Pregnancy-Associated Plasma Protein-A Accelerates Atherosclerosis by Regulating Reverse Cholesterol Transport and Inflammation

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Background: Recent studies have suggested that pregnancy-associated plasma protein-A (PAPP-A) is involved in the pathogenesis of atherosclerosis. This study aims to investigate the role and mechanisms of PAPP-A in reverse cholesterol transport (RCT) and inflammation during the development of atherosclerosis.

Methods and Results: PAPP-A was silenced in apolipoprotein E (apoE−/−) mice with administration of PAPP-A shRNA. Oil Red O staining of the whole aorta root revealed that PAPP-A knockdown reduced lipid accumulation in aortas. Oil Red O, hematoxylin and eosin (HE) and Masson staining of aortic sinus further showed that PAPP-A knockdown alleviated the formation of atherosclerotic lesions. It was found that PAPP-A knockdown reduced the insulin-like growth factor 1 (IGF-1) levels and repressed the PI3K/Akt pathway in both aorta and peritoneal macrophages. The expression levels of LXRα, ABCA1, ABCG1, and SR-B1 were increased in the aorta and peritoneal macrophages from apoE−/− mice administered with PAPP-A shRNA. Furthermore, PAPP-A knockdown promoted RCT from macrophages to plasma, the liver, and feces in apoE−/− mice. In addition, PAPP-A knockdown elevated the expression and secretion of monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), tumor necrosis factor-α, and interleukin-1β through the nuclear factor kappa-B (NF-κB) pathway.

Conclusions: The present study results suggest that PAPP-A promotes the development of atherosclerosis in apoE−/− mice through reducing RCT capacity and activating an inflammatory response.

Key Words: Atherosclerosis; Inflammation; Pregnancy-associated plasma protein-A; Reverse cholesterol transport

Atherosclerosis is one of the leading causes of mortality worldwide. Dysregulation of lipid metabolism and inflammation are the pivotal etiologies for the progression of atherosclerosis. When vascular endothelial cells are damaged, macrophages are recruited to the lumen and then transmigrate to the subintimal space, where they take up oxidized low-density lipoprotein (ox-LDL) and undergo foam cell formation, leading to the development of atherosclerosis. Notably, macrophages accumulated in the sub-endothelium also secrete inflammatory factors to accelerate atherosclerosis. Reverse cholesterol transport (RCT) is the process whereby the excess cholesterol is removed from peripheral tissues, such as macrophages by high-density lipoprotein (HDL), and eventually delivered to the liver.
in vivo, remains unclear. Therefore, we used shRNA to knock down the expression of PAPP-A in apoE−/− mice and then examined the effects of PAPP-A knockdown on RCT, inflammation, and atherosclerosis in vivo and the underlying mechanisms.

**Methods**

**Animal Studies**

Male apoE−/− mice (8 weeks old) were purchased from Changzhou Cavens Laboratory Animal Co. LTD (Jiang Su, China). ApoE−/− mice were injected via the tail vein with 1×10¹¹ genome copies (GCs) of AAV-shRNA (TCT CGCTTGGGCGAGAGTA) or AAV-PAPP-A shRNA (GCAACAGATCCA CGCTACT) (Genechem, China) once at the beginning of the experiment. Animals were fed a Western-type diet and then examined the effects of PAPP-A knockdown on RCT, inflammation, and atherosclerosis in vivo and the underlying mechanisms.
Role of PAPP-A on RCT and Atherosclerosis

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)
To evaluate the gene expression in tissues or cells from mice, total RNAs were extracted using TRIzol reagent (Beyotime, China), following the manufacturer’s instructions. The complementary DNA was synthesized with a high-capacity cDNA reverse transcription kit (Takara, China). qRT-PCR analyses were performed on the iCycler IQ Real-Time Detection System (Bio-Rad, U.S.A) using the following primer pairs: ABCA1 Primers, Forward 5'-GGGTGGTGTTCTTCCTCATTAC-3' and Reverse 5'-GAATGACGAGGATGAGGATGTG-3'; ABCG1 Primers, Forward 5'-CCTGACACATCTCGAGAACATCA-3' and Reverse 5'-GAGGAACAGCATGGAGAAGAA-3'; SR-BI Primers, Forward 5'-TTTGGATTGTGGTAGTAAAAAGGGC-3' and Reverse 5'-TGACATCAGGGACTCAGAGTAG-3'; LXRα Primers, Forward 5'-CTCAATGCCTGATGTTTCTCCT-3' and Reverse 5'-TCCAACCCTATCCCTAAGGAAAAAGGGC-3' and Reverse 5'-TCCAACCCTATCCCTAAGGAAAAAGGGC-3'; Reverse 5'-TCCAACCCTATCCCTAAGGAAAAAGGGC-3' and Reverse 5'-TCCAACCCTATCCCTAAGGAAAAAGGGC-3'; GAPDH Primers, Forward 5'-TGGATTTGGACGCATTGGTC-3' and Reverse 5'-TTTGCACTGGTACGTGTTGAT-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control.

Western Blotting Assay
Total proteins were extracted from cells or tissues using RIPA buffer (Beyotime) containing phenylmethylsulfonyl fluoride (Beyotime) to inhibit proteases. Protein concentrations were measured using a BCA Protein Assay Kit (Beyotime). The primers were obtained from Bio-Rad, USA. The primers were used for the following primer pairs: ABCA1 Primers, Forward 5'-GGGTGGTGTTCTTCCTCATTAC-3' and Reverse 5'-GAATGACGAGGATGAGGATGTG-3'; ABCG1 Primers, Forward 5'-CCTGACACATCTCGAGAACATCA-3' and Reverse 5'-GAGGAACAGCATGGAGAAGAA-3'; SR-BI Primers, Forward 5'-TTTGGATTGTGGTAGTAAAAAGGGC-3' and Reverse 5'-TGACATCAGGGACTCAGAGTAG-3'; LXRα Primers, Forward 5'-CTCAATGCCTGATGTTTCTCCT-3' and Reverse 5'-TCCAACCCTATCCCTAAGGAAAAAGGGC-3'; GAPDH Primers, Forward 5'-TGGATTTGGACGCATTGGTC-3' and Reverse 5'-TTTGCACTGGTACGTGTTGAT-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control.

Atherosclerosis Analysis
The aortas and hearts were collected immediately from euthanized mice and fixed using 4% paraformaldehyde. The whole aortas were stained with Oil Red O and the en face staining of the aortas was photographed with a digital camera. The aortic roots were frozen with optimal cutting temperature compound (O.C.T. compound; Sakura Finetek, USA, Inc., Torrance, CA, USA) and serial sections (8-μm thick) were cut throughout the three aortic valves. The sections were stained with Oil Red O, HE, and Masson’s trichrome staining for the quantification of plaque cross-sectional area. Lesion areas and percentages were quantified with Image Pro Plus software.

Plasma Lipid Profiles
Blood samples from the apoE−/− mice were collected into Ethylene Diamine Tetraacetic Acid (EDTA)-coated tubes, and plasma was isolated by centrifugation (5,000 rpm, 4°C, 10 min). The levels of total cholesterol (TC), triglycerides (TG), and HDL-C in the plasma samples were detected in the clinical laboratory of the Affiliated Nanhua Hospital, University of South China (Hengyang, China). The levels of low-density lipoprotein cholesterol (LDL-C) were calculated by subtracting the levels of HDL-C and 0.2×TG from that of TC.

Reverse Cholesterol Transport Assay
ApoE−/− mice were treated with control or PAPP-A shRNA and fed a Western-type diet for 8 weeks. J774 macrophages were treated with 50 μg/mL acetylated low-density lipoprotein (ac-LDL) (Yiyuan Biotechnologies, China) and loaded with 5μCi/mL [3H] cholesterol for 24h. J774 cells labeled with [3H] cholesterol (~4.5×10^8 cells/mouse, n=5 mice/group) were intraperitoneally injected into an individual mouse, which was randomly selected from each group. After injection, mice were housed individually and plasmas were collected from mice at 6, 24, and 48 h after injection, followed by the measurement with a scintillation counter. Feces were collected continuously until 48 h, and dissolved in ethanol for counting. Mice were euthanized 48 h after the injection, and lipids were extracted from the liver for measurement. The data were shown as the percentage of counts.

Figure 2. The reverse cholesterol transport (RCT) is increased in the apoE−/− mice with PAPP-A knockdown. [3H]-cholesterol and acetylated LDL-loaded J774 macrophages were injected into apoE−/− mice (n=5 mice/group). The amounts of [3H]-tracer in plasma (A), the liver (B), and feces (C) were determined by scintillation counting. Data are presented as the mean±SD. *P<0.05 (**P<0.01) indicates a significant difference compared with Control groups at the same time. Abbreviations as in Figure 1.

Advance Publication
The absorbance at 450 nm was determined using a Bio-
Rad iMark microplate reader.

**Immunofluorescence (IF)**

The frozen sections of aortic roots were fixed in 4% para-
formaldehyde and permeabilized with 0.1% Triton X-100
for 20 min. No permeabilization was performed for ABCA
1 and ABCG1 staining. The samples were blocked in goat
serum for 30 min at room temperature, followed by an
incubation with primary antibodies for CD68 (ab955, mouse
monoclonal antibody, 1:200; ABCAM, UK), HSP70
(AF0189, rabbit polyclonal antibody, 1:200, Beyotime,
China), ABCA1 (ab18180, mouse monoclonal antibody,
1:200; ABCAM), or ABCG1 (ab52617, rabbit polyclonal
antibody, 1:100; ABCAM) for 3.5 h at room temperature

**Enzyme-Linked Immunosorbent Assay (ELISA)**

The ELISA kits were used to determine the levels of inflam-
mation factors (MCP-1, IL-6, TNF-α and IL-1β)
(TermoFisher Scientific, USA) and IGF-1 (R&D Systems,
UK) according to the standard protocol. Briefly, serum
samples from apoE−/− mice or cell culture medium were
added into each well and incubated at room temperature
for 120 min. Then, the biotin-conjugated antibody was
added, followed by incubation for 90 min. Thereafter,
the substrate solution was added into the well and incubated
for 30 min. After washing five times with 0.01 mol/L tris
buffered saline (TBS), 3,3’5,5’tetramethylbenzidine
(TMB) was added and incubated for 30 min in the dark.

**Figure 3.** Expression of ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette transporter G1 (ABCG1) and scav-
egenger receptor class B type 1 (SR-B1) is enhanced through the insulin-like growth factor (IGF)-1-Pi3K-Akt pathway in the aorta of
the apoE−/− mice with PAPP-A knockdown. Total RNA was isolated from the aorta and then subjected to quantitative real-time
polymerase chain reaction (qRT-PCR) to quantify mRNA levels of ABCA1 (A), ABCG1 (B), SR-B1 (C), and LXRα (G). The levels of
each target were normalized to that of GAPDH at the same condition. The same amount of protein lysate isolated from aorta
homogenate was subjected to western blot analysis to detect ABCA1 (A), ABCG1 (B), SR-B1 (C), p-PI3K (E), p-Akt (F) and LXRα
(G). The levels of IGF-1 in serum (D) measured by enzyme-linked immune sorbent assay are shown. Data are presented as the
mean±SD (n=5 mice/group). *P<0.05 (**P<0.01) indicate significant difference compared with Control groups. Abbreviations as
in Figure 1.

**Table.** Effects of the PAPP-A Knockdown on the Plasma Lipids of Western Diet-Fed Apolipoprotein E (apoE−/) Mice

<table>
<thead>
<tr>
<th></th>
<th>BW</th>
<th>TC</th>
<th>HDL-C</th>
<th>LDL-C</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33.19±0.78</td>
<td>30.22±3.59</td>
<td>1.54±0.39</td>
<td>28.39±3.69</td>
<td>1.44±0.36</td>
</tr>
<tr>
<td>shRNA</td>
<td>32.11±0.91</td>
<td>29.56±3.16</td>
<td>1.50±0.42</td>
<td>27.76±3.22</td>
<td>1.50±0.41</td>
</tr>
<tr>
<td>PAPP-A shRNA</td>
<td>33.75±1.01</td>
<td>31.38±4.04</td>
<td>2.08±0.33</td>
<td>28.97±4.17</td>
<td>1.67±0.34</td>
</tr>
</tbody>
</table>

Enzymatic methods were used to measure the levels of plasma lipids (mmol/L) including TC, total cholesterol; LDL-C, low-density lipoprotein
cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglyceride. BW, body weight (g); PAPP-A, pregnancy-associated plasma protein
A. All values are shown as mean± SD (n=10), *P<0.05 vs. Control .
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Statistical Analysis

All data were expressed as means±SD and evaluated using Student’s t-test of two groups or one-way ANOVA with Tukey’s post hoc test or Dunnett’s post hoc. Statistical analyses were performed using GraphPad software (version 7.0). The difference was significant when P values were less than 0.05.

Results

PAPP-A Knockdown Attenuates Atherosclerotic Plaque Formation in ApoE−/− Mice

To explore the role of PAPP-A in atherosclerosis in vivo, we knocked down the PAPP-A expression in apoE−/− mice using adeno-associated virus-short hairpin RNA (AAV-
shRNA). The mice were then fed a Western-type diet for 8 weeks. Injection of AAV-shRNA did not cause any changes in mortality (data not shown). The plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also comparable among different groups (Supplementary Figure 1), indicating that AAV-shRNA did not cause obvious liver damage. The levels of PAPP-A in the plasma (Supplementary Figure 2), the aorta, and peritoneal macrophages (Supplementary Figure 3) were significantly increased in apoE−/− mice on the Western-type diet compared to the wild-type mice on the Western-type diet and apoE−/− mice on a regular chow diet. The expression of PAPP-A in the aorta and peritoneal macrophages was measured to confirm knockdown efficiency. We found that the levels of PAPP-A were dramatically reduced in mice injected with AAV-PAPP-A shRNA (Figure 1A,B). AAV-shRNA also significantly reduced plasma levels of PAPP-A in apoE−/− mice on the Western-type diet (Supplementary Figure 2). Addition, we also measured the blood pressure, blood glucose and insulin of AAV-PAPP-A shRNA treated mice and the results showed that their levels didn’t change (Supplementary Table). We then examined the effects of PAPP-A knockdown on the development of atherosclerosis. Oil Red O staining revealed that the lipid-laden plaque areas in the aortic arch regions (Figure 1C) and the entire en face aorta (Figure 1D,E) were significantly decreased in apoE−/− mice injected with PAPP-A shRNA. Consistently, HE, Oil Red O, and Masson staining of cross-sections of the aortic root showed that PAPP-A knockdown significantly reduced plaque formation in apoE−/− mice (Figure 1F,G). CD-68-positive macrophages were also significantly reduced in the plaques of apoE−/− mice injected with PAPP-A shRNA (Supplementary Figure 4). These findings suggest that PAPP-A plays an important role in atherosclerotic plaque formation.

PAPP-A Knockdown Increases the Efficiency of RCT in ApoE−/− Mice

The dysregulation of lipid metabolism plays a critical proatherogenic role and the efficiency of RCT is associated with the progress of atherosclerosis. So, we examined the effect of PAPP-A on RCT in apoE−/− mice. [3H]-cholesterol-loaded J774 cells were injected into Western diet-fed mice transduced with PAPP-A shRNA. The plasma levels of macrophage-derived [3H]-tracer in mice with PAPP-A shRNA were significantly increased 24h and 48h after injection (Figure 2A). The level of tracer was also increased in the liver and feces of PAPP-A knockdown apoE−/− mice 48h after injection of [3H]-cholesterol-loaded J774 cells (Figure 2B,C). Taken together, these observations indicate that PAPP-A reduces the efficiency of RCT to accelerate atherosclerosis in apoE−/− mice.

PPAP-A Knockdown Increases the Expression of ABCA1, ABCG1, and SR-B1 in ApoE−/− Mice

Considering that the HDL is a major player of RCT and that cholesterol efflux is the first rate-limiting step in RCT, we detected plasma levels of lipids in mice and found that PAPP-A knockdown had no detectable effect on plasma levels of TC, LDL-C, and TG, but significantly increased the levels of HDL-C (Table). Given that ABCA1, ABCG1, and SR-B1 play important roles in HDL metabolism, we measured their expression in apoE−/− mice and found that knockdown of PAPP-A expression significantly increased their expression in the aorta (Figure 3A–C). In contrast, the expression of LXRα and its target genes such as CYP7a1, SREBP1c, and IDOL was increased in the liver of PAPP-A knockdown apoE−/− mice (Supplementary Figure 5). We then detected the levels of cholesterol efflux in the primary peritoneal macrophages and found that knockdown of PAPP-A promoted cholesterol efflux (Figure 4H,I). Our data suggest that PAPP-A reduces the levels of HDL-C and the expression of ABCA1, ABCG1, and SR-B1 to suppress the cholesterol efflux and RCT, leading to the promotion of atherosclerosis.
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and the nuclear translocation of p65. Our results revealed that both expression and secretion of MCP-1 (Figure 6B), TNF-α (Figure 6C), IL-6 (Figure 6D) and IL-1β (Figure 6E) were reduced in mouse peritoneal macrophages (MPMs) isolated from apoE−/− mice with PAPP-A knockdown in response to lipopolysaccharide (LPS). In addition, we found that the nuclear translocation of p65 was blocked when the PAPP-A was downregulated (Figure 6F), suggesting the involvement of the NF-κB pathway in PAPP-A-induced inflammation.

Discussion

Lipid accumulation and local inflammation in the vascular wall play a pivotal role in the progression of atherosclerosis. Therefore, inhibiting inflammation and elevating RCT efficiency could limit the plaque lesion formation and maintain plaque stability which are potential strategies to treat atherosclerosis. It was reported that the high PAPP-A levels are associated with atherosclerosis, and PAPP-A has been proposed as a new marker of acute coronary syndromes. It was also reported that PAPP-A is highly expressed in atherosclerotic plaques and can accelerate lipid accumulation in cells. The current study revealed that PAPP-A aggravates the development of atherosclerosis in apoE−/− mice through inhibiting the expression of cholesterol transports including ABCA1, ABCG1 and SR-B1, reducing RCT efficiency, and enhancing the expression and secretion of pro-inflammatory factors. Together with our previous findings that recombinant PPAP-A can activate the IGF-1/PI3K/Akt signal pathway to inhibit the expression of ABCA1, ABCG1 and SR-B1 in THP-
l-1-derived macrophages, our results strongly support the conclusion that PAPP-A contributes to plaque formation in atherosclerosis. Thus, novel strategies aimed at PAPP-A, which is a relatively accessible extracellular enzyme, might prevent atherosclerosis.

It is well known that enhanced macrophage RCT is one of the crucial mechanisms by which HDL exerts its atheroprotective effect. There are three processes in RCT. Cholesterol efflux is the first step and a key component in RCT, which plays an important role in reducing the accumulation of lipids in the arterial wall and preventing the development of atherosclerosis. Cholesterol efflux depends on the expression of specific transport proteins including ABCA1, ABCG1, and SR-B1 to mediate the efflux of excess cellular cholesterol from macrophages to apoA-I and nascent HDL. However, ABCG1 possesses the function to redistribute intracellular cholesterol to plasma membrane domains, which are accessible for removal by HDL, but not lipid-poor apoA-I. SR-BI mediates cholesterol efflux to HDL and cholesterol influx from HDL as well, but it does not alter cellular cholesterol mass. In addition, SR-BI mediates cholesterol efflux to large but not smaller HDL particles. The second step of RCT is that free cholesterol in HDL is esterified by lecithin cholesterol acyl transferase (LCAT) to form cholesteryl ester (CE), which can be directly transferred to hepatocytes through SR-BI or indirectly transferred to apoB-containing lipoproteins via CE transfer protein (CETP) and then delivered to the liver via the low-density lipoprotein Receptor (LDLR). Finally, hepatic cholesterol can be converted to bile salts or directly excreted to the bile. When ABCA1, ABCG1 and SR-B1 expression was increased, the levels of HDL-C were increased. In the current study, we indeed found that the expression of ABCA1, ABCG1, and SR-B1 was increased in peritoneal macrophages, atherosclerotic plaque, and aorta from the apoE−/− mice treated with PAPP-A shRNA. The expression of ABCA1, ABCG1 and SR-B1 in other peripheral cells such as smooth muscle cells and endothelial cells were also upregulated when the PAPP-A was knocked down in the apoE−/− mice. Further, the expression of ABCA1 but not SR-B1 was increased in the liver of PAPP-A knockdown apoE−/− mice. In this study, we observed that the expression of inflammatory proteins and cytokines that are possibly mediated by inhibiting the expression of ABCA1, ABCG1, and SR-B1 was increased in atherosclerotic plaque.

In summary, we have addressed the missing link between PAPP-A and atherosclerosis by providing evidence that the downregulation of PAPP-A is positively associated with RCT and negatively related with the inflammation, which is possibly mediated by inhibiting the expression of cholesterol transporters. Meanwhile, PAPP-A releases IGF-1 from IGF binding proteins and activates the PI3K/Akt pathway by IGF-1R. The activated signaling may inhibit the expression of ABCA1, ABCG1, SR-B1 by suppressing LXRα, thereby reducing the efficiency of RCT and leading to the atherosclerotic plaque formation. In contrast, the PI3K/Akt pathway can trigger NF-κB activation to facilitate the secretion of pro-inflammatory cytokines, accelerating atherosclerosis. Thus, the selective modulation of PAPP-A may provide the foundation to develop a novel therapeutic approach.

Acknowledgments
The authors gratefully acknowledge the financial support from the Natural Science Foundation of Hunan Province (2017JJ4076), the National Natural Sciences Foundation of China (81770461) and Hunan Provincial Innovation Foundation for Postgraduate (CX2018B61).

References

**Supplementary Files**