Atorvastatin Reduces Plaque Vulnerability in an Atherosclerotic Rabbit Model by Altering the 5-Lipoxygenase Pathway

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Key Words

Atorvastatin • Atherosclerosis • 5-Lipoxygenase-activating protein

Abstract

Objective: The 5-lipoxygenase catalyzed formation of leukotriene lipid mediators is a mediator for inflammatory response in arteries. The present study investigated the relationship between atorvastatin and the 5-lipoxygenase pathway in an atherosclerotic rabbit model. **Methods:** Thirty male New Zealand White Rabbits were randomized into negative control, positive control and atorvastatin groups. At week 4, the rabbits were subjected to carotid balloon-dilation injury or carotid balloon-dilation injury, followed by treatment with atorvastatin. At week 12, all the animals were sacrificed. Plasma lipids, LTD₄, and 15-epi-lipoxin A₄ were measured using the enzymatic endpoint method and ELISA, respectively. RT-PCR was performed to detect the gene expression of 5-lipoxygenase-activating protein and cysLT1R in rabbit carotid arteries. Finally, histological analysis was used to evaluate the pathophysiological changes of rabbit carotid arteries. **Results:** The results showed atorvastatin markedly lowered serum lipids and LTD₄ levels compared with the control group. Similarly, mRNA expression of 5-lipoxygenase-activating protein and cysLT1R was significant-

ly inhibited by atorvastatin. Decreased carotid plaque instability was evident in atorvastatin-treated animals, as demonstrated by a thickened elastic layer, less neointima hyperplasia and macrophage proliferation. **Conclusions:** Atorvastatin may stabilize carotid plaque by regulating the 5-lipoxygenase pathway in atherosclerotic rabbits and delay the progression of atherosclerosis by exerting anti-inflammatory effects.

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Introduction

Stroke is the third leading cause of morbidity and long-term disability worldwide. Plaque vulnerability, rather than arterial stenosis, plays a crucial role in the pathophysiology of recurrent atherothrombotic stroke [1]. The characteristics of vulnerable plaque include a large lipid core, thin fibrous cap, inflammatory cell aggregation and secretion of metalloproteinases and cytokines. Atorvastatin, a hydroxymethylglutaryl-CoA reductase inhibitor, can reduce plaque destabilization by downregulating matrix metalloproteinase and proinflammatory cytokine levels (e.g. growth-related oncogene, CX3CL1, FasL, etc.) [2].

The 5-lipoxygenase (5-LO)-catalyzed formation of leukotriene (LT) lipid mediator pathways contributes to inflammatory events and is associated with cardiovascular disease (CVD) [3]. 5-LO is involved in the metabolism of arachidonic acid, which leads to elevated levels of LTs in arterial lesions. By selectively transferring arachidonic acid to 5-LO, the 5-lipoxygenase-activating protein (FLAP) facilitates the conversion to LTA₄, which is then converted to either LTB₄ or cysteinyl leukotrienes (cysLTs). LTB₄ and cysLTs, which can bind to their corresponding receptors (i.e., BLT1, BLT2, cysLT1R and cysLT2R), which in turn increase the inflammatory response of carotid plaque formation and contribute to plaque destabilization [4]. Our previous study also showed that the cysLT1R antagonist montelukast exerted anti-atherogenic effects by downregulating monocyte chemoattractant protein-1 (MCP-1) in a rabbit balloon injury model [5]. With aggravation of the inflammatory response, carotid plaque is at a high risk of rupture, which can cause cerebral infarction or a transient ischemic attack. In recent years, emphasis has been placed on the roles of the 5-LO pathway in stroke. For example, the gene polymorphism of ALOX5AP encoding FLAP was found to be strongly associated with CVD in different populations [3, 6, 7]. However, little is known about the relationship between the antiatherogenic effects of atorvastatin and the 5-LO pathway.

The rabbit carotid balloon injury model has been widely used in studies of coronary artery restenosis prevention. Smooth muscle cell (SMC) accumulation occurs in the presence of endothelium denuded by balloon dilatation [8, 9]. Here, we report that atorvastatin attenuates carotid plaque vulnerability in a rabbit carotid balloon injury model by altering the 5-LO pathway.

Methods and Materials

Carotid Balloon-Dilation Injury Model

The present study conformed to the principles outlined in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health [10]. The animal experiments were approved by the animal care committee of Nanjing University. Thirty male New Zealand White Rabbits (aged 3 months, weighing 2.2–2.8 kg) were obtained from the model animal center of Nanjing University. They were randomly divided into the following groups: negative control group (n = 10), positive control group (n = 10) and atorvastatin group (n = 10). The rabbits in the negative control group were fed a chow diet, whereas those in other groups were fed a hypercholesterolemic diet (containing 1% cholesterol, 5% axungia porci and 7.5% yolk powder) for 12 weeks. Each rabbit was fed with a pellet of 120 g daily. Water was supplied ad libitum. At week 4, all rabbits except those in the negative con-

trol group were anesthetized with an intramuscular injection of ketamine (50 mg/kg) and droperidol (5 mg/kg). After the right carotid bifurcation was exposed, a 3-french Fogarty balloon catheter (2.5 × 8 mm, Cordis, Bridgewater, N.J., USA) was inserted into the right common carotid artery. To injure the carotid intima, the balloon was then dilated at 6 atm and moved 3 times. For the atorvastatin group, atorvastatin (1.5 mg/kg per day, Pfizer) was orally administered to the rabbits shortly after the operation daily for 8 weeks. At the end of week 12, all the animals were sacrificed. Subsequently, their right carotid arteries were excised for reverse transcription PCR (RT-PCR) and histological immunostaining.

Lipid Measurements

At weeks 0, 8 and 12, blood samples were drawn from the rabbit auricular veins and then centrifuged to obtain sera within an hour after blood collection. Determinations of serum total cholesterol, triglyceride, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were performed using the enzymatic endpoint method (Randox, Hitachi, Japan).

Serum LTD₄ and 15-Epi-Lipoxin A₄ Measurement by ELISA

Enzyme-linked immunosorbent assay (ELISA) kits (R&D Laboratory Research, Neogen Corp.) were used in accordance with the manufacturer's instructions for measuring serum LTD₄ and 15-epi-lipoxin A₄ concentration at week 12. The standards were serially diluted for constructing a standard curve. The serum samples were incubated for 90 min at 37°C in plate wells. The plates were then washed and biotin anti-rabbit LTD₄ antibody (or anti-rabbit 15-epi-lipoxin A₄ antibody) was added to all plate wells and incubated with the samples for 60 min at 37°C. Afterwards, horseradish peroxidase (HRP) diluent was applied to the wells and incubated for 30 min at 37°C. Tetramethylbenzidine substrate was used for ELISA detection and incubated for 15 min at 37°C. The final results were quantified at 450 nm using a spectrophotometer.

Reverse Transcription PCR

Total mRNA was extracted from the rabbit carotid artery using trizol reagent (Invitrogen Inc., Md., USA) according to the manufacturer's instructions. The synthesis of cDNA was achieved from 1 µg total mRNA by using the M-MLV reverse transcription system (Promega, Madison, Wisc., USA). For each target gene, PCR amplification (a total of 25 µl reaction volume containing 8 µl template cDNA) was performed using specific primers (table 1). PCR products were electrophoresed on a 2% agarose gel. The homologous bands were photographed under ultraviolet transillumination. Exclusion of reverse transcriptase yielded negative results, which meant there was no DNA contamination. The gene expression was semi-quantitated after normalizing with glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Histological Analysis

The carotid artery tissues were embedded in paraffin and then cut into 3-µm slices. For the purpose of studying histological changes of carotid intima and the elastic layer, the sections were subjected to hematoxylin and eosin (H&E) staining, and Van Gieson (elastic fiber maker) staining. Immunohistochemical analysis was undertaken to evaluate the expression of macrophages, and SMCs. After routine deparaffinization and rehydration, the sec-

Table 1. Primers for the target genes in the 5-LO pathway

Target genes	Primers	Accession No.	T _m (°C)	Product size (bp)
FLAP	Sense 5'-GGGACCCTTGCTTTTGAG-3' Antisense 5'-GTGGTGCTGATCGTCTTTATGT-3'	M96556	55	459
cysLT1R	Sense 5'-ATGACAGCCATGAGCTTTTTC-3' Antisense 5'-CATTCTAAGGACAGAATCACA-3'	NM_006639	57	459
GAPDH	Sense 5'-ACGGTGCACGCCATCACTGCC-3' Antisense 5'-GCCTGCTTCACCACCTTCTTG-3'	NM_001082253	63	266

tions were incubated with 3% hydrogen peroxide for 10 min, washed three times with PBS and blocked with 10% goat blood serum for 30 min at room temperature. Subsequently, they were labeled with monoclonal mouse anti-rabbit macrophage RAM-11 (DAKO, 1:200) antibody and monoclonal mouse anti-rabbit SMC HHF-35 antibody (DAKO, 1:100; Santa Cruz, Calif., USA, 1:200), respectively, for 12 h at 4°C, and then with goat-anti-mouse or goat-anti-human biotin-labeled IgG antibody (R&D Laboratory Research, Santa Cruz, Calif., USA, 1:200) for 30 min at room temperature. After being incubated with Streptavidin/HRP (BD Bioscience, 1:200) for 30 min at room temperature, the sections were visualized under light microscopy using a DAB Peroxidase Substrate Kit (Vector Laboratories), according to the manufacturer's instructions. All the photographs were analyzed using Image-Pro Plus 6.0 (Media Cybernetics).

Statistical Analysis

Data were shown as means $\pm$ SEM. A one-way ANOVA test was used to evaluate the differences of carotid intimal hyperplasia, macrophage aggregation and inflammatory cytokines, as well as the related cell receptor expression between groups. A Dunnett T3 test together with Dunnett C test were performed for the comparison of means of these parameter values between two groups. $p < 0.05$ was considered to be statistically significant. SPSS (version 15.0) was used for all the analyses.

Results

Atorvastatin Treatment Regulates Serum Lipids and LTD₄ Level after Carotid Balloon-Dilation Injury

Serum lipids in rabbits were measured at weeks 0, 8 and 12. Compared with the positive control group, total cholesterol, together with triglyceride, was markedly decreased at weeks 8 and 12 after atorvastatin administration (43.02 ± 1.66 mmol/l vs. 40.80 ± 1.24 mmol/l, 41.72 ± 1.47 mmol/l vs. 30.31 ± 1.55 mmol/l, $p < 0.001$; 5.56 ± 0.04 mmol/l vs. 5.33 ± 0.11 mmol/l, 5.91 ± 0.05 mmol/l vs. 3.64 ± 0.04 mmol/l, $p < 0.001$). Atorvastatin led to an appreciable reduction in LDL levels ($19.62 \pm$

1.01 mmol/l vs. 13.63 ± 0.33 mmol/l, $p < 0.001$) and an increase in HDL levels (1.58 ± 0.03 mmol/l vs. 1.65 ± 0.03 mmol/l, $p < 0.05$) at week 12, compared to the positive control group (fig. 1). ELISA was performed to test whether atorvastatin treatment decreases serum LTD₄ level. There was a significant decline in serum LTD₄ level in the atorvastatin-treated group compared to the positive control group (104.84 ± 89.38 vs. 23.96 ± 5.52 , $p < 0.001$; fig. 2a) at the end of week 12. 15-epi-lipoxin A₄ level markedly augmented after arterial intimal injury compared with the negative control group (119.92 ± 3.45 vs. 58.58 ± 2.23 , $p < 0.001$). However, atorvastatin had no effect on 15-epi-lipoxin A₄ expression (fig. 2b).

Atorvastatin Treatment Regulates Arterial FLAP, and cysLT1r Gene Expression after Carotid Balloon-Dilation Injury

RT-PCR was performed to measure gene expression of FLAP and cysLT1R (cysteinyl leukotriene receptor 1) in rabbit carotid arteries after carotid balloon injury. Atorvastatin significantly decreased FLAP mRNA expression in rabbit arteries (0.55 ± 0.02 vs. 0.12 ± 0.03 , $p < 0.001$). A downregulation of cysLT1R mRNA levels compared to positive control animals (0.93 ± 0.17 vs. 0.15 ± 0.03 , $p < 0.001$; fig. 3) was observed. These data suggest that atorvastatin attenuates carotid plaque vulnerability by altering the 5-LO pathway.

Atorvastatin Treatment Stabilized Carotid Plaque and Decreased Vascular Inflammation after Carotid Balloon-Dilation Injury

Histological analysis was undertaken to investigate the pathophysiological features of carotid arteries in rabbits. Compared with the positive control group, neointima hyperplasia was significantly inhibited by atorvastatin treatment after carotid balloon injury (8.46 ± 0.64

Fig. 1. Atorvastatin treatment regulates serum lipids. **a** Levels of serum total cholesterol (TC) at weeks 0, 8 and 12 in rabbits. **b** Levels of serum triglyceride (TG) at weeks 0, 8 and 12 in rabbits. **c** Levels of serum HDL at weeks 0, 8 and 12 in rabbits. **d** Levels of serum LDL at weeks 0, 8 and 12 in rabbits. PC = Positive control; NC = negative control, n = 10. ** p < 0.001 vs. PC group; * p < 0.05 vs. PC group.

Fig. 2. Atorvastatin treatment decreases plasma LTD₄ and 15-epi-lipoxin A₄ level. Measurement of optical densities (OD) of serum LTD₄ and 15-epi-lipoxin A₄ at 450 nm in rabbits. PC = Positive control; NC = negative control, n = 10. ** p < 0.001 vs. PC group; * p < 0.05 vs. PC group.

Fig. 3. Atorvastatin treatment decreases arterial FLAP and cysLT1R gene expression. **a** Gel electrophoresis images of target genes and their internal control in rabbits. **b** mRNA levels of FLAP between groups in rabbits. **c** mRNA levels of cysLT1R between groups in rabbits. PC = Positive control; NC = negative control, n = 10. ** p < 0.001 vs. PC group; * p < 0.05 vs. PC group.

vs. 4.18 ± 0.70 , $p < 0.001$). SMC accumulation was markedly increased by atorvastatin treatment after carotid balloon injury compared to the positive control (0.14 ± 0.04 vs. 0.24 ± 0.03 , $p = 0.001$). In contrast, atorvastatin stabilized carotid plaque by inducing thickening of the elastic layer, compared to the positive control (3.73 ± 0.09 vs. 1.02 ± 0.34 , $p < 0.001$). Compared to the positive control, atorvastatin treatment significantly inhibited vascular inflammation by decreasing macrophage accumulation after carotid balloon injury (0.41 ± 0.03 vs. 0.24 ± 0.08 , $p < 0.05$; fig. 4).

Discussion

In this study, we found that atorvastatin treatment produced a prominent lipid-lowering effect and down-regulated FLAP, LTD₄ and cysLT1R expression in rabbit

carotid plaques as well as reduced carotid plaque vulnerability after carotid balloon injury. To the best of our knowledge, this is the first study to show that atorvastatin can attenuate carotid plaque vulnerability in a rabbit carotid balloon injury model by altering the 5-LO pathway.

Atorvastatin Treatment Regulates Lipid Metabolites and Stabilizes Carotid Plaques, and Decreases Vascular Inflammation

Statins, a HMG-CoA (3-hydroxy-3-methylglutaryl co-enzyme A) reductase inhibitor, are widely used in the treatment and prevention of atherosclerosis as a cholesterol-lowering agent [11]. Angiotensin-converting enzyme (ACE) accelerates the progression of atherosclerosis by inducing macrophage-mediated LDL oxidation and synthesis of redox-sensitive cytokines, chemokines and growth factors [12]. Atorvastatin exhibits antioxidant activity by inhibiting ACE expression in differentiating

Fig. 4. Atorvastatin treatment stabilizes carotid plaque and decreases vascular inflammation. **a** Pathological images of carotid tissue sections in rabbits. **b** Measurement of carotid intima-media thickness between groups in rabbits. **c** Thickness of elastic layer between groups in rabbits. **d** Level of SMC between groups in rabbits. **e** Level of macrophage between groups in rabbits. Mean density = integrated optical density/area. PC = Positive control; NC = negative control, n = 10. Bar = 5 μm. ** p < 0.001 vs. PC group; * p < 0.05 vs. PC group.

macrophages and reduces atherosclerotic plaque vulnerability [13]. In addition, many biological effects of statins, such as anti-inflammatory actions and atherosclerotic plaque stabilization, are independent of cholesterol-lowering mechanisms [14]. Statins reduce the incidence of CVD by exerting pleiotropic effects beyond its lipid lowering effect [15]. Atorvastatin treatment elevates serum levels of HDL, which also reduces the risk of CVD. These effects are attributed to an atorvastatin-mediated lowering of the content of cholesterol ester transfer protein in serum and the subsequent decline in cholesterol ester transfer protein-mediated cholesterol transfer from HDL [16]. In addition, atorvastatin downregulates MCP-1 expression independently of its lipid-lowering effects [17], reducing macrophage recruitment into carotid lesions [18]. Moreover, atorvastatin enhances the plaque stability by suppressing matrix metalloproteinase expression, platelet activity and membrane lipid peroxidation [19–21]. Our data show that atorvastatin treatment may decrease LDL levels, increase HDL levels, stabilize carotid plaque and decrease vascular inflammation. By reducing

the plaque burden, statins may minimize the risk of atherosclerotic plaque rupture or arterial occlusion [22]. However, it is not clear whether atorvastatin may induce stabilized carotid plaque and decrease vascular inflammation via lipid-lowering dependent or independent effects.

Atorvastatin Attenuates Carotid Plaque Vulnerability by Altering the 5-LO Pathway

The emerging at-risk alleles for CVD in the 5-LO pathway raise a concern about the relationship between this inflammatory pathway and plaque instability [3]. Inflammatory cell accumulation is intensified in atherosclerotic lesions [23]. 5-LO is over-expressed in inflammatory cells such as macrophages, foam cells and mast cells, and induces the formation of unstable plaque [24]. Bäck et al. [25] showed an elevated expression of FLAP in macrophages of ApoE^{-/-} × CD4dnTβRII mice, followed by an increase in LTB₄ production. MK886, the FLAP antagonist, attenuates plaque vulnerability by reducing atherosclerotic lesion size and T cell accumulation. LTD₄

stimulates activation and chemotaxis of human monocytes and monocyte-derived immature dendritic cells by mediating innate immune reactions [26]. cysLTs has a potent influence on vasopermeability, which may facilitate inflammatory cell or cytokine recruitment into arterial lesions [27]. A recent clinical study suggested that atorvastatin (80 mg/day) caused a nonsignificant decrease in serum LTB₄ levels [28]. It has been reported that fluvastatin blocked the production of LTC₄ [29]. In accordance with the previous results, our data showed atorvastatin significantly downregulated the level of serum LTD₄. Inhibition of the LTD₄ receptor (cysLT1R) by montelukast abrogated vascular reactive oxygen species production and improves endothelial function and plaque stability [30]. Recently, 15-epi-lipoxin A₄ was proven to be an anti-inflammatory cytokine in the progression of atherosclerosis [31]. Interestingly, atorvastatin had no effect on serum 15-epi-lipoxin A₄ concentration in the study, which probably attributed to the relatively low dose of atorvastatin [32]. Platelet-activating factor lipids derived from oxidized LDL stimulate 5-LO expression in leukocytes, and is accompanied by biosynthesis of MCP-1 and LTB₄. Inhibition of MCP-1 results in a decline of LTB₄ production [33]. The metabolic products derived from the 5-LO pathway aggravates atherosclerosis. Taken together, all these factors contribute to plaque destabilization. Blockade of the 5-LO pathway attributed to the reduction in the number of macrophages in arterial lesions [18, 23]. A recent study by Ye et al. [31] has suggested that atorvastatin increases the phosphorylation of 5-lipoxygenase at Ser-523 by PKA. This phosphorylation prevents the translocation of 5-LO to the membranous fraction, thus inhibiting the interaction between 5-LO and cPLA2.

Instead, P-5-lipoxygenase interacts with COX2 to produce 15-epi-lipoxin A₄, a potent anti-inflammatory mediator. In this study, we found that atorvastatin treatment decreases LTD₄, FLAP and cysLT1R expression in atherosclerotic lesions, as well as attenuates carotid plaque vulnerability. Interestingly, a recent study showed that high dose simvastatin treatment induced over-expression of FLAP in patients' muscle [34]. There are two probable explanations for the conflicting results: they could be attributable to the dosage forms of statins or species differences.

In conclusion, atorvastatin may attenuate the progression of atherosclerotic lesions by regulating the 5-LO pathway, which may provide a new interpretation of its pleiotropic effects. However, the exact mechanisms of these beneficial effects are unclear, thus warranting further studies.

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