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Protective effect of simvastatin on arterial plaque instability induced by p-cresyl sulfate

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Abstract. – OBJECTIVE: To study the protective effect of simvastatin on arterial plaque instability induced by p-cresyl sulfate (PCS).

MATERIALS AND METHODS: Apolipoprotein E (ApoE)$^{-/-}$ mice were selected as objects of this study. All mice were randomly divided into three groups: 1) the control group, 2) the PCS group and 3) the PCS + simvastatin group. After successful modeling, the levels of plasma cholesterol, triglyceride, low-density lipoprotein, high-density lipoprotein, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-β (TNF-β) were detected. The gross specimen of coronary artery was stained. Meanwhile, oil red O staining and Sirius red staining were performed for coronary arterial sections to observe the lipid and collagen components. The expression levels of smooth muscle cells and macrophages were observed by immunohistochemistry. In addition, the expression levels of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and monocyte chemoattractant protein-1 (MCP-1) in tissues were detected by Western blotting.

RESULTS: Simvastatin could improve atherosclerotic plaque growth and atherosclerotic plaque instability induced by PCS. Moreover, simvastatin could also improve the changes of MMPs and TIMPs caused by PCS as well as the inflammatory status in mice.

CONCLUSIONS: Simvastatin can improve the inflammatory status in mice, eventually improving the arterial plaque instability caused by PCS.

Key Words:
PCS, Simvastatin, Atherosclerosis.

Introduction

Chronic kidney disease (CKD) can be induced by many diseases, such as diabetes mellitus, hypertension and glomerulonephritis. The incidence rate of CKD in the United States is as high as 15%, leading to a significant threat to public health[1]. Cardiovascular disease (CVD) is the leading cause of deaths in CKD patients, especially for those receiving long-term dialysis[2]. The mortality rate of CKD patients with atherosclerosis has been significantly increasing[3]. In addition, if the renal function deteriorates, the progression of coronary atherosclerotic plaques in CKD patients will be accelerated[4]. The possible reason may be that heart and kidneys have a close cooperative relationship in maintaining hemodynamics through complex networks[5]. However, the potential mechanism of these two diseases remains to be fully elucidated.

In the process of CKD, there is the retention of various uremic toxins. It's known to all that two kinds of protein-binding toxic molecules, including p-cresol sulfate (PCS) and indoxyl sulfate, exhibit great influences on the occurrence and development of CVD[6]. Due to its protein-binding characteristics, PCS cannot be cleared by traditional hemodialysis. However, it will constantly accumulate in CKD patients. Therefore, PCS is closely related to the mortality rate of CVD, which can also be used to predict the mortality rate of CKD-related CVD[7]. At present, researches[8] have reported that PCS will affect atherosclerosis. Statins, known as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are widely used to reduce the level of serum cholesterol. Statins can lower total cholesterol and low-density lipoprotein (LDL) levels, thus reducing the long-term risk and mortality rate of coronary heart diseases[9]. In addition to lowering blood lipid, statins also exert other effects, such as anti-inflammation and the function of protecting endothelial cells[10]. Many works[11] have indicated statins also have an effect on arterial plaque stability. Therefore, in this work, we investigated the protective effect of simvastatin on atherosclerosis accelerated by PCS, and studied the effect of simvastatin and PCS on atherosclerotic plaque stability.

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Materials and Methods

Reagents

PCS was synthesized according to the previously reported method[12]. The concentration and purity (>99%) of PCS were confirmed by nuclear magnetic resonance spectrum. Macrophage-3 (MAC-3), smooth muscle cell (SMC) and monocyte chemoattractant protein-1 (MCP-1) antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oil red O staining, Sirius red staining, triglyceride, low-density lipoprotein, high-density lipoprotein, cholesterol, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-β (TNF-β) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Beyotime (Shanghai, China).

Animals

A total of 45 clean adult male apolipoprotein E (ApoE)$^{-/-}$ mice (8 weeks in age and 24.20±0.25 g in weight) were obtained from the Laboratory Animal Center of Nanjing University. The nutritional status and the mental state of all animals were normal. All mice were fed with high-fat diet supplemented with 0.25% cholesterol and 20% lard oil. All mice were grown under the following conditions: temperature of (21±2)°C, relative humidity of (50±15)% and light/dark cycle of 12 h. They were randomly divided into 3 groups: 1) the control group (n=15); 2) the PCS group (n=15): mice in this group were treated with gavage using 17 μL/g distilled water containing PCS (100 mg/kg per day); and 3) the PCS + simvastatin group (n=15): mice in this group were treated with gavage using 17 μL/g distilled water containing PCS (100 mg/kg per day), and using simvastatin dissolved in normal saline (10 mg/kg) simultaneously. Body weight, triglyceride, cholesterol, low-density lipoprotein and high-density lipoprotein levels of the ApoE$^{-/-}$ mice were shown in Table I. Mice were sacrificed 20 weeks later. After fasting overnight, chest of the mice was opened under anesthesia to expose the heart. Subsequently, the blood sample was collected from the heart by using a syringe and needle. The levels of triglyceride, low-density lipoprotein, high-density lipoprotein, cholesterol, IL-6, IL-1 and TNF-β were detected by ELISA in accordance with the kit instructions. This study was approved by the Ethics Committee of Yantai Yuhuangding Hospital. Informed consents were obtained from all the participants before the study.

Oil Red O and Sirius Red Staining

After the chest was opened in mice, the heart, aortic root and aorta were peeled off, rinsed with phosphate-buffered saline (PBS) at 4°C and fixed with 4% paraformaldehyde. All the tissues were embedded in paraffin. Subsequently, paraffin-embedded tissues were sliced into 4-μm-thick sections serially and placed at 60°C overnight. Then, the sections were dewaxed by xylene and dehydrated by gradient alcohol, followed by Sirius red staining and oil red O staining. Medical image analysis software (Image-Pro Plus IPP. Mediaplayer, USA) was used to detect 3 cross sections of each sample.

Immunohistochemical Staining

After xylene dewaxing and gradient alcohol dehydration, the paraffin-embedded arterial tissue sections were incubated with warm deionized water containing 0.3% H_2O_2 for 30 min. After the removal of endogenous peroxides, the sections were sealed in serum and relevant primary antibodies were added at 4°C overnight. On the next day, immunoglobulin G (IgG) antibody-horseradish peroxidase (HRP) was added for incubation. Next, the sections were incubated with a mixed solution prepared by the avidin-biotin-peroxidase complex (ABC) kit. After 10 minutes of coloring by diaminobenzidine (DAB), hematoxylin re-staining was performed, followed by washing, dehydration, transparency and observation under a light microscope.

Table I. Body weight, triglyceride, cholesterol, low-density lipoprotein and high-density lipoprotein levels in ApoE-/- mice.

	Control	PCS	PCS+statin
Weight (g)	28.68±0.73	30.23±0.74	30.55±0.95
TC (mmol/L)	19.25±0.60	20.15±0.73	18.17±0.54
TG (mmol/L)	2.33±0.11	2.42±0.06	2.21±0.10
LDL-C (mmol/L)	5.27±0.17	5.30±0.15	5.19±0.15
HDL-C (mmol/L)	1.73±0.06	1.71±0.05	1.69±0.07

Gross Staining

After fasting for 12 h, chest of the mice was opened under anesthesia to expose the heart. The heart, aortic root, and aorta were peeled off, rinsed with PBS at 4°C and fixed with 4% paraformaldehyde. The specimens were tiled on a wax plate under a microscope, and were fixed with pins after oil red O staining. After photography with the charge-coupled Device (CCD) camera, images were integrated by Adobe Photoshop Version 7.0. Finally, Image J Software was used for calculation.

Western Blotting

Extracted tissues were ground with liquid nitrogen, diluted with normal saline and placed on ice. Then the supernatant was collected and centrifuged at 4°C for 5 min, and the supernatant was discarded. Radio-immunoprecipitation assay (RIPA) lysis solution (Beyotime, Shanghai, China) containing phenylmethanesulfonyl fluoride (PMSF) was used for sediment re-suspension and lysis, followed by centrifugation at 4°C, 16000 g for 15 min. The supernatant was collected for protein quantification. The proteins were added to the loading buffer and heated for denaturation. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred onto a membrane. Subsequently, the membrane was incubated with 5% skim milk for 2 h, followed by primary antibody incubation at 4°C overnight. After that, the membrane was washed with tris-buffered saline and Tween-20 (TBST) for 3 times (10 min/time). Corresponding secondary antibody was added for incubation at room temperature for 1 h, followed by washing with TBST for 3 times (10 min/time). Finally, the protein expression levels of different samples were detected by using the enhanced chemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 Software (IBM, Armonk, NY, USA) were used for data analysis, and GraphPad Software (La Jolla, CA, USA) was applied for plotting. Data were presented as mean ± standard deviation, paired or unpaired t-test was used for data analysis. One-way analysis of variance was used for comparing the differences among groups, followed by Student-Newman-Keuls (SNK) post hoc test for the pairwise comparison. $p<0.05$ was considered statistically significant.

Results

Simvastatin Improved the Growth of Atherosclerotic Plaque Induced by PCS

To evaluate the effect of PCS on atherosclerosis in ApoE$^{-/-}$ mice, oil red O staining was performed for the gross specimens of aorta in each group, and the area of atherosclerotic plaque was compared among the three groups (Figure 1). Compared with the control group, PCS increased the atherosclerotic plaque area. Meanwhile, simvastatin treatment significantly inhibited the atherosclerotic plaque growth induced by PCS. Moreover, the levels of body weight, triglyceride, cholesterol, high-density lipoprotein and low-density lipoprotein in each group were detected, respectively. Results showed that low-dose simvastatin could not significantly reduce the blood lipid level in mice, and the effect of simvastatin on atherosclerosis was independent of its lipid-lowering effect.

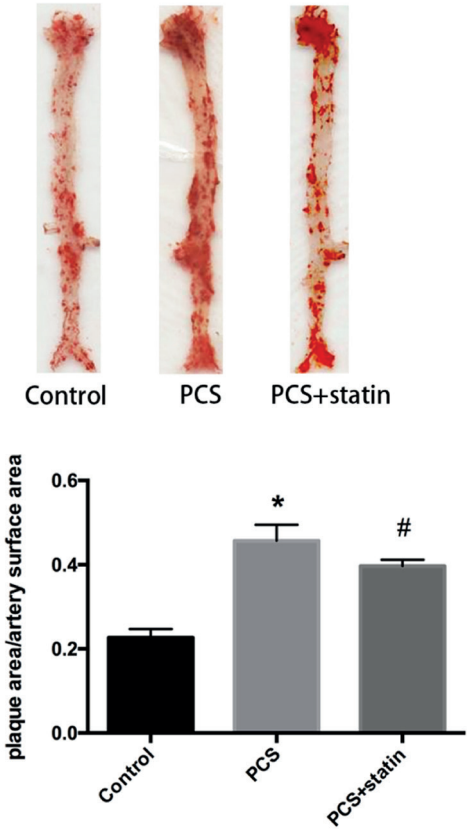

Figure 1. Oil red O staining for gross specimens of aorta in the control group, the PCS group and the PCS + simvastatin group (scale: 0.5 cm). ***(B)*** The degree of aortic lesion was presented as the percentage of oil red O staining in artery. *$p<0.05$ vs. the control group, #$p<0.05$ vs. the PCS group (n=5).

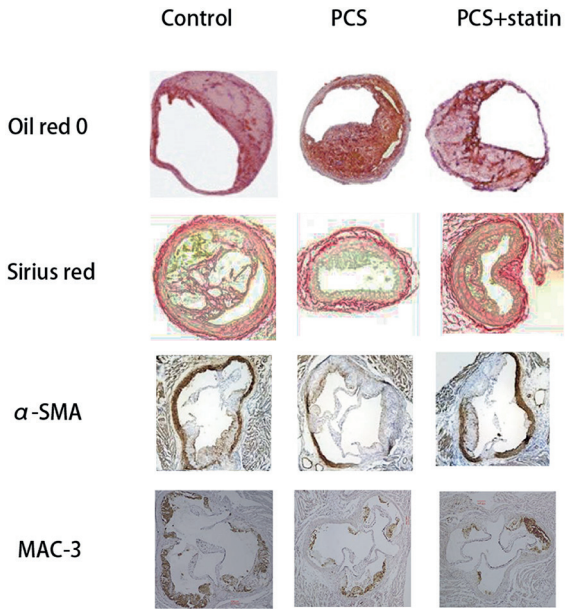

Figure 2. Histological staining of arterial sections of ApoE$^{-/-}$ mice in the control group, the PCS group and the PCS + simvastatin group. Staining of macrophages (MAC-3), vascular SMCs (α-actin), collagen (Sirius red) and lipids (oil red O) in carotid cross sections in different groups (scale: 100 μm).

Simvastatin Improved Plaque Instability Induced by PCS

To further investigate the effect of simvastatin and PCS on atherosclerotic plaque stability, the aortic sections of mice were stained. The aortic macrophages, vascular smooth muscle cells, collagen and lipid were labeled with MAC-3 antibody, alpha-smooth muscle actin (α-SMA), Sirius red and oil red O, respectively. In the PCS group, more macrophage infiltration and lipid deposition in the arterial plaque were found. Destruction of the vascular smooth muscle layer was increased, whereas the collagen was decreased (Figure 2). The treatment of simvastatin could improve the PCS-induced lipid deposition and macrophage infiltration, increase the number of collagen and protect the smooth muscle layer.

According to the staining results, we further analyzed the stability of plaques. The percentage of each component in aorta plaque was shown in Table II. The vulnerability index (VI) of plaque was calculated. It was found that the plaque stability was weakened in the PCS group; however, it was increased in the simvastatin group.

Simvastatin Improved the Changes of MMPs and TIMPs Caused by PCS

MMPs could hydrolyze collagen fibers, thereby affecting the stability of plaques. Our results indicated that MMP-2 and MMP-9 were significantly increased in the PCS group, while the expression levels of TIMP-1 and TIMP-2 were decreased. In the simvastatin group, the expression levels of MMPs were decreased, whereas the levels of TIMPs were increased (Figure 3).

Statins Improved the Inflammatory Status in Mice

MMPs and IL are secreted by macrophages and foam cells. Meanwhile, macrophage infiltration is an important initiating link for the formation of atherosclerotic plaques, which is mediated by MCP-1. Therefore, we measured the levels of serum IL-1 and IL-6, as well as TNF-β and MCP-1 levels in arterial tissues of mice. It was found that PCS increased the MCP-1 level in mice, as well as the increase of IL-1 and TNF-β levels. In the statin group, the levels of MCP-1, IL and TNF-β were decreased (Figure 4).

Discussion

As a toxic substance that is not easy to be removed by dialysis, PCS can bind to relevant proteins. The effect of PCS on the cardiovascular system

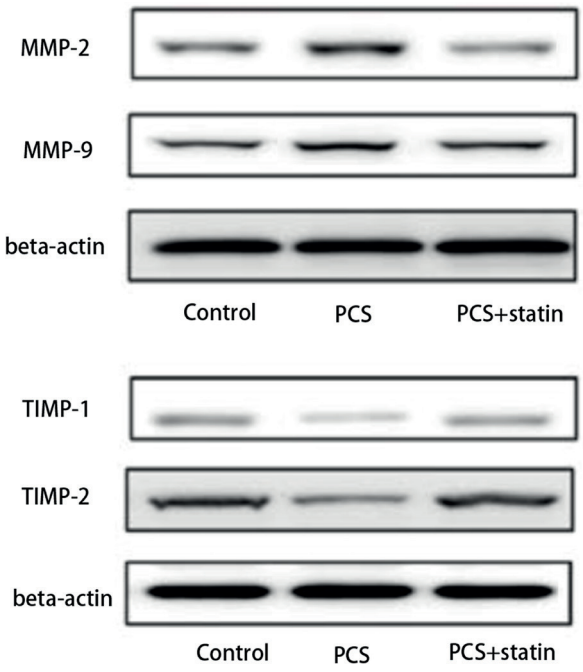

Figure 3. Expression levels of MMP-2, MMP-9 and TIMP in the arterial tissues of ApoE$^{-/-}$ mice and mice in the PCS group and the PCS + simvastatin group.

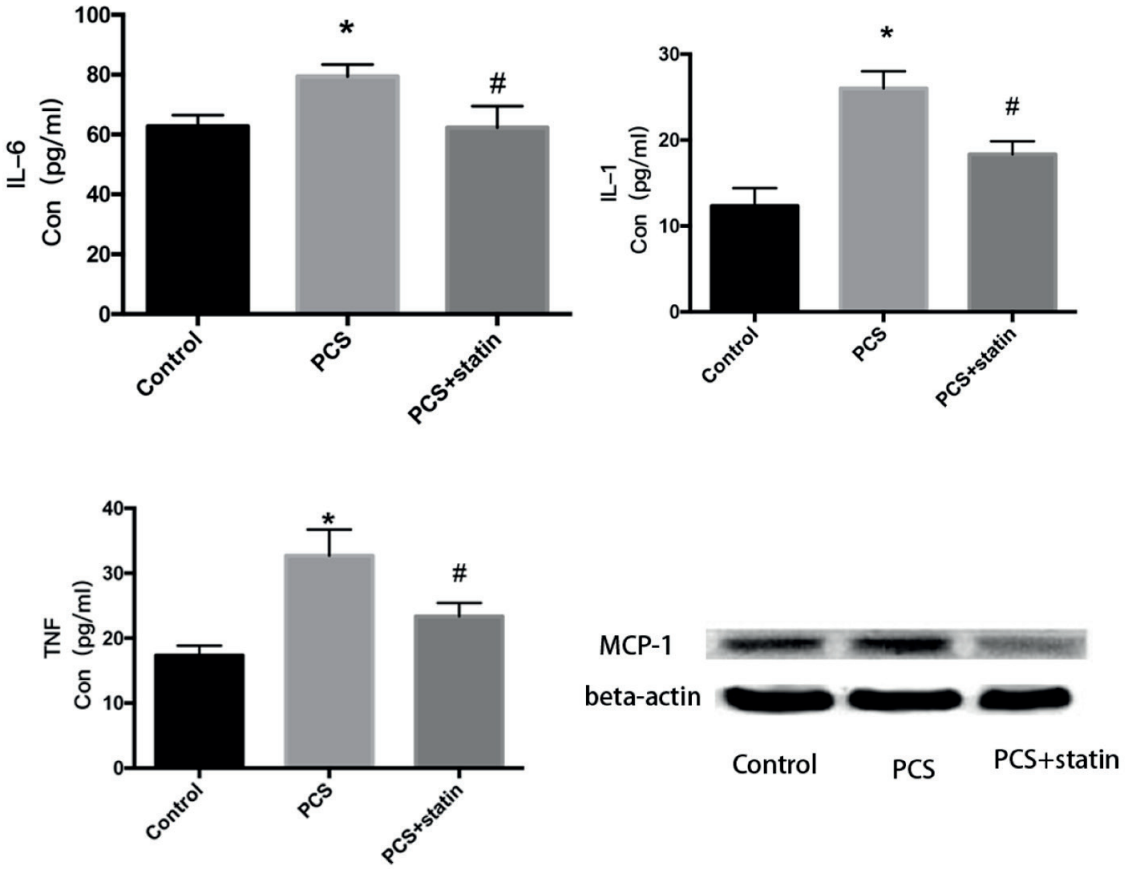

Figure 4. Levels of serum IL-1 (A), IL-6 (B), TNF-β (C) and MCP-1 (D) in the arterial tissues of ApoE$^{-/-}$ mice and mice in the PCS group and the PCS + simvastatin group.

has also been repeatedly demonstrated by clinical studies. Meanwhile, the relationship between serum PCS and the mortality rate of CVD has always been the focus of concern[13].

Clinical evidence has shown that most acute cardiovascular events (ACS) can be attributed to the erosion and rupture of vulnerable plaques[14]. Vulnerable plaques are usually characterized by atrophic fibrous caps, large necrotic core, accumulation of inflammatory cells, and imbalance between the synthesis and degradation of extracellular matrix (ECM)[15]. During the development of arterial plaque, produced vascular SMCs may proliferate and migrate from the tunica media to the intima. In this process, vascular SMCs will produce a large number of ECM proteins. However, these newly-generated vascular SMCs and ECM will eventually lead to fibro-fatty degeneration[16].

In the study of unstable plaque, MMPs have always been the hotspot of research. MMPs can

Table II. Area percentages of plaque, lipid, macrophages, collagen and SMCs in the aortic section, and VI = (lipid + macrophage)/ (SMCs + fiber).

	Control	PCS	PCS+statin
Plaque area (%)	27.33±3.06	47.33±4.04*	31.67±4.51#
Lipid (%)	31.00±3.61	59.67±3.51*	47.33±3.51#
Macrophage (%)	18.00±2.01	10.33±1.15*	15.01±1.03#
Collagen content (%)	8.10±0.54	5.36±0.35*	11.13±1.63#
α-SMA (%)	11.57±0.45	7.45±0.77*	13.49±1.12#
Vulnerability index	2.45±0.26	5.48±0.49*	2.56±0.45#

*p<0.05 vs. control group, #p<0.05 vs. PCS group (n=5).

mediate immune cell infiltration and fibroblast migration, and may even trigger the remodeling of myocardial tissues after cardiac injury to a great extent[17]. MMPs are a group of calcium-dependent endopeptidases, which can decompose ECM proteins. However, TIMPs can block the activation of MMPs. The dynamic balance between MMPs and TIMPs in the body plays an important role in the degradation of ECM[18]. Our study showed that PCS interfered with the balance between MMPs and TIMPs, whereas statins could improve this imbalance.

It's known to all that MMPs are mainly secreted by mononuclear macrophages in arterial plaques. Recruitment and aggregation of blood monocytes are important initiating links in the occurrence process of vascular inflammation. Therefore, the decrease of macrophages can reduce foam cells formation, improve lipid deposition, and reduce collagen degradation[19]. Chemokines and their ligands are responsible for the recruitment of monocytes. C-C motif chemokine ligand 2 (CCL-2), also known as MCP-1 and C-X3-C motif chemokine ligand 1 (CX3CL1), is an important monocyte chemokine in the body. CCR2, a ligand of MCP-1, is mainly expressed in peripheral blood monocytes[20]. Scholars[21] have demonstrated that the susceptibility to atherosclerosis in CCR2-deleted animals is reduced. In the present work, the expression levels of MMPs were decreased in the statin group. Immunohistochemistry results showed that the number of macrophages in the statin group was smaller than that of the PCS group. Meanwhile, we also measured the expression of MCP-1, and found that it was decreased, which was consistent with our conjecture. In addition to lowering the serum cholesterol level, statins including HMG-CoA reductase inhibitors[22] also have an anti-inflammatory effect[23]. It was speculated that statins could improve the aggregation of macrophages and the secretion of MMPs through improving the inflammatory response. Subsequently, IL-1, IL-6 and TNF were further detected to determine the level of inflammation in mice. Results showed that statins could improve the PCS-induced changes in the inflammatory state *in vivo*.

Conclusions

Atherosclerosis is a long-term and chronic inflammatory change of the arterial wall, which is initiated by macrophage infiltration. Meanwhile, a large number of vascular SMCs may proliferate and migrate from the tunica media to the intima. The proliferation of vascular SMCs leads to the large-scale synthesis of ECM, thereby exacerbating local inflammation that is a positive feedback cycle. Simvastatin can break this positive cycle and improve the arterial plaque vulnerability induced by PSC. We provided new insights into protein-bound uremic toxins and excavated the potential of statins in the treatment of cardiovascular complications of CKD, suggesting that patients with higher levels of PCS may benefit from the direct vascular protective effect of statins.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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