

Protective effect of rivaroxaban on arteriosclerosis obliterans in rats through modulation of the toll-like receptor 4/NF- κ B signaling pathway

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Abstract. The aim of the present study was to explore the pharmacological role of rivaroxaban in rats with arteriosclerosis obliterans (ASO) and the potential mechanism of its action. A total of 60 adult male Sprague Dawley (weighing 210-250 g) were randomly assigned into either the sham group, model group or Riv group. Rats in the sham group were fed a normal diet, whereas those in model group and Riv group were fed a high-fat diet for 8 weeks. After establishment of the ASO model, rats in the Riv group were intragastrically administered 10 mg/kg rivaroxaban, whereas those in the sham group and the model group were administrated with the same volume of 0.9% saline for 4 weeks. At the end of animal procedures, a blood sample and the femoral artery of the rats were harvested. The results of the present study revealed that rats in the model group presented with an irregularly narrowed femoral artery lumen, disordered endothelial cells, internal elastic plates and smooth muscle cells. By comparison, the arterial wall structure and stenosis of the femoral artery of rats in Riv group recovered and all the pathological changes were alleviated after rivaroxaban treatment. Levels of total cholesterol, triglycerides and low-density lipoproteins decreased, whereas the level of high-density lipoproteins increased in the Riv group compared with the model group. Rivaroxaban treatment significantly reduced serum levels of interleukin-1, tumor necrosis factor- α and monocyte chemoattractant protein-1 (MCP-1), and increased the serum level of transforming growth factor- β (TGF- β). Rats in the Riv group had reduced expression of toll-like receptor 4 (TLR4), NF- κ B and MCP-1, and increased expression of TGF- β in femoral

artery tissues compared with the model group. Therefore rivaroxaban may have exerted its anti-atherosclerotic effects by regulating the expression of genes in the TLR4/NF- κ B signaling pathway and the activation of the downstream molecules.

Introduction

In arteriosclerosis obliterans (ASO), lipids are persistently deposited in the intima of arteries to form atheromatous plaques (1). The intima and middle layers of the arteries are deteriorated and then proliferate, leading to thickening, stiffness and distortion of arterial walls (1,2). The gradual loss of elasticity, enlargement of atherosclerotic plaque and secondary thrombosis result in narrowing or even obstruction of the arterial lumen, leading to corresponding ischemic symptoms at the distal end of arteries (1,2).

There are numerous hypotheses regarding the etiology of ASO, including lipid infiltration, thrombosis and inflammatory injury response (3-5). Although these hypotheses do not comprehensively explain all the pathological phenomena of ASO, they do demonstrate that atherosclerosis (AS) is initiated by form damaging stimuli, such as dysregulation of lipid metabolism, hemodynamic damage, heredity, infection, and physical or chemical stimuli (6,7). Multiple inflammatory factors and associated cytokine networks co-operatively act on the vascular wall, leading to a persistent state of vascular dysfunction (8). The gradual formation and development of AS plaques in blood vessels, accompanied by the rupture of unstable plaques and thrombosis ultimately result in different degrees of stenosis or occlusion of the arteries, leading to clinical events of acute and chronic limb ischemia (8).

Recent studies have suggested that toll-like receptor 4 (TLR4) and its associated signal transduction pathways are critical for formation of AS (9,10). TLR4 expression is high in the atherosclerotic plaque, resulting in the synthesis and release of various cytokines or chemokines associated with AS (11,12). TLR4 activates nuclear translocation of NF- κ B by mediating the myeloid differentiation primary response protein MyD88 (Myd88)-dependent early response pathway, thus initiating a series of inflammatory responses by producing pro-inflammatory factors and monocyte

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chemoattractants (12). Transforming growth factor- β (TGF- β) is inhibited by the p38 mitogen activated protein kinase (MAPK) pathway (13-15). Increased expression of monocyte chemoattractant protein-1 (MCP-1) accelerates the progression of AS, and its absence can slow the progression of AS (16). TGF- β downregulated the levels of cytokines during the atherosclerotic inflammatory response, including tumor-necrosis factor- α (TNF- α), interferon (IFN) γ and interleukin (IL)-1 (17). IL-1 β and TNF- α are proinflammatory cytokines implicated in the pathogenesis of autoimmune diseases such as rheumatoid arthritis, whereas TGF- β is an anti-inflammatory cytokine, which has been reported to serve an anti-inflammatory role in autoimmune diseases such as multiple sclerosis and mediate the beneficial effect of IFN β in multiple sclerosis (18-20).

At present, the primary treatment options used for treating ASO are drug therapy and surgical treatment for inhibiting arterial intimal hyperplasia (1). However, surgical treatment has certain risks such as angina pectoris, myocardial infarction and pulmonary infection, is expensive, and the middle-aged and elderly patients may refuse surgery (21). Therefore patients with ASO are frequently treated with drug therapy including antiplatelet drugs, vasodilators and drugs that promote collateral circulation. Rivaroxaban is a novel anticoagulant with the advantages of easy absorption, a quick onset of effect and fewer and less egregious side effects (22). Rivaroxaban is used at present to prevent the formation of venous thrombosis and pulmonary embolism after hip or knee joint replacement and may also be used to prevent the recurrence of coronary artery syndrome (22). Rivaroxaban may reduce NF- κ B activity through the Myd88-dependent pathway of TLR4, thereby preventing AS by reducing the expressions and release of downstream pro-inflammatory cytokines (23). The present study developed a rat model of ASO and the pharmacological role of rivaroxaban was determined when used to treat ASO.

Materials and methods

In vivo ASO model. A total of 60 adult male Sprague Dawley rats (age, 6-8 weeks; weight, 210-250 g), were obtained from Charles River Laboratories. The rats were housed in a temperature-controlled room (21 \pm 2°C) with 40-70% relative humidity and a 12-h light/dark cycle. All rats had free access to water and food. The rats were randomly assigned into three groups, a sham group, model group and the Riv group, with 20 rats in each group. Rats in the sham group were fed with a normal diet, whereas those in the model group and Riv group were fed a high-fat diet for 8 weeks. After establishment of the ASO model by surgery, rats in the Riv group were intragastrically administered with 10 mg/kg rivaroxaban as described previously (24), whereas those in sham group and model group were administered with the same volume of 0.9% saline for 4 weeks. The present study was approved by The Animal Ethics Committee of Zhejiang Chinese Medical University Animal Center (Hangzhou, China).

Construction of ASO model in rats. After 8 weeks of high fat diet and prior to rav administration, rats were anesthetized with

intraperitoneal injection of 10% chloral hydrate (300 mg/kg). After routine disinfection of the bilateral inguinal region and the skin on the inner side of the hind limbs, a longitudinal incision of ~2 cm in length was made from the bilateral groin to the knee joint. The surrounding tissue of the vascular nerve sheath was isolated, and the femoral artery and its branches were ligated for 30 sec. Rats in the sham group were only cut open but the ligation was not performed. After the incision was closed, 2 ml saline was subcutaneously injected for fluid infusion. Rats were returned to the cage until their vital signs were stable.

Sample collection. After a total of 4 weeks of Rav/saline treatment, the rats received anesthesia with 10% chloral hydrate (0.4 g/kg) and were sacrificed by cervical dislocation prior to blood collection. A 2.5-ml blood sample was harvested from the tail vein and centrifuged at 1,000 x g for 15 min at 4°C. The supernatant was collected and preserved at -80°C. A part of rat femoral artery was resected and washed with PBS. Half of the femoral artery was preserved at -80°C, and the other half was fixed in 4% paraformaldehyde or 2.5% glutaraldehyde at room temperature for 24 h.

Hematoxylin and eosin (H&E) staining. The rat femoral artery fixed with 4% paraformaldehyde was embedded in paraffin and sectioned into slices (4- μ m-thick). Artery slices were unfolded in warm water at room temperature for 60 min and transferred on to a slide. After staining with hematoxylin for 5 min at room temperature and eosin for 3 min at room temperature, the femoral artery was observed using a light microscope (magnification, x400).

Transmission electron microscopy. Rat femoral arteries fixed with 2.5% glutaraldehyde were washed with PBS and re-fixed with 1% osmic acid at room temperature for 2 h. After washing with PBS and different concentrations of ethanol (30-100%), the femoral artery was dehydrated using 100% acetone and embedded in Epon 812 at 45°C for 12 h. The artery was sectioned in to 70-nm thick slices. Sections were stained with lead citrate for 10 min and uranium acetate for 30 min at room temperature, and finally imaged using transmission electron microscope (magnification, x25,000).

Determination of the serum lipid levels. Serum levels of total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) in rats were measured using an automatic serum biochemical analyzer (BK-200; BIOBASE).

ELISA. Serum samples were warmed in room temperature. Serum levels of IL-1 (cat. no. SRLB00), TNF- α (cat. no. SRTA00), MCP-1 (cat. no. DY3144-05) and TGF- β (cat. no. SMB100B) in rats were determined using commercial ELISA kits (R&D Systems, Inc.).

Western blotting. The femoral artery tissues were homogenized after the addition of RIPA lysis buffer (Beyotime Institute of Biotechnology). Then the supernatant was centrifuged at 5,000 x g for 10 min at 4°C. BCA assay kit (cat. no. p0011; Beyotime Institute of Biotechnology) was

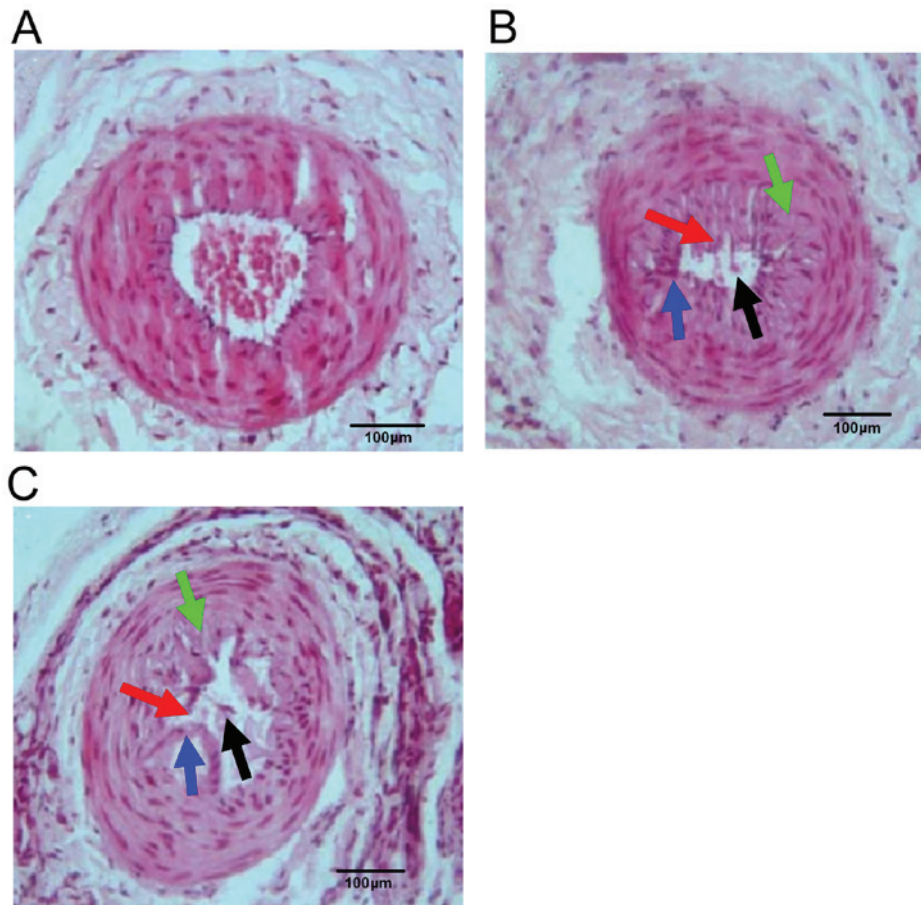


Figure 1. Pathological changes of the rat femoral artery. Magnification, x200. Pathological changes of the rat femoral artery in the (A) sham group, (B) model group and (C) the rivaroxaban group. Black arrow, irregularly narrowed femoral artery lumen; red arrow, disordered endothelial cells; blue arrow, defective internal elastic lamina; green arrow, proliferative smooth muscle cells.

used to determine total protein content. A total of 10 µg protein was loaded per lane and separated by SDS-PAGE (12% gel). After being separated, the proteins were transferred to a PVDF membrane (Roche Diagnostics), which was blocked with 5% skim milk for 1 h at room temperature. The specific primary antibodies including TLR4 (1:500; cat. no. ab217274; Abcam), NF-κB (1:500; cat. no. ab32360; Abcam), MCP-1 (1:500; cat. no. ab25124; Abcam), TGF-β (1:500; cat. no. ab92486; Abcam) and GAPDH (1:500; cat. no. ab181602; Abcam), were used to incubate with the membrane overnight at 4°C. The membrane was washed with TBS with Tween-20 (TBST) five times, and the goat anti-rabbit IgG H&L secondary antibody (1:1,000; cat. no. ab150077; Abcam) was used to incubate the membrane for 2 h at room temperature. After washing with TBST for 1 min, SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) was used to visualize the signals. Quantity One software (version 4.0, Bio-Rad Laboratories, Inc.) was used for quantification.

Statistical analyses. All statistical analyses were performed in SPSS version 17.0 (SPSS Inc.). Data are presented as the mean ± standard deviation. Differences among multiple groups were analyzed by one-way ANOVA, followed by a least significant difference post-hoc test. Each experiment

was repeated three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Pathological changes of rat femoral artery. Femoral artery endothelial cells, inner elastic plate and smooth muscle cells of rats in the sham group were regularly arranged, and the vascular lumen was not narrowed (Fig. 1A). Rats in model group presented irregularly narrowed femoral artery lumen (black arrow), disordered endothelial cells (red arrow), defective internal elastic lamina (blue arrow) and proliferative smooth muscle cells (green arrow) (Fig. 1B). These characteristics were present in the Riv group, but to a lesser extent compared with the model group indicating that the arterial wall structure and stenosis of the femoral artery of rats in Riv group were partially recovered (Fig. 1C).

Transmission electron microscopy of the rat femoral artery. Endothelial cells of rats in the sham group were intact and covered the vascular surface. The internal elastic lamina was clear and complete (Fig. 2A). In the model group, the endothelial cells were irregular and the incomplete endothelium was exposed to the luminal surface (blue arrow). The inner elastic plate was partially deformed (red arrow). Smooth muscle cells, which had migrated to the vascular intima, showed deformation

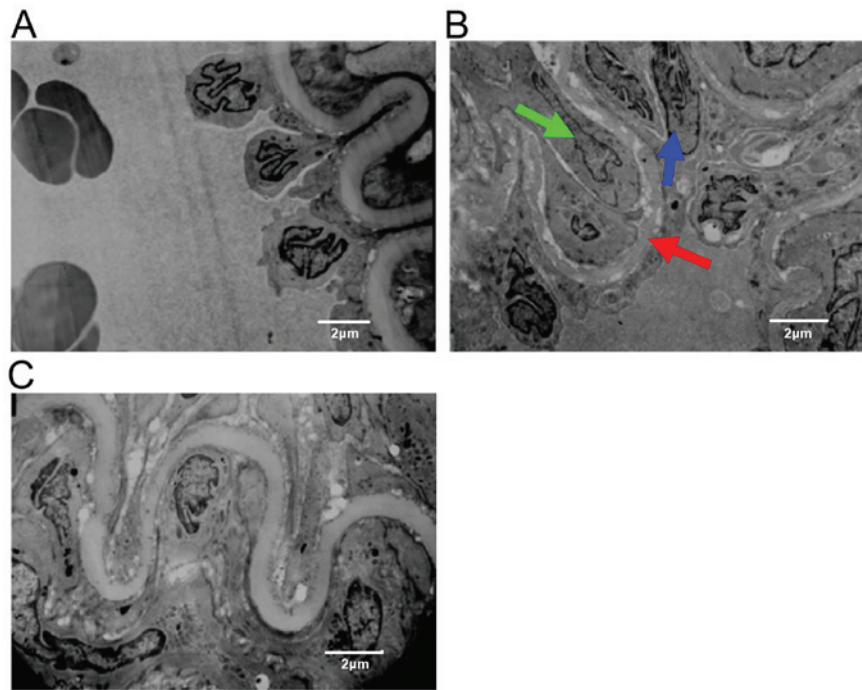


Figure 2. Transmission electron microscopy of the rat femoral artery. Magnification, x20,000. Transmission electron microscopy of the rat femoral artery in the (A) sham group (B) model group and (C) the rivaroxaban group. Blue arrow, irregular endothelial cells and incomplete endothelium exposed to the luminal surface; red arrow, partially deformed elastic plate; green arrow, deformation and nuclear condensation of smooth muscle cells which had migrated to the vascular intima.

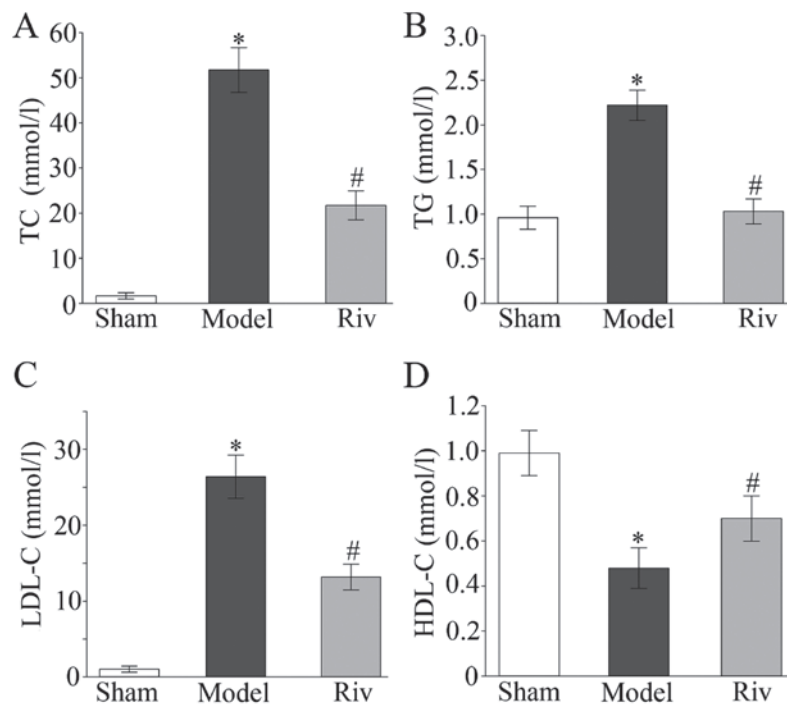


Figure 3. Serum levels of lipids. Serum levels of (A) TC, (B) TG, (C) LDL-C, (D) HDL-C in rats of the different groups * $P < 0.05$, compared with sham group; # $P < 0.05$, compared with model group. TC, total cholesterol; TG, triglyceride; LDL-C, low density lipoproteins; HDL-C, high density lipoproteins; Riv, rivaroxaban.

and nuclear condensation (green arrow) (Fig. 2B). Rats in Riv group presented regular arterial endothelial cells and smooth muscle cells, as well as a continuous elastic plate (Fig. 2C).

Serum lipid levels. Compared with the sham group, rats in the model group exhibited significantly higher levels of TC, TG and LDL-C, as well as significantly lower levels of HDL-C (all

$P < 0.05$; Fig. 3). The levels of TC, TG and LDL-C were significantly decreased, and the levels of HDL-C were significantly increased in the Riv group compared with the model group (all $P < 0.05$; Fig. 3).

Serum levels of inflammatory factors in rats of different groups. ELISA data demonstrated that rats in the model group

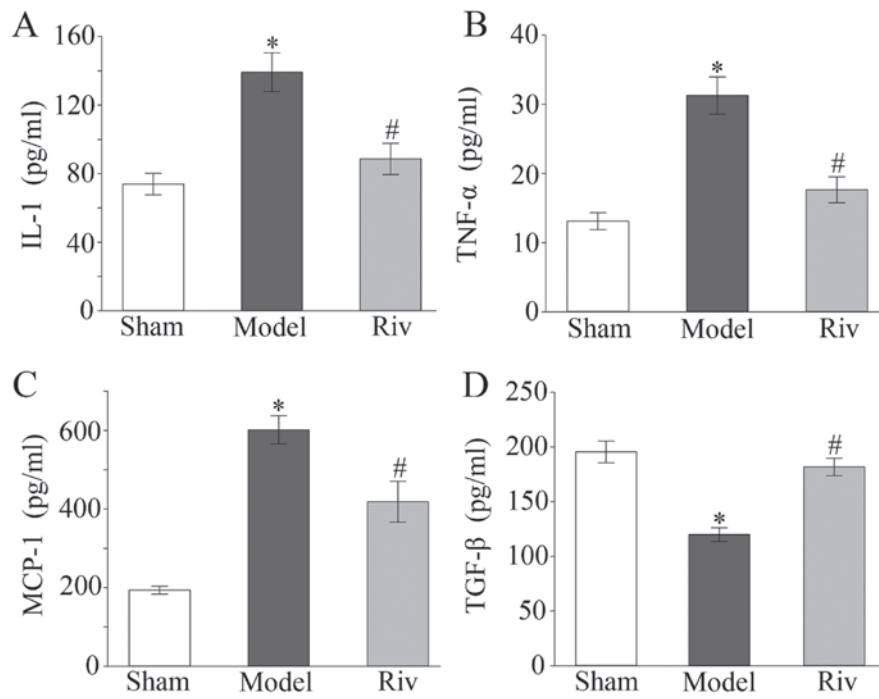


Figure 4. Serum levels of inflammatory factors. Serum levels of (A) IL-1, (B) TNF- α , (C) MCP-1, (D) TGF- β in rats of the different groups *P<0.05, compared with sham group; #P<0.05, compared with model group. IL-1, interleukin-1; TNF- α , tumor necrosis factor α ; MCP-1, monocyte chemoattractant protein-1; TGF- β , transforming growth factor β .

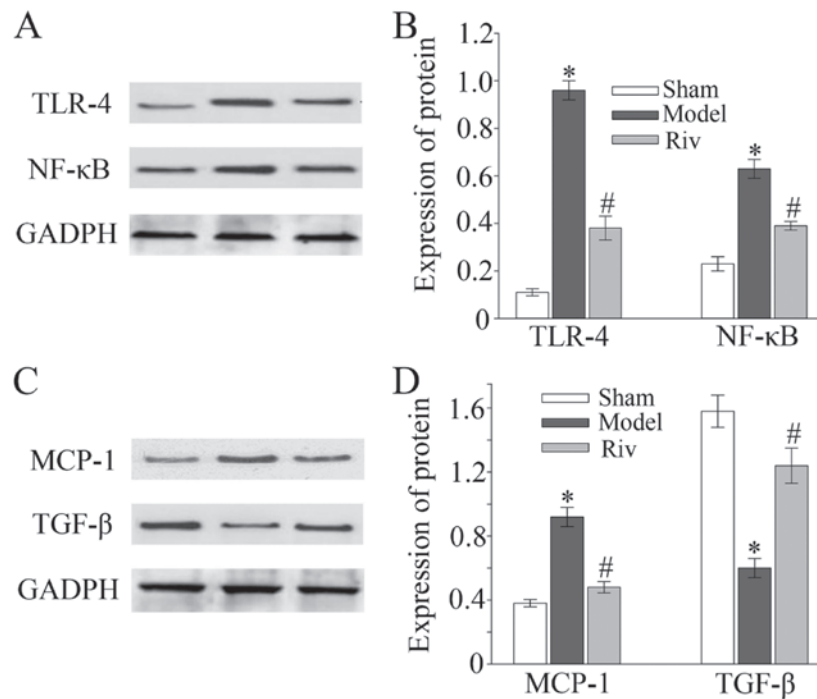


Figure 5. Protein expression of TLR4, NF- κ B, MCP-1 and TGF- β in the rat femoral artery. Protein expression of (A) TLR4, (B) NF- κ B, (C) MCP-1 and (D) TGF- β in the rat femoral artery *P<0.05, compared with sham group; #P<0.05, compared with model group. TLR-4, toll-like receptor-4; NF- κ B, nuclear factor- κ B; MCP-1, monocyte chemoattractant protein-1; TGF- β , transforming growth factor- β .

exhibited significantly higher serum levels of IL-1, TNF- α and MCP-1 compared with the sham group, whereas the TGF- β level was significantly lower (all P<0.05; Fig. 4). Rivaroxaban treatment significantly decreased the serum levels of IL-1, TNF- α and MCP-1, and increased the serum levels of TGF- β compared with the model group (all P<0.05; Fig. 4).

Protein expression of TLR4, NF- κ B, MCP-1 and TGF- β in rat femoral artery. Upregulated protein expression levels of TLR4, NF- κ B and MCP-1 were detected by western blot analysis the in femoral artery tissues of rats in the model group compared with those in sham group (all P<0.05; Fig. 5). However, rats in the Riv group exhibited decreased expression of TLR4,

NF- κ B and MCP-1 in the femoral artery tissues compared with the model group (all $P < 0.05$; Fig. 5). Protein expression levels of TGF- β were lower in the femoral artery tissues of rats in the model group compared with the sham group, and were increased in the Riv group compared to the model group (all $P < 0.05$, Fig. 5).

Discussion

The occurrence of ASO is a complex process, and its cause has not been fully elucidated. Genetics, sex, age, abnormal lipid metabolism, obesity, smoking, mechanical damage of blood vessel walls and imbalance of trace elements are recognized as factors affecting the occurrence of ASO (25). Additionally, long-term mental stimulation and emotional stress result in contraction of the arteries (26). Increased blood pressure results in dystrophy of the blood vessel wall and deposition of certain substances in the blood vessels, eventually leading to the occurrence of ASO (25-27).

The inflammatory response is an important factor affecting the occurrence of ASO. TLRs are not only key molecules of the inflammatory process, but are also the initial link between the recognition of exogenous antigens and initiation of the inflammatory response (28). TLRs are a family of receptors expressed on the cell membrane. As transmembrane signal transduction receptors, TLRs link both innate and specific immunity, when molecular components of certain microorganisms are recognized (29). *In vitro* studies have shown that TLR4 expression is lower in human vascular endothelial cells under physiological conditions (14,15). However, stimulation of inflammatory factors markedly upregulates TLR-4 expression in tunica media vascular smooth muscle cells, exerting a significant role in vascular reconstruction (30). TLR4 is expressed and increased in human atherosclerotic plaques, and is involved in the proliferative regulation of smooth muscle cells (28). Plaques subsequently migrate to the tunica intima under stimulation of cytokines, which is the primary step in the formation of an atherosclerotic plaque (31,32). NF- κ B is an essential multi-channel nuclear transcription factor involved in the inflammatory process, cell proliferation and differentiation (33,34). TLR4 not only activates NF- κ B, but also stimulates macrophage aggregation and inflammatory response by upregulating MCP-1 through the Myd88-dependent signaling pathway (16,35). MCP-1 is involved in the formation and transformation of macrophages and may promote the formation of atherosclerotic plaques by regulating inflammatory factors (35).

Rivaroxaban is a highly selective oral drug that directly inhibits factor Xa (FXa), which has an antithrombotic effect in an *in vivo* arteriovenous thrombosis model. Rivaroxaban not only inhibits free FXa, but also inhibits the activity of FXa in the prothrombin complex (36). In the coagulation cascade, FXa is involved in regulating the conversion of prothrombin to thrombin on the surface of vascular cells (36). A previous study demonstrated that FXa activates the acute inflammatory response (37). In endothelial cells, FXa can activate NF- κ B, resulting in the release of inflammatory factors such as IL-6 and MCP-1 (38). Activation of inflammatory pathways is closely associated with the coagulation reaction (37,38). Previous studies have found that anticoagulant therapy

efficiently inhibits coagulation activation and the inflammatory response, suggesting that anticoagulant therapy may be applied in treating ASO (39).

In the present study, the levels of IL-1, TNF- α , MCP-1, TLR-4 and NF- κ B in the rats of the model group were significantly increased compared with those in the sham group, whereas TGF- β levels decreased. TGF- β expression may be inhibited by Myd88-dependent TLR4/NF- κ B signal transduction by activating the p38MAPK pathway, thus attenuating the anti-inflammatory effect of TGF- β (9). Levels of IL-1, TNF- α , MCP-1, TLR-4 and NF- κ B in the Riv group were lower compared with those in the model group, while the TGF- β level increased. Therefore, rivaroxaban may suppress transcriptional activity of NF- κ B and synthesis of MCP-1 by inhibiting TLR4 expression. TGF- β expression was increased in the Riv group, which in turn negatively regulated Myd88-dependent TLR4/NF- κ B signal transduction decreasing the inflammatory response. Specific TLR4 inhibitors, such as VGX-1027 and eritoranor, were used to treat a number of inflammatory diseases, with positive results (40-42). The results of the present study highlight the possibility of using specific TLR4 inhibitors to treat ASO.

In conclusion, an ASO model in rats was developed by crush injury of the femoral artery and feeding the rats with a high-fat diet. The TLR4/NF- κ B pathway and its downstream inflammatory factors were inhibited following rivaroxaban treatment. Therefore, rivaroxaban may prevent ASO through inhibiting inflammatory response.

The TLR-4/NF- κ B signaling pathway is an important signal transduction mechanism and may be a key regulatory pathway in AOS. Rivaroxaban may significantly inhibit inflammation and serve an anti-atherosclerotic role by inhibiting the TLR-4/NF- κ B signaling pathway and downstream inflammatory factors.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

XL and XY designed the study, performed the experiments, analyzed the data, and prepared the manuscript. XL, JC and ZY established the animal models and collected the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of Zhejiang Chinese Medical University Animal Center (Hangzhou, China; approval no. 20180322).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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