-Eosin (B, arrow to necrotic cores, ×40), Masson's Trichrome (MT, C, ×40), and CD68 immunohistochemical staining (D, the positive area is brown, ×400). (E–J) Quantification of the lesion areas (E); the cross lesion area was expressed as the percentage of total aortic section area (F); the fibrous cap area was expressed as the percentage of the total plaque area (G); quantification of necrotic cores and the CD68 positive area were shown as the percentage of total plaque area (H, I); and protein oxidation was shown as nitrotyrosine concentration using the ELISA kit (J). All data are presented as mean±SEM (3 equally spaced sections per mouse; n=3 mice per group). Original magnification, ×100. *P<0.01 vs. the control group; #P<0.01 vs. the AOPPs group.
effects of AOPPs and AG-490 on hepatic injury and lipids accumulation in the livers of apoE-KO mice

In the current view of macrophage RCT, macrophage-derived cholesterol is delivered to the liver because the liver plays an important role in RCT. We determined hepatic injury and lipid accumulation by H&E (Figure 2A) and Oil red O staining (Figure 2B). We found that the liver structure was damaged, as indicated by liver lobule structure disorder and increased lipid droplet (Figure 2C) in AOPP groups, but those liver damages were alleviated in mice injected with both AOPPs and AG-490.

AOPPs Decrease the Expression of ABCA1, ABCG1 and LXRα in apoE-KO Mouse Aorta, Liver and Small Intestine

We determined the efficacy of the AOPP and/or AG-490 treatments by measuring the expression of ABCA1, ABCG1 and LXRα in mouse aorta, liver and small intestine. Quantitative PCR and Western blot analysis revealed that the above tissues from apoE-KO mice showed significant downregulation of ABCA1, ABCG1 and LXRα expression in the AOPPs-treated group, but not in the group treated with both the JAK inhibitor AG-490 and AOPPs. Instead, the expression of ABCA1, ABCG1 and LXRα was upregulated in mice treated with both AG-490 and AOPPs, compared with the group treated with AOPPs alone (Figure 3).

Effects of AOPPs and AG-490 on Hepatic Injury and Lipids Accumulation in the Livers of apoE-KO Mice

In the current view of macrophage RCT, macrophage-derived cholesterol is delivered to the liver because the liver plays an important role in RCT. We determined hepatic injury and lipid accumulation by H&E (Figure 2A) and Oil red O staining (Figure 2B). We found that the liver structure was damaged, as indicated by liver lobule structure disorder and increased lipid droplet (Figure 2C) in AOPP groups, but those liver damages were alleviated in mice injected with both AOPPs and AG-490.

AOPPs Accelerate Atherosclerosis in apoE-KO Mice, Which Is Attenuated by AG-490

Continuous accumulation of atherosclerotic plaque is one of the major risk factors for cardiovascular disease. We examined the development of atherosclerotic lesions by analysis of cross-sections of the aortic roots of mice. We found that atherosclerotic lesion areas and necrotic core areas were significantly increased in the aortic roots of mice injected with AOPPs, compared with those in control groups. We also found that atherosclerotic lesions and necrotic areas were reduced in the aortic roots of mice injected with AOPPs and AG-490 compared with those in the groups injected with AOPPs alone (Figures 1A,B). Further analysis of atherosclerotic lesions revealed that AOPPs increased the fibrous cap areas, macrophage accumulation and protein oxidation, while AG490 attenuated the effects of AOPPs in apoE-KO mice fed a HFHC diet (Figures 1C,D).
AOPPs Exacerbates Atherosclerosis

We analyzed the $[{}^3{}^H{}]$-labeled counts of hepatic, bile and feces for secreting secretion from the liver to bile and feces. Compared with control mice, hepatic $[{}^3{}^H{}]$-labeled counts revealed an increase and bile counts were markedly reduced in mice treated with AOPPs. A reduction in fecal counts was also observed in mice treated with AOPPs alone, hepatic $[{}^3{}^H{}]$-labeled counts were shown to increase (Figure 4B), but bile and fecal counts revealed a decrease in mice treated with both AOPPs and AG-490 (Figures 4C,D).

Effects of AOPPs and AG-490 on Macrophage-Specific RCT In Vivo

The effects of AOPPs and AG-490 on $[{}^3{}^H{}]$-cholesterol movement from intraperitoneal macrophages into the plasma were determined by analysis of plasma $[{}^3{}^H{}]$-labelled counts. The results showed a reduction in plasma counts in AOPPs-treated mice compared with the control mice. While compared with mice treated with AOPPs only, plasma $[{}^3{}^H{}]$-labeled counts increased in mice treated with both AOPPs and AG-490 (Figure 4A).

Figure 3. ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1) and liver X receptor (LXRα) expression in the aorta, liver and small intestine of apolipoprotein E knockout (apoE-KO) mice. The mRNA expression of ABCA1, ABCG1 and LXRα was assessed by using quantitative polymerase chain reaction in the aorta (A), liver (C) and small intestine (E). The protein expression of ABCA1, ABCG1 and LXRα was measured by Western blotting in the aorta (B), liver (D) and small intestine (F). All data represent the mean±SEM from 3 independent experiments, each performed in triplicate. *P<0.05 vs. the control group; #P<0.05 vs. the AOPPs group.
Effects of AOPPs and AG-490 on ABCA1 and ABCG1 Expression, and Cholesterol Efflux in PMs

The effects of AOPPs on ABCA1 and ABCG1 expression were examined by real-time quantitative PCR and Western blot assays in PMs. AOPPs downregulated ABCA1, ABCG1, LXRα mRNA and protein expression (Figures 5A,B). In the PMs treated with AG-490 and AOPPs, however, the expression of ABCA1, ABCG1 and LXRα was upregulated when compared with the AOPPs only group (Figures 5A,B). Furthermore, cellular cholesterol efflux was decreased in cells treated with AOPPs, but co-treatment with AOPPs and AG-490 increased the cellular cholesterol efflux (Figure 5E). Meanwhile, AOPPs increased cellular cholesterol, TG and phospholipid (CM, SM and PC) contents, but it was decreased those in mice co-treatment with AG-490 and AOPPs in comparison of AOPPs treating alone (Tables 2,3).

In addition, cells were transfected with JAK siRNA and/or treated with AOPPs (100 μmol/L), respectively. In the cells treated with AOPPs-puls-JAK siRNA, however, the expressions of ABCA1, ABCG1 and LXRα were upregulated in comparison with the AOPPs alone (Figures 5C,D). Then, we further examined the cholesterol efflux in PMs by using liquid scintillation count. Cellular cholesterol efflux was decreased in cells treated with AOPPs, then it was increased in cells treated with AOPPs-puls-JAK siRNA (Figure 5F).

Discussion

Advanced oxidation protein products have emerged as a novel class of renal pathogenic mediators. Plasma AOPPs are mainly carried by albumin, and their concentration closely correlates with the level of dityrosine, a hallmark of oxidized protein. Therefore, AOPPs have been considered as the markers of oxidant-mediated protein damage. AOPPs are also involved in the further development of oxidative stress and inflammation by the activation of immune cells. Accumulation of AOPPs has been reported in various pathologies and is also associated with impaired carbohydrate metabolism.

The lipid metabolism plays a key role in atherogenesis and contributes to atherosclerotic plaque formation in vivo. The previous studies demonstrate a strong relationship between levels of plasma lipids and the incidence of atherosclerotic...
AOPPs Exacerbates Atherosclerosis

AOPPs accelerate the progression of atherosclerosis, through a mechanism that might involve increasing the plasma levels of TG, TC and LDL-C in apoE-KO mice fed an atherogenic diet.

Both ABCA1 and ABCG1 play a pivotal role in the removal of excess intracellular cholesterol and phospholipids to lipid-poor apolipoprotein acceptors in the initial step of RCT.

In the present study, we also evaluated the effects of AOPPs on the plasma lipid profiles in the atherosclerosis-prone apoE-KO mice fed an atherogenic diet. The levels of plasma lipids were shown in Table 1. Significant increases were found in TC, TG and LDL-C of apoE-KO mice treated with AOPPs, compared with the control mice. Meanwhile, AOPPs promoted the increase in lipids accumulation in the aortic sinus plaques and livers in apoE-KO mice, as shown in Figures 1A, 2B by Oil red O staining. Additionally, we found that AOPPs exacerbated the atherosclerotic lesions in the aortic sinus, as shown in Figure 1. These findings suggest that there are significant correlations between changes of atherosclerotic lesions and plasma lipid levels in AOPPs-treated mice. AOPPs accelerate the progression of atherosclerosis, through a mechanism that might involve increasing the plasma levels of TG, TC and LDL-C in apoE-KO mice fed an atherogenic diet.

Both ABCA1 and ABCG1 play a pivotal role in the removal of excess intracellular cholesterol and phospholipids to lipid-poor apolipoprotein acceptors in the initial step of RCT. In
leads to the formation of fatty streaks, complex lesions, and from the accumulation of excess cholesterol in macrophages, 32,33. These findings suggest that AOPPs impair the RCT in vivo and found that AOPPs can decrease the specific RCT in vivo and found that AOPPs can decrease the reverse cholesterol transport (RCT) and inter-cellular adhesion molecule 1 (ICAM-1) at both gene and protein levels, 33 suggesting that overexpression of adhesion molecules is the pro-inflammatory pathway of AOPPs in vECs.

Reverse cholesterol transport is a complex process ensuring the efflux of cholesterol from peripheral cells and its transport back to the liver for its metabolism and biliary excretion. The abnormalities of the various components of RCT accelerate atherosclerosis. 34 Therefore, we assayed the macrophage-specific RCT in vivo and found that AOPPs can decrease the [1H]-cholesterol counts in plasma, liver, bile and feces of apoE-KO mice. These results suggest that AOPPs impair the RCT at multiple steps in the RCT pathway, including the macrophage cholesterol efflux and the cholesterol flux through the liver to bile and feces. The impairment of RCT might contribute to the accumulation of lipids and exacerbation of atherosclerosis induced by treatment with AOPPs in apoE-KO mice.

To gain further insights into the relationship between AOPPs and ABCA1 in modulation of atherosclerosis and cholesterol efflux of cells, we evaluated the mechanism underlying the effects of AOPPs in apoE-KO mice. LXR forms a heterodimer with the retinoid X receptor (RXR), which binds to the LXR-
response element in the promoter sequence of ABCA1. LXR can upregulate ABCA1 expression through forming heterodimers with RXR. In the current study, we found that the LXRe expression was significantly downregulated in AOPPs-treated apoE-KO mice and PMs. These results suggested that LXRe was involved in the downregulation of ABCA1 and ABCG1 induced by AOPPs.

The JAK/STAT pathway is considered a stress-responsive signaling cascade that transduces signals from cell surface receptors to the nucleus, thereby modulating gene expression as the compensatory mechanism. To date, various vascular stress factors are described linking activation of the JAK/STAT signaling pathway to vascular diseases. Our previous data showed that IFN-γ might decrease the expression of ABCA1 through the JAK/STAT signaling pathway in THP-1 macrophage-derived foam cells. In the study, we investigated the effects of the JAK inhibitor (AG-490) on ABCA1 and ABCG1 expression and atherosclerosis in AOPPs-treated apoE-KO mice. We found that the plasma lipid levels were decreased, the ABCA1, ABCG1 and LXRα expressions in the aorta were upregulated, the lesions in the aortas were decreased, and the lipid content in aortic sinus plaques and livers were decreased in the AOPPs and AG-490 group mice in comparison with the AOPP group, suggesting a role of the JAK/STAT signaling pathway in the effects of AOPPs on ABCA1 and ABCG1 expression and lipid accumulation.

In summary, our study showed that AOPPs can exacerbate lipid accumulation and atherosclerosis in apoE-KO mice, downregulate the ABCA1 and ABCG1 expression, and inhibit the cholesterol efflux in PMs. Expression of LXRs is also inhibited by AOPPs, which can be reversed by a JAK inhibitor, AG-490. The main conclusion that can be drawn from our studies is that there is an interrelationship between AOPPs and cholesterol efflux, in which AOPPs might first downregulate the expression of LXRs through the JAK/STAT signal pathway and then decrease the ABCA1 and ABCG1 expression and cholesterol efflux. These data suggest that treatment with pharmacological agents that decrease the AOPP levels may be an effective approach for reducing atherosgenic risk in humans.

Acknowledgments
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**Supplementary Files**

**Supplementary File 1**

**Methods**

**Results**

**Figure S1.** SR-BI, SREBP-1 and CETP expression in the aorta and peritoneal macrophages (PMs) of apolipoprotein E knockout (apoE-KO) mice.

**Figure S2.** The role of advanced oxidation protein products (AOPPs) on ATP-binding cassette transporter A1 (ABCA1) and ATP-binding cassette transporter G1 (ABCG1) expression in vascular smooth muscle cells (vSMCs) and vascular endothelial cells (vECs).

**Figure S3.** Effect of advanced oxidation protein products (AOPPs) on cholesterol efflux in peritoneal macrophages (PMs) treated with small interference RNA (siRNA).