

Contents lists available at [ScienceDirect](http://www.sciencedirect.com)

Asian Pacific Journal of Tropical Medicine

journal homepage: www.elsevier.com/locate/apjtm

Document heading doi:

Effect of atorvastatin on expression of TLR4 and NF- κ B p65 in atherosclerotic rabbits

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ARTICLE INFO

ABSTRACT

Article history:

Received 10 March 2013
 Received in revised form 15 April 2013
 Accepted 15 May 2013
 Available online 20 June 2013

Keywords:

Atherosclerosis
 Atorvastatin
 TLR4
 NF- κ B p65

Objective: To study the effect of atorvastatin on atherosclerotic rabbits. **Methods:** A total of 60 New Zealand male rabbits were randomly divided into the normal group, model group and atorvastatin group. The replication rabbit atherosclerotic model with immune injury combined with a high fat diet feeding was used. All rabbits were sacrificed after 3 months. TLR4 and NF- κ B p65 were observed by HE staining, immunohistochemistry and western blotting. **Results:** The expression of TLR4, NF- κ B p65 were significantly increased in the model group compared with the normal group. The expression of TLR4 and NF- κ B p65 decreased significantly in the atorvastatin group, and there was no difference compared with the normal group. **Conclusions:** The effect of atorvastatin on atherosclerosis may be achieved by the inhibition of the expression of TLR4 and NF- κ B p65.

1. Introduction

The inflammatory reaction is an important reason for the formation of the atherosclerosis^[1], atorvastatin which inhibit vascular inflammatory response can effectively intervene atherosclerosis^[2]. Based on this, the replication rabbit atherosclerotic model with immune injury combined with a high fat diet feeding was used as research objects in this study. The changes of TLR4 and NF- κ B p65 during the formation of atherosclerosis were detected by HE staining, immunohistochemistry and western blotting methods, and atorvastatin intervention were also studied in order to provide new ideas for drug intervention of atherosclerosis.

2. Materials and methods

2.1. Animals

A total of 60 general male New Zealand white rabbits were selected, weighting 2.0–2.2 kg, aged three months, provided by Shanghai Shengwang Experimental Animal Breeding Co., Ltd., certification number: SCXX (Shanghai) 2012–0007. They were fed, modeled and observed by the Experimental Animal Center of Shanghai University of TCM, with the feeding temperature 18–20 °C, and the relative humidity 50%–70%.

2.2. Main reagents

Bovine serum albumin (Sigma, USA), Atorvastatin (Beijing Jialin Pharmaceutical Co., Ltd., Batch number: H199990258). Mouse anti-rabbit TLR4 monoclonal antibody was purchased from Thermo Fisher Scientific Inc; NF- κ B

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Foundation project: It is supported by the National Natural Science Foundation of China (No. 81202731), Shanghai Municipal Health Bureau Fund (No. 20114047).

p65 mouse anti-rabbit monoclonal antibody was purchased from BD Company; Anti- β -glyceraldehyde phosphate dehydrogenase antibody was purchased from the Shanghai Kangchen Biological-Technical Company; Fluorescently labeled goat anti-mouse secondary antibody were purchased from Gene Company.

2.3. Model preparation and grouping

All New Zealand rabbits were randomly divided into 3 groups ($n = 20$), normal group ($n = 20$), model group ($n = 20$) and atorvastatin group ($n = 20$). After one week of adaptive feeding, to establish atherosclerotic model, rabbits were injected bovine serum albumin 250 mg/kg through ear vein, combined with daily fed of high fat diet (1 g/rabbit) for 3 months. The animals were given the diet of high fat feed formulation (80.5% basic diet + 5% egg yolk powder + 0.5% cholesterol + 4% lard) respectively^[3], processed into pellets by Shanghai Shengwang Experimental Animal Breeding Co., Ltd. The normal group were fed with conventional normal diet, the model group were fed with conventional high fat diet, the atorvastatin group were fed with processed medicated diet based on a high fat diet (calculated atorvastatin tablets in accordance with the 60 kg adult weight equivalent dose).

2.4. Sample collection and treatment

Three months after modeling, all rabbits underwent pentobarbital sodium (50 mg/kg) ear vein injection, 2 mL blood samples were collected from heart after anesthesia, then put into common tube, cultured at 4 °C. A total of 0.8 mL supernatant was collected by centrifuge (3 000 rpm, 20 min), then stored in -20 °C for blood-lipid detection. The animals were killed by jugular venesection, then aorta was isolated from the roots to the abdominal aortic bifurcation for disconnection. The aorta about 2 cm long was harvested with a distance of about 2.5 cm to the root of aorta, cut longitudinally, washed with saline and then fixed in 10% formalin for the preparation of pathological specimens. The rest of the aorta tissues were immediately washed with saline, saved in the tube after absorbed the excess liquid by filter paper, immediately frozen in the liquid nitrogen, and then moved to -80 °C refrigerator for western blotting analysis.

2.5. Index detection

TC, TG, HDL-C, LDL-C were detected by lipid testing. After aortic paraffin imbedding and routine HE staining, the aortic histopathological changes was observed under light microscope.

2.5.1. Immunohistochemical

The aortas were embedded in paraffin, made in 4 μ m slices, tissue sections was dewaxed (4 μ m), rinsed with 3% phosphate buffer, and were under microwave heat repairing. The first antibody was added (TLR4, NF- κ B p65 antibodies were diluted at 1:100) and incubated, horseradish peroxidase conjugated second antibody was added and incubated, ABC chromogenic reagent was used for the colour reaction. Meanwhile, the PBS (pH 7.4) was used as a negative control instead of the first antibody.

Five randomly vision (200 \times) of each tissue section was observed and analyzed by IPP software. The color range was divided into 4 grade: + color range <1/4 vision; ++ color range 1/4 to 1/2 vision; +++ color range 1/2-3/4 vision; ++++ color range >3/4 vision. The degree of coloration was divided into weak (+), moderate(++), strong (+++). The color range and the color degree were converted into the color rendering index = color degree \times color range. + is 1, ++ is 2, +++ is 3, ++++ is 4.

2.5.2. Western blotting detection

Tissues of aorta were collected, total protein was extracted to made protein qualification. It was denatured at 95-100 °C for 5 min, then underwent 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2.5 h, and BSA blocking. The first antibody solution (TLR4 and NF- κ B p65 which were diluted at 1:500) was added, incubated overnight at 4 °C. After washing, the second antibody was added (diluted to 1:5 000), incubated for 1 h at room temperature, washed, and scanned by the Odyssey fluorescence system. The gray integral value of each stripe were automatically read and recorded by the computer, statistical analysis was conducted based on sample integral value/internal reference ratio.

2.6. Statistical analysis

Data in every group were expressed with Mean \pm SD. All the data were managed by EPI Info 5.01a and analyzed by SPSS 13.0 software. The means of two groups were analyzed by *t* test and ANOVA. $P < 0.05$ was considered as statistical significance.

3. Results

3.1. Comparison of the blood lipid levels

Compared with the control group, the TC, TG and LDL-C levels in serum of the model group were significantly increased, while the HDL-C level was significantly reduced

($P<0.05$); Compared with the model group, TC, TG and LDL-C levels of the atorvastatin group were decreased significantly ($P<0.05$) (Table 1).

Table 1

Comparison of the blood lipid levels (mmol/L) (mean \pm sd).

Groups	TC	TG	HDL-C	LDL-C
Normal group	2.85 \pm 0.17	0.43 \pm 0.08	0.46 \pm 0.11	0.61 \pm 0.24
Model group	35.33 \pm 3.58*	3.75 \pm 0.67*	2.95 \pm 0.46*	29.08 \pm 3.57*
Atorvastatin group	26.28 \pm 1.65* Δ	2.91 \pm 0.56* Δ	3.13 \pm 0.58*	18.73 \pm 2.38* Δ

Note: Compared with the normal group, * $P<0.01$; compared with the model group, $\Delta P<0.01$.

3.2. Aorta HE staining

In normal group, the endothelium of aortic intima were smooth, and the smooth muscle were clear, there were no obvious abnormalities. In model group, there was significant plaque formation in the aortic intima, the vessel wall is not clear, with the structural disorder. In atorvastatin group, the aortic intima was smooth with a small amount of plaque and the neat arrangement of smooth muscle cells, and the plaque apparently disappeared compared with the model group.

3.3. Immunohistochemical detection for the TLR4, NF- κ B p65 expression

Compared with the normal group, the TLR4 and NF- κ B p65 expression level in the model group were significantly higher ($P<0.05$); compared with the model group, the TLR4 and NF- κ B p65 expression levels of the atorvastatin group were decreased significantly ($P<0.05$) (Table 2).

Table 2

Immunohistochemical detection for the TLR4, NF- κ B p65 expression.

Groups	TLR4	NF- κ B p65
Normal group	0.85 \pm 0.07	0.79 \pm 0.05
Model group	3.96 \pm 0.56**	3.82 \pm 0.24**
Atorvastatin group	2.82 \pm 0.64* Δ	2.78 \pm 0.31* Δ

Note: Compared with the control group, * $P<0.05$, ** $P<0.01$; compared with the model group, $\Delta P<0.05$.

3.4. Western blotting detection for TLR4, NF- κ B p65 protein levels

Electrophoresis scanning found that the TLR4, NF- κ B p65 expression in the model group were significantly higher than that of the normal group ($P<0.01$); Compared with the model group, the TLR4 and NF- κ B p65 expression of the atorvastatin group were decreased significantly ($P<0.05$) (Table 3).

Table 3

TLR4 and NF- κ B p65 protein expression of the aorta.

Groups	TLR4	NF- κ B p65
Normal group	1.35 \pm 0.27	1.42 \pm 0.35
Model group	3.97 \pm 0.68**	3.78 \pm 0.64**
Atorvastatin group	2.16 \pm 0.74* Δ	2.64 \pm 0.73* Δ

Note: Compared with the normal group, * $P<0.05$, ** $P<0.01$; compared with the model group, $\Delta P<0.05$.

4. Discussion

The research about the relationship between the inflammatory response and the development of the atherosclerosis has been popular, but its internal mechanism has not been elucidated. It is generally believed that the infiltration of inflammatory cells to the vascular endothelial can lead to vascular endothelial injury, which can also induce the formation of foam cells, and then form the atherosclerosis. Currently the wide range clinical use of atorvastatin can delay the atherosclerosis disease progression by reducing blood lipid levels, controlling systemic inflammatory response and the plaque stabilization, thereby intervene in the atherosclerosis effectively, lower the atherosclerosis mortality[4]. Previous studies have shown that atorvastatin has the function of lipid-lowering and stabilizing plaque, and it can also inhibit the atherosclerosis. In this study, we explore the possible anti-atherosclerosis mechanism by investigating atorvastatin's intervention to atherosclerosis rabbits.

NF- κ B is an important multi-directional nuclear transcription factor, it involved in the process of immune response and cell proliferation and differentiation, plays an important role in the immune response process. Toll-like receptors (TLRs) is an ancient innate immune receptors which widely present in the endothelial cell surface, as an immune receptors which first sense the pathogen and deliver the extracellular antigen identification information to the intracellular. When TLRs stimulated by a variety of factors, TLR4 of the endothelial cell surface was activated, the signal can be passed down and activated the nuclear transcription factor NF- κ B, thereby inducing target gene expression, activated the inflammatory response[5]. Research reported that TLR is the core of the immune response process, it is the initiating links and core for the recognition and signal transduction of the exogenous antigen and the endogenous antigen[6]. There are some other studies believe that TLR4/NF- κ B signaling pathway play an important role in the development process of atherosclerosis, and TLR4 pathways can activate NF- κ B through the MyD88-

dependent signal^[7-9].

Our results suggest that the lipid levels, TLR4 and NF- κ B p65 expression were significantly increased after immune injury combined with a high fat diet for rabbits, while after atorvastatin statin therapy, the blood lipids levels, TLR4 and NF- κ B p65 expression were significantly lower. Because a large number of inflammatory mediators plays a critical role in the process of atherosclerosis, atorvastatin can reduce the level of inflammatory factor, and reduce the damage of chronic inflammatory response to the coronary of the patients, so as to delay the process of pathological changes for the coronary^[10]. Thus, we believe that the atorvastatin can achieve effective intervention on atherosclerosis by regulating the lipid metabolism, affecting the TLR4/NF- κ B signaling pathway and inhibiting of the inflammatory response.

In summary, by the effective regulation of lipids and the inhibition for the expression of the key factor TLR4 and NF- κ B p65 in TLR4 / NF- κ B signaling pathways, atorvastatin may achieve the purpose of effective intervention on atherosclerosis. In view of statins concentration-response relationship has been popular in the atherosclerosis research^[11,12], we will further discuss that in the follow-up study.

Conflict of interest statement

We declare that we have no conflict of interest.

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