Pravastatin polarizes the phenotype of macrophages toward M2 and elevates serum cholesterol levels in apolipoprotein E knockout mice

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Abstract

Objective: Statins are clinically used for protection against cardiovascular disease with lipid-lowering and anti-inflammatory properties. These properties tip the balance of macrophage polarization, which is an essential process in the development and progression of atherosclerosis. This study aimed to investigate the effect of pravastatin on atherosclerosis of the aorta in apolipoprotein E knockout (apoE-KO) mice without high lipid feeding.

Methods: Six 8-week-old apoE-KO male mice were randomly divided into two groups: a control group and a pravastatin (40 mg·kg⁻¹·day⁻¹)-treated group. At 35 weeks, the mice were sacrificed and the size of plaques on the aorta was assessed by Oil Red O staining. M1 and M2 macrophages were identified by inducible nitric oxide synthase and arginase-I, respectively, using immunohistochemistry.

Results: Pravastatin increased the size of atherosclerotic plaques in apoE-KO mice without high lipid feeding. The ratio of M1/M2 macrophages increased in atherosclerotic plaques, which might slow the process of atherosclerosis, while blood cholesterol levels were elevated.

Conclusion: Our study suggests that pravastatin polarizes the phenotype of macrophages toward M2 in atherosclerotic lesions, despite an increase in serum cholesterol levels in ApoE-KO mice.

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Introduction

Atherosclerosis has been recognized as an inflammatory process. Inflammatory cells, especially macrophages, are assumed to play critical roles in the initial stages and further progression of atherosclerotic inflammation. Macrophages represent a heterogeneous cell population, with classically activated M1 (pro-inflammatory) macrophages and alternatively activated M2 (anti-inflammatory) macrophages representing the two extremes of the phenotypic spectrum. Pro-atherogenic factors, such as Th1 cytokines and lipopolysaccharide, induce a “classical” activation profile (M1). M1 macrophages are characterized by the expression of a broad spectrum of pro-inflammatory cytokines and chemokines and the release of inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO). By contrast, the M2 macrophage phenotype is induced by Th2 cytokines and leads to secretion of anti-inflammatory factors. M2 macrophages show reduced NO production because of upregulation of arginase-I (Arg-I) activity, which converts arginine to ornithine and urea. The presence of both M1- and M2-polarized macrophages in the arterial wall and the switch from an M2 to an M1 phenotype have been recently reported to occur during atherosclerotic lesion development.

Statins, or 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, are widely used in clinical practice for the primary and secondary prevention of cardiovascular disease. Besides the well-known lipid-lowering effects of statins, their protective action has also been associated with their anti-inflammatory effects. However, the extent to which the protective action of statins depends on their lipid-lowering effects remains unclear. This is partly because of the difficulty in separating lipid-related effects from non-lipid-related effects of statins in clinical studies.

We addressed this issue by using an apolipoprotein E-knockout (apoE-KO) mouse model of atherosclerosis in this study. In this model, advanced, unstable, atherosclerotic plaques show many of the morphological features of atherosclerotic plaques observed in humans. However, statins do not lower plasma cholesterol levels in this mouse model, and this can facilitate separation of the lipid-related effects from non-lipid-related responses. We examined the effect of pravastatin administration to this mouse model on the M1/M2 macrophage ratio, plaque calcification, blood cholesterol levels, and atherosclerotic plaque size.

Material and methods

Animal model

Eight 8-week-old male apoE-KO mice in a C57BL/6J background were purchased from Beijing Biocytogen Co. Ltd. (Beijing, China).

All mice underwent a 2-week acclimatization period and were maintained on a normal chow diet. At 10 weeks old, the mice were randomly divided into two groups, with four mice in the control group and four in the treatment group. All mice in the control group were fed a normal chow diet every day, whereas the mice in the treatment group were fed a diet supplemented with 40 mg/kg of...
pravastatin per day until 35 weeks old. The protocols for animal handling were previously approved by our institutional Animal Ethics Committee at the Affiliated Hospital of Guilin Medical University (approval number 2014GZR-20).

**Serum lipid measurement and tissue preparation**

Blood from each mouse was drawn from the heart directly for tests of serum lipid levels. Serum lipid levels were measured using a full-automatic biochemistry analyzer (Roche Cobas C702, Manheim, Germany). The mice were then sacrificed, and the aorta (the arch of the aorta, abdominal aorta, and common iliac arteries in some cases) were dissected. Three specimens were used for Oil Red O staining and pathological analysis. One specimen was used for western blot and real-time reverse transcription-polymerase chain reaction assay (not included in this paper). Aortic specimens were thoroughly rinsed with phosphate-buffered saline and then fixed for 18 hours with 4% paraformaldehyde. After fixation, sterile water was added to gently rinse the specimen.

For Oil Red O staining, 60% isopropanol was added to the aortic specimen and discarded after reacting for 5 minutes, and then Oil Red O was added. The specimen was slowly rotated to spread the dye evenly. After 5 minutes, the specimen was rinsed with tap water until the water ran clear. The specimen was photographed (Olympus digital camera; Olympus Optical Co., Tokyo, Japan) and the images were analyzed.

**Histopathological and immunohistochemical detection of plaques and macrophages**

To observe the pathological process of development of atherosclerotic plaques, every plaque in the aorta of six mice from the two groups was cut into 4-µm-thick sections from paraffin blocks and subjected to hematoxylin and eosin staining and immunohistochemistry. M1 and M2 macrophages in the atherosclerotic plaques were investigated immunohistochemically using antibodies to iNOS (rabbit, Cat. No. PA1-37925; Thermo Fisher Scientific Inc., Rockford, MD, USA) and Arg-I (goat, Cat. No. SC-18351; Santa Cruz Biotech, Dallas, TX, USA), respectively. The slides were baked at 60°C for several hours and then deparaffinized and rehydrated with xylene and ethanol. Antigen retrieval was conducted according to the primary antibody (heat-induced antigen retrieval for 3 min for iNOS; boiled in Tris/EDTA, pH 9.0 buffer [Cat. No. MVS-0098; Fuzhou Maxim Biotechnology, Fuzhou, China] for 20 min for Arg-I). Endogenous hydroperoxidase was blocked in 0.3% H2O2 for 10 minutes. The specimens were then incubated with the primary antibody for 1 hour and then with the secondary antibody (Cat. No. KIT-5920 and ABD-0030, respectively; Fuzhou Maxim Biotechnology) for 30 minutes at 37°C. Freshly prepared 3,3′-diaminobenzidine chromogenic reagent (Cat. No. DAB-0031; Fuzhou Maxim Biotechnology) was applied (for 3–5 min) until a color change was noted. The slides were then dehydrated, set with a cover slip, and held for fixation.

**Quantification of atheromatous plaques with or without calcification and the M1/M2 cell ratio**

Plaques were sectioned onto 4-µm-thick slides to identify atheromatous plaques and calcified atheromatous plaques. Atheromatous plaques were identified as those with an extracellular lipid core with or without cholesterol crystals. The percentage of calcified atheromatous plaques was also calculated. To count M1 and M2 cells, maximal sections were submitted to three pathologists for
histological assays. Only the strongly stained cells were regarded as M1 or M2 cells.

Statistical analysis
All data are expressed as the mean ± standard error of the mean. Significant differences between mean serum cholesterol levels and lesion numbers were determined by the Student’s two-tailed t-test. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Pravastatin elevates plasma cholesterol levels in apoE-KO mice
The pravastatin-treated group showed significantly higher plasma cholesterol levels, including total cholesterol, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol, compared with the control group (Figure 1, all P < 0.05).

Pravastatin increases the area of atherosclerotic plaques
After administration of pravastatin (40 mg/kg per day) or normal chow only (control) for 35 weeks, all apoE-KO mice developed atherosclerotic plaques in the aorta. However, the pravastatin-treated group developed more and larger-sized atherosclerotic lesions compared with the control group, which is consistent with the results of elevated cholesterol levels (Figure 2).

Effects of pravastatin on plaque calcification
Coronary calcification is considered to play a role in the process of atherosclerosis, which may protect against rupture of plaques. In apoE-KO mice, pravastatin treatment resulted in significantly higher plaque calcification than in the control group (P < 0.05), which indicated a plaque-stabilizing effect of statins (Figure 3).

Effects of pravastatin on the balance of M1 and M2 macrophages
The pravastatin-treated group showed a significantly higher number of M1 cells and a significantly higher number of M2 cells (P < 0.05), and a significantly lower M1/M2 macrophage ratio (P < 0.05) compared with the control group (Figure 4). Our findings suggested that pravastatin promoted a switch from an M1 to an M2 phenotype of macrophages.

Discussion
Several previous studies have showed that treatment with statins decrease serum lipid levels and then inhibit atherosclerosis. However, in this study, pravastatin increased serum cholesterol levels and the size of atheromatous plaques in the apoE-KO mouse model. Wang et al.11 reported that simvastatin elevated serum total cholesterol levels and increased the aortic plaque area in apoE-KO mice. These authors proposed that therapeutic effects of simvastatin may depend on the presence of functional apolipoprotein E. Pravastatin showed similar results in our study.
Several previous studies have shown that treatment with statins reduces the macrophage content of atherosclerotic lesions.\textsuperscript{9,10} Macrophages are predominant inflammatory cells within plaques, and these cells are versatile and can polarize to different phenotypes depending on the local microenvironment. Different macrophage subsets (M1 and M2) can be detected at every stage of atherosclerotic development. Additionally, the macrophage phenotype is reversible and can switch mutually in response to different microenvironment signals.\textsuperscript{4} In the present study, we aimed to determine whether pravastatin can change the phenotypes of macrophages in apoE-KO mice. We found that the M1/M2 macrophage ratio was lower in atherosclerotic plaques in pravastatin-treated apoE-knockout mice compared with controls.

In a Wistar-Kyoto rat model of anti-glomerular basement membrane glomerulonephritis, statin administration induced M2 polarization by suppression of M1 development and augmentation of M2 development with upregulation of the anti-inflammatory cytokine interleukin-10.\textsuperscript{12} Atherosclerosis is a chronic inflammatory vascular disease and macrophages play a pivotal role in the initiation and perpetuation of this inflammatory process. Stöger et al.\textsuperscript{13} showed that M1-specific cell markers were predominantly detected in rupture-susceptible shoulder regions, whereas M2-specific markers were preferentially found in the adventitia. Additionally, Chinetti-Gbaduidi et al.\textsuperscript{14}
identified M2 macrophages in more stable plaque regions outside of the lipid core.

Secretion of a series of cytokines and chemokines by M1 macrophages activates macrophages and endothelial and smooth muscle cells, and destroys the integrity of the arterial wall. M1 macrophages also secrete matrix metalloproteinases, which can degrade the extracellular matrix in fibrous caps. This is thought to lead to thinning and rupture of the arterial wall.\textsuperscript{15} By contrast, M2 macrophages play a major role in tissue remodeling and inflammation resolution, which lead to plaque stabilization. Therefore, the differential distribution and balance of M1 and M2 macrophages within atheromatous plaques can be a determinant of plaque status. Moreover, the results from several studies\textsuperscript{16} using mouse models of atherosclerotic regression have shown the dynamic nature of the M1/M2 balance in plaques. M1 predominates during disease progression and M2 dominates during the regression phase. In a previous study of advanced atherosclerotic plaques of apoE-KO mice, injections of human apoA-I led to atherosclerotic regression.\textsuperscript{17} There was also a significant reduction in the plaques of inflammatory M1 macrophage markers and an increase in anti-inflammatory M2 macrophage markers.

In the present study, we found an increase in plaque calcification, despite an increase in the size of atherosclerotic plaques with pravastatin administration. Calcification was originally thought to enhance the risk of plaque rupture.\textsuperscript{18} However, more recent research suggests that a low proportion of plaques containing microcalcification actually rupture.\textsuperscript{19} Calcification is a part of the atherosclerotic process, which makes the plaque stiffer and less prone to rupture. Indeed, plaque calcification is a marker for carotid plaque stability.\textsuperscript{20} Clinical studies\textsuperscript{5,6} have shown that statins reduce cardiovascular events, and this may be due to their effects on stabilizing vulnerable atherosclerotic plaques following a decrease in

![Figure 3. Calcification of atherosclerotic plaques in apolipoprotein E knockout mice after 35 weeks of (a) a normal chow diet only (control group) and (b) pravastatin treatment (40 mg/kg per day). Hematoxylin and eosin stain, magnification ×200. (c) Percentage of calcified plaques. *P < 0.05, compared with the control group.](image-url)
lipids. Recent studies have suggested that microcalcification rendered by statins considerably reduces vessel wall stress, contributing to plaque stability.\(^{21}\) Additionally, statin-mediated atheroma calcification may improve plaque stability independent of the effects of lipid regression within plaques following long-term potent statin therapies in clinical practice.\(^{22}\) Recently, an in vivo study showed that statins exert pro-calcific effects related to the intensity of therapy, despite the association with plaque regression.\(^{23}\) This finding suggests that the pro-calcific effects of statins are associated with plaque stabilization.

The mechanism by which pravastatin induces plaque calcification and plaque stabilization remains unknown. One likely explanation is the anti-inflammatory properties of statins. M1 macrophages secrete inflammatory factors to induce inflammation, and M2 macrophages decrease inflammation and play a major role in tissue remodeling, leading to plaque stabilization. Therefore, the balance of M1/M2 macrophages within atheromatous plaques may be a major determinant of plaque status. A lower M1/M2 macrophage ratio after pravastatin treatment favors resolution of inflammation and plaque stabilization.
Nevertheless, the association between polarization of macrophages and plaque stabilization requires clarification.

Development of atherosclerotic lesions in apoE-KO mice is similar to that in humans. However, in contrast to humans, simvastatin does not decrease plasma cholesterol levels in apoE-KO mice. We obtained similar results with pravastatin in apoE-KO mice. We did not expect pravastatin treatment of ApoE-KO mice to elevate serum cholesterol levels. However, this observation is consistent with reports by Wang et al. and Bea et al. The reason why chronic treatment of ApoE-KO mice with pravastatin increased serum cholesterol levels is unclear. One possible explanation for our finding is that, in the absence of functional apolipoprotein E, pravastatin had stimulatory effects on the expression and activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in the liver and/or the effects of inhibiting protein prenylation in this animal model.

In summary, although pravastatin elevates serum cholesterol levels and promotes atherosclerosis in ApoE-KO mice, pravastatin still shows abilities in stabilizing plaques. This results from increased calcification of plaques and the M2 phenotype of macrophages. Our study provides direct support for the lipid-independent pleiotropic effects of pravastatin and helps to explain the beneficial effects of statins that have been observed in clinical trials.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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