European Review for Medical and Pharmacological Sciences 2014; 18: 242-246

Atorvastatin suppresses Toll-like receptor 4 expression and NF-κB activation in rabbit atherosclerotic plaques

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Abstract. – BACKGROUND: Toll-like receptor 4 (TLR4) plays an essential role in the pathogenesis and progression of atherosclerosis, which overexpresses in atherosclerotic lesions and mediates the production of inflammatory factors. The aim of this study was to investigate the effects of atorvastatin on TLR4 protein and mRNA expression and its downstream factor NF-κB activation in rabbit atherosclerotic plaques.

MATERIALS AND METHODS: Rabbits continuously fed with high-fat diet for 24 weeks were randomly divided into two groups, the drug-treated group orally administrated with atorvastatin (2 mg/kg/day) three weeks after high-fat diet feeding and the model group with no treatment. The expression of TLR4 protein and mRNA, the level of activated NF-κB (p65) were respectively detected by western blotting, quantitative RT-PCR, and ELISA.

RESULTS: The results showed that atorvastatin treatment reduced the expression of TLR4 protein and mRNA by 24.1% ($p < 0.05$) and 46.9% ($p < 0.01$), respectively, and also inhibited NF-κB activation by 76.0% ($p < 0.001$) in the atherosclerotic plaques.

CONCLUSIONS: Thus, it was suggested that atorvastatin could exert an anti-atherosclerotic activity besides inhibiting cholesterol biosynthesis.

Key Words:
Atorvastatin, Toll-like receptor 4, NF-κB, Atherosclerosis plaque.

Introduction

Atherosclerosis, also known as arteriosclerotic vascular disease, is a progressive and inflammatory disease characterized by the accumulation of lipids and fibrous elements in the large arteries[1]. Atherosclerotic plaques contain many blood-borne inflammatory and immune cells, including the antigen presenting cells (APCs)[2]. APCs can express many pattern recognition receptors on their surfaces including Toll-like receptors (TLRs) which are required for host defense against infection[3]. TLR-4, the first of the TLRs described, primarily functions as a sensor of microbes or tissue damage, thus, serving as a first-line defense against infections or issue injury[4]. Recently, TLR4 has been linked to the pathogenesis and progression of atherosclerosis, and it could affect atherosclerosis in multiple ways[5]. TLR4 overexpresses in human atherosclerotic lesions and its activation can induce the production of cytokines and chemokines[4,6], thus, promoting the inflammation which is an inherent component of atherosclerosis. Therefore, it is recently recognized that blocking of TLR4 activation and its downstream inflammatory signaling pathways could attenuate atherosclerosis initiation or progression, and will have potential clinical benefits in patients suffering from atherosclerosis[7].

Statins, potent inhibitors of the cholesterol biosynthesis, have been reported to be widely used in the treatment of hypercholesterolemia and prevention of atherosclerotic diseases[8,9]. Statins, apart from their initially lipid-lowering effects, also have other pleiotropic effects including atherosclerotic plaque stabilization and anti-inflammatory effects[7,10]. Recent reports have shown that statins could down-regulate TLR4 protein or mRNA expression in monocytes and endothelial cells[11,12]. However, few studies have been reported the effects of statins on TLR4 expression and its downstream signaling including activation of NF-kB *in vivo*.

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In this study, we aimed to investigate the potential effects of atorvastatin on TLR4 protein and mRNA expression and NF-kB activation in rabbit atherosclerotic plaques.

Materials and Methods

Animals and Drug Administration

Adult New Zealand white rabbits were purchased from Shanghai Songjiang Songlian (Shanghai, China) and cared for in accordance with the Guide to the Care and Use of Experimental Animals.

The rabbit models of atherosclerosis[13] were established by the researchers in Department of Laboratory Animal Science, Fudan University. Rabbits were randomly assigned into two groups (10 rabbits in each group): the model group, fed with high-fat diet containing 1% cholesterol, 8% lard, and 11% egg yolk powder for 24 weeks continuously; the drug-treated group, orally administrated of atorvastatin (Pfizer, New York, NY, USA, 2 mg/kg/day) three weeks after high-fat diet feeding. The atherosclerosis plaque formation was assessed by using color Doppler ultrasound at 8 and 16 weeks. Twenty four weeks later, the rabbits were sacrificed by air injection from marginal vein of ear. The aorta samples were removed and rinsed with sodium chloride, then deep frozen in liquid nitrogen and stored at –80°C until protein or mRNA extraction.

Protein Extraction and Western Blotting

Total tissue lysates were prepared in RIPA (radioimmunoprecipitation assay) extraction buffer (Beyotime, Haimen, China) containing 100 μg/mL phenylmethylsulfonyl fluoride (PMSF, Biocolor, Shanghai, China). Protein concentration was determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). A total of 20 μl total protein were loaded in each well and separated by 10% SDS/PAGE, then transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were saturated and blocked with 10% fat-free milk at room temperature for 1 h, before being incubated with rabbit anti-TLR4 (1:200, Boster, China) and b-actin antibodies (1:1000, Boster, China). After extensive washing, the second antibody (goat anti-rabbit HRP, Santa Crutz, CA, USA) was added. Specific antibody-antigen complexes were detected using a chemiluminescence (ECL) western blotting detection kit (Tian Biotech, China). Immunoreactive bands on membranes were quantified for the level of specific induction using Leica Qwin software, Bensheim, Germany.

Real-Time Quantitative PCR

Total mRNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 1 μg RNA was converted to cDNA and then stored at –80°C. The sequences of the primers were shown in Table I. Twenty microlitres of reaction mixture was prepared for each sample, containing 10 μl SYBR Green Realtime PCR Master Mix, 1 μl primer mixture, 1 μl cDNA, and 8 μl RNase free water. The PCR amplification cycle was set as 95°C for 3 min for initial denaturation, then performed for 45 cycles under the following conditions: 15 s min at 95°C, 15 s at 61°C, and 20 s at 72°C, and finally the reaction mixture was performed at 95°C for 15 min, at 60°C for 10 s, and then at 95°C for 15 s. Copy numbers were obtained through the extrapolation of Ct values of the test samples against the corresponding standard curve, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the amount of mRNA from each sample. The data were analyzed by ABI Prism 7300 SDS Software.

Enzyme-Linked Immuno Sorbent Assay

Nuclear extract were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) following the manufacturer's instruction. The aorta samples (100 mg) were mixed with 300 μL complete lysis buffer and homogenized for 1-2 min, then incubated on ice for 30 min. After 10 min centrifugation at 10000 rpm under 4°C, the supernatant was collected for further detection.

NF-κB (p65) activation was quantified using commercially available ELISA kits (Active Motif) according to the manufacture's instruction. Briefly, nuclear extract samples were added into the wells with or without appropriate oligonucleotide binding to activated p65. Control extract in the kits and the lysis buffer were used as posi-

Table I. Primer sequences for analyzing target genes.

Primer	5′-3′
TLR-L	GCCGAAAGGTGATTGTTGTGGTGT
TLR-R	ACTGCCAGGTCTGAGCAATCTCAT
GAPDH-L	ACTCTGGCAAAGTGGATGTTGTCG
GAPDH-R	TTGATGACCAGCTTCCCGTTCTCA

tive control and blank. After 1 hour incubation at room temperature with mild agitation and proper washing, primary antibody was added to each well and incubated for 1 hour. The wells were washed again and added with horseradish peroxidase (HRP) conjugate antibody, then incubated for another 1 hour. Developing solution was added after washing. Five minutes later, the absorbance was read at 450 nm.

Statistical Analysis

All the data analysis was carried out using the statistical analysis software SPSS 10.0 for windows (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation (SD). Significant differences between the groups were analyzed using paired Student's *t*-test, and differences were considered significant at $p < 0.05$.

Results

In-vivo Effect of Atorvastatin on TLR4 Protein Expression in Rabbit Atherosclerotic Plaques

Six rabbits in the model group and seven in the atorvastatin-treated group were successfully survived to the end of this study. The expression of TLR4 protein in rabbit atherosclerotic plaques was determined by western blotting. As shown in Figure 1, the relative expression of TLR4 was reduced by 24.1% in the atorvastatin-treated group ($p < 0.05$).

In-vivo Effect of Atorvastatin on TLR4 mRNA Expression in Rabbit Atherosclerotic Plaques

In the present study, the levels of TLR4 mRNA in rabbit atherosclerotic plaques were detected by Real-Time Quantitative PCR. Compared with the model group, TLR4 mRNA expression was significantly suppressed in the atorvastatin-treated group (Figure 2, 1.12±0.23 vs. 2.11±0.41, $p < 0.01$).

In-vivo Effect of Atorvastatin on NF-κB Activation in Rabbit Atherosclerotic Plaques

Activated NF-κB (p65) protein was determined by ELISA. As shown in Figure 3, significant difference of activated NF-κB in rabbit atherosclerotic plaques was observed between the model group (0.25±0.05) and the atorvastatin-treated group (0.06±0.01) ($p < 0.001$).

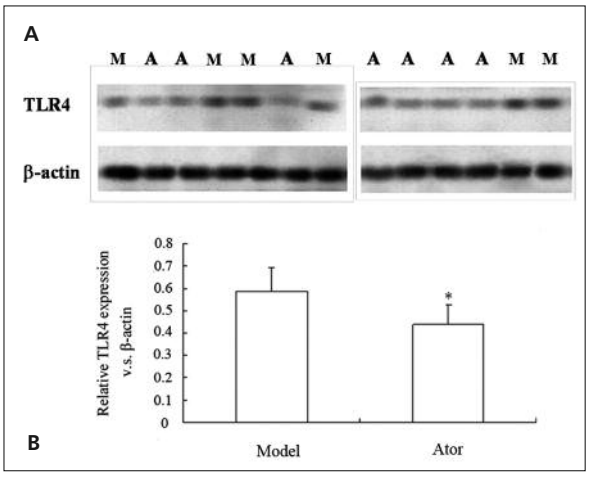

Figure 1. The *in-vivo* effect of atorvastatin on TLR4 protein expression in rabbit atherosclerotic plaques. Rabbits were randomly divided into two groups: the model group fed with high-fat diet containing 1% cholesterol, 8% lard, and 11% egg yolk powder for 24 weeks continuously and the atorvastatin-treated group with oral administration of atorvastatin (Ator, 2 mg/kg/day) three weeks after high-fat diet feeding. The expression of TLR4 protein was detected by western blotting, and the β-actin was used as the loading control (Figure 1a). Levels of TLR4 and β-actin were determined by densitometric analysis (Figure 1b). Data represent mean ± SD of six or seven rabbits. *$p < 0.05$, the model group versus the atorvastatin-treated group.

Discussion

Results of numerous studies both *in vitro*[12] and in the peripheral blood[14] support the additional activity of statins beyond their serum cholersterol-lowering effects. However, to date, the anti-

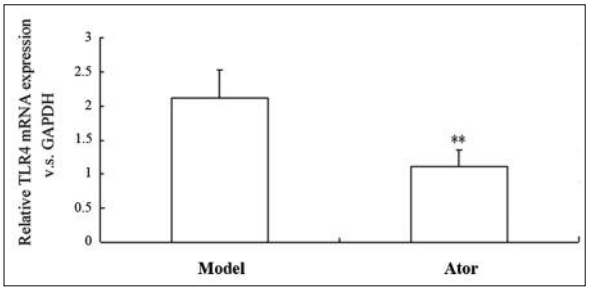

Figure 2. The *in-vivo* effect of atorvastatin on TLR4 mRNA expression in rabbit atherosclerotic plaques. Rabbits were randomly divided into two groups: the model group fed with high-fat diet containing 1% cholesterol, 8% lard, and 11% egg yolk powder for 24 weeks continuously and the atorvastatin-treated group with oral administration of atorvastatin (Ator, 2 mg/kg/day) three weeks after high-fat diet feeding. The levels of TLR4 mRNA were measured by Quantitative real-time PCR analysis. Threshold cycle values were normalized by GAPDH expression and represented as mean ± SD. **$p < 0.01$, the model group versus the atorvastatin-treated group.

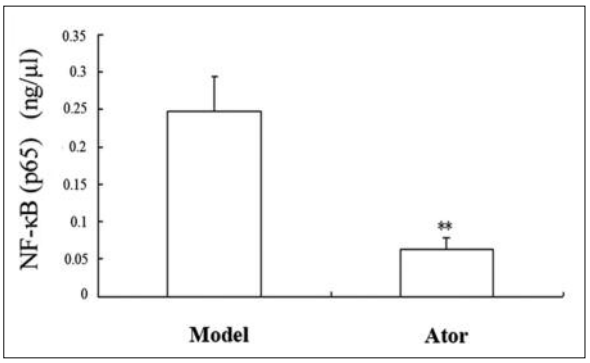

Figure 3. The *in-vivo* effect of atorvastatin on NF-κB activation in rabbit atherosclerotic plaques. Rabbits were randomly divided into two groups: the model group fed with high-fat diet containing 1% cholesterol, 8% lard, and 11% egg yolk powder for 24 weeks continuously and the atorvastatin-treated group with oral administration of atorvastatin (Ator, 2 mg/kg/day) three weeks after high-fat diet feeding. The levels of activated NF-κB were measured by ELISA. Data represent mean ± SD of six or seven rabbits. $^{**}p < 0.01$, the model group versus the atorvastatin-treated group.

inflammatory mechanism by which atorvastatin acts on atherosclerotic plaques *in vivo* has not been fully explored with regard to the intracellular signaling pathway. In this study, we reported that atorvastatin exerted its anti-inflammatory effects by suppressing TLR4 protein and mRNA expression, finally causing the inhibition of NF-κB activation for the first time.

Toll-like receptors (TLRs) are a family of structurally related type-I transmembrane receptors expressed on the cell membrane after lipopolysaccharide (LPS) stimulation. They function as the crucial components of innate immunity that recognize and react to a wide variety of pathogen-associated molecules in mammals[15]. TLR4, the first described TLR, was identified as the signaling receptor for LPS[16]. Activation of TLR4 signal is related to its downstream expression of proinflammatory cytokines in patients with acute coronary syndrome[17]. Recently, several reports have suggested an important role of TLR4 in atherosclerosis and cardiovascular disease[6,18], and inhibition of TLR4 activation involved in the LPS signaling can decrease the plaque sizes or macrophage infiltration[19].

Statins could inhibit the activity of 3-hydroxy-3-methylglutaryl CoA reductase, thus, resulting in the increased clearance of LDL cholesterol from the bloodstream[7]. It has been reported that statins could also inhibit TLR4 surface protein expression and downstream signaling in many types of cells *in vitro*. Statins have been proposed to have direct anti-inflammatory activity. It can reduce the number of inflammatory cells within the atherosclerotic plaque. These critical functions have made statins to be widely used in the treatment of hypercholesterolemia which contributes to the atheromatous plaque formation, finally in turn preventing the atherosclerosis. However, little is known about the mechanisms underlying these anti-inflammatory effects of statins, especially *in vivo*.

In the present study, we established rabbit models of atherosclerosis. Our results showed that atorvastatin-treatment could significantly decrease the TLR4 protein and mRNA expression in atherosclerosis plaques. Compared with the model group, the expression of NF-κB, the important composition of TLR4 downstream pathway, was also significantly decreased in the atorvastatin-treatment group. These results suggested that atorvastatin is a potential anti-atherosclerotic drug independent of its cholesterol-lowering function.

There are some potential criticisms to our study, since the exact mode of statin action on TLR4 protein and mRNA expression remains to be further explored. Thus, further studies are needed to examine the exact molecular mechanisms of statin-dependent regulation of TLR4 expression *in vivo*. Moreover, the effects of atorvastatin on NF-κB protein expression and inflammatory factors (e.g. IL-1b, IL-6, IL-12, and TNF-a) modulated by NF-κB are required to clarify.

Conclusions

Atorvastatin inhibits TLR4 protein and mRNA expression, and suppresses TLR4-dependent NF-κB activation *in vivo*. These findings may provide a better understanding of the molecular mechanism of the beneficial effects of statin therapy in atherosclerotic disease.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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