



Atorvastatin suppresses vascular hypersensitivity and remodeling induced by transient adventitial administration of lipopolysaccharide in rats

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Background: The phenotypic transition of vascular smooth muscle cells (VSMCs) from a contractile to a proliferative state markedly affects the pathophysiology of cardiovascular diseases. The adventitial inflammation can promote neointimal formation and vascular remodeling. We used direct administration of lipopolysaccharide (LPS) into the periphery of the carotid artery to investigate the influence of transient adventitial inflammation on vascular remodeling and its potential mechanism.

Methods: Male 15-week-old Wistar rats were randomly assigned to four groups with six rats in each group. The rats of groups I and II were administered distilled water, and group III and IV were treated with fasudil and atorvastatin respectively. All treatments were given daily for 11 days. On day 8, the adventitia in group I was injected with 5 μ L sterile saline, and the group II–IV were injected with 5 μ L sterilized LPS. The carotid blood flow and femoral blood pressure were measured *in vivo*, and the thickness of vascular intima and middle layer was measured *in vitro*. Serum interleukin-6 (IL-6) and tumor necrosis factor α (TNF α) were determined using enzyme-linked immunosorbent assay (ELISA) assay. And the Rho-associated coiled-coil-containing protein kinase 2 (ROCK2), myosin phosphatase target subunit 1 (MYPT1), myosin light chain (MLC), myocardin, SM- α actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected by western blot. The comparisons were made by one-way analysis of variance with Bonferroni's post hoc test. A value of $P < 0.05$ was considered to represent a statistically significant difference.

Results: Transient adventitial inflammation induced by LPS caused no obvious change in basal blood flow, but did lead to vascular hypersensitivity to serotonin. Morphological examinations revealed that the medial layer was the only domain affected, and showed VSMC proliferation and rearrangement. LPS increased serum IL-6 and TNF α contents, ROCK2 expression and activity, and caused changes in the expression levels of some stereotypical VSMC genes. Similar to the Rho-kinase inhibitor fasudil, atorvastatin completely restored the morphological alterations, even increased blood flow.

Conclusions: Our study confirms the beneficial effect of atorvastatin on the vascular system in terms of morphology and function.

Keywords: Lipopolysaccharide (LPS); vascular smooth muscle cells (VSMCs); myocardin; Rho-associated coiled-coil-containing protein kinase 2 (ROCK2)

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Introduction

Blood vessel walls are composed of the intima, media, and adventitia. The media is mainly composed of vascular smooth muscle cells (VSMCs) in an extracellular matrix (ECM). VSMCs are not terminally differentiated and possess the potential to change phenotype in response to alterations in the local environment (1). Phenotypic switching of VSMCs from a differentiated (contractile) to a dedifferentiated (proliferative) state plays a critical role in the pathophysiology of cardiovascular diseases, including atherosclerosis, hypertension, and restenosis after angioplasty (1,2). The transition of VSMCs between contractile and proliferative phenotypes is influenced by microenvironmental cues, such as growth factors, ECM components, cell-cell contacts, and neuronal inputs, which lead to alterations in the expression of stereotypical VSMC genes.

Myocardin is a transcriptional coactivator of serum response factor (SRF), which controls the expression of most genes for smooth muscle (SM) contractile proteins (3). As a master coactivator of SRF, myocardin plays a critical role in the transcription of SM contractile genes, such as SM α -actin, SM22, calponin, SM myosin heavy chain (SM-MHC), and SM myosin light chain kinase (SM-MLCK) (4-6), which presumably increases the contractility of cardiac and SM. Deregulation of myocardin protein and/or activity has been implicated in numerous cardiovascular diseases, including atherosclerosis and restenosis (7-9).

RhoA is a member of a small monomeric GTPases family that is involved in SM contraction and has been implicated in certain SM-dependent processes, such as cell proliferation, adhesion, motility, and migration (10-12). The best-characterized downstream effectors of RhoA are Rho-associated, coiled-coil-containing protein kinases (ROCKs), which are directly involved in SM contraction (13). RhoA/ROCK signaling induces myocardin and subsequently stimulates the transcription of SM-specific genes, such as SM- α actin, SM-MHC, and desmin (14,15).

For many years, atherosclerosis has been regarded as an inflammatory disease (16). As arterial injuries are generally initiated at the interface with circulating blood, most studies have focused on the innermost intimal layer rather than on the outermost adventitial layer. However, increasing evidence suggests that the adventitia is a mediator of atherosclerosis and vascular dysfunction (17-19). Additionally, adventitial inflammation occurs in blood vessels of atherosclerosis patients and the infiltration

of inflammatory cells into the vascular adventitia is positively correlated with vascular membrane lesions of atherosclerosis (17-19).

Here, we directly added the bacterial product lipopolysaccharide (LPS) to the adventitia of rat carotid arteries to induce transient adventitial inflammation. LPS-induced transient adventitial inflammation resulted in vascular hypersensitivity to serotonin, proliferation, and disordered arrangement of VSMCs. We also demonstrated that LPS addition activated the RhoA/Rho-kinase ROCK2. Importantly, fasudil and atorvastatin inhibited ROCK2, completely blocked hypercontraction in response to serotonin, and prevented the proliferation and disordered rearrangement of VSMCs. Our findings indicate that the activation of the RhoA/Rho-kinase signaling pathway plays an important role in LPS-induced alteration of vascular contractility and VSMC proliferation. Both fasudil and atorvastatin prevented the LPS-induced increases in myocardin levels, suggesting the involvement of ROCK2 activation in induced myocardin expression. In summary, our data indicated that transient adventitial inflammation caused proliferation and disordered arrangement of VSMCs, as well as dysregulated expression of some stereotypical VSMC genes. Additionally, activation of the RhoA/Rho-kinase signaling pathway played an important role in the LPS-induced alteration of vascular contractility and proliferation.

Methods

The experimental protocols for animal use and care were approved by the Animal Care and Use Committee of China Medical University. All Male Wistar rats used in experiments were obtained from the Laboratory Animal Center of China Medical University. All animal experiments were conducted in strict accordance with the university guidelines. All surgeries were performed under anesthesia by intraperitoneal injection of chloral hydrate (30 mg/kg), and all efforts were made to minimize suffering throughout the course of the study.

Male 15-week-old Wistar rats (300–350 g) were randomly assigned to four groups with six rats in each group. In groups I and II, rats were administered distilled water (1 mL/kg, intragastrically). In group III, rats received fasudil (15 mg/kg, intraperitoneally). In group IV, rats were treated with atorvastatin (30 mg/kg, intragastrically). All treatments were given daily for 11 days. On day 8, both sides of the common carotid artery of each rat from each

group were separated surgically and cleared of adherent connective tissue along the longitudinal axis (15–18 mm in length). The exposed bilateral common carotid artery adventitia was then infused with 5 μ L sterile saline in group I rats, and with 5 μ L sterilized LPS (10 μ g/mL) in group II–IV rats. In brief, groups I–IV were termed as control (I), LPS (II), fasudil + LPS (III), and atorvastatin + LPS (IV) groups.

On day 8–13, the left common carotid and right femoral arteries of rats from each group were exposed surgically and a transonic volume flow-probe (2PSB; Transonic Systems Inc., Ithaca, NY, USA) and a hand-made tube were placed around the carotid or femoral artery, respectively. Carotid blood flow and femoral blood pressure were measured using a transonic perivascular flowmeter (TS420; Transonic Systems Inc., Ithaca, NY, USA) and recorded using a computerized acquisition system (MP150; Biopac Systems, Goleta, CA, USA). The system provided real-time volume flow measurements with a resolution of 0.05 mL/min. The carotid was minimally manipulated to avoid vasospasm. Vascular responses were examined after the topical application of serotonin. The carotid artery was gently wrapped with a cotton mesh and 5 μ L 1% serotonin was administered flush to the cotton mesh. Carotid artery responses to serotonin were expressed as the percent changes of blood flow induced by serotonin compared with the resting blood flow. Then, rats were humanely killed with an overdose of chloral hydrate and the left carotids were excised and fixed in 4% paraformaldehyde for 24 h. Arterial segments were embedded in paraffin and cut into 4- μ m-thick sections at the midpoint of the common carotid.

Morphological analyses were performed on hematoxylin and eosin stained (H&E stained) paraffin-embedded sections of the previously described arterial segments using a light microscope coupled to a digital camera (BX51; Olympus, Japan) imaging system. The thickness of intima and media were measured using DP2-BSW software (Olympus, Japan).

Total RNA was isolated from the pooled arteries (n=6 per group) using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA). Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously reported (18). The following primers were used: myocardin, (F) 5'-GCCTACCGTATTCCTATTAAGC-3' and (R) 5'-AGAGACTCGGGCAATCTGTGT-3'; myosin phosphatase target subunit 1 (MYPT1), (F) 5'-GCTTCCAGAACATACGATGAGAC-3' and (R) 5'-TACGGGAGTAGGCAGAGGTT-3'; ROCK2, (F) 5'-AACAGTCCGTGGGTGGTTCA-3' and (R)

5'-CGTTATCGGGCTTCACATCTC-3'; tumor necrosis factor α (TNF α), (F) 5'ACCACCAAGTGGAGGAGCAG-3' and (R) 5'-TGTCCCTTGAAGAGAACCTG-3'; interleukin-6 (IL-6), (F) 5'-GTGCAATGGCAATTCTGATTG-3' and (R) 5'-TAGCTATGGTACTCCAGAAG-3'; and β -actin, (F) 5'-CTCATCCACGA AACCACCTAT-3' and (R) 5'-CGCCGATCCAGACAGAATA-3'. Results were normalized to β -actin expression levels and presented as arbitrary units.

The pooled carotids arteries (n=6 per group) were lysed using lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100] containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Extracted proteins were quantified using the bicinchoninic acid (BCA) protein assay kit. Equivalent amounts of protein (50 μ g) were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blotting was performed using primary antibodies against ROCK2 (Abcam, Cambridge, UK), MYPT1 (pThr696) (Abcam, Cambridge, UK), myosin light chain (MLC) (pSer19, CST, Danvers, MA, USA), myocardin (Abbiotec, San Diego, CA, USA), SM- α actin (Abcam, Cambridge, UK), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Millipore, Billerica, MA, USA). Horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse were used as secondary antibodies (Amersham Biosciences, Amersham, UK). Immunoreactivity was visualized using an enhanced chemiluminescence (ECL) western blotting detection kit (Millipore, Billerica, MA, USA). Quantitative assessments of band densities were performed by scanning densitometry.

The ROCK2 kinase activity of the pooled carotids arteries (n=6 per group) were measured using tissue ROCK2 kinase activity quantitative assay kit (GenMed Scientifics Inc., USA) according to the manufacture's instruction.

All results are expressed as the mean \pm standard deviation (SD) or the mean \pm standard error of the mean (SEM) where appropriate. Comparisons were made by one-way analysis of variance with Bonferroni's post hoc test. A value of P<0.05 was considered to represent a statistically significant difference.

Results

Microscopic examinations indicated that compared with group I control rats (*Figure 1A,B*), the media of the carotid arteries appeared thicker and SM cells had proliferated and

become disorganized after LPS infusion into the adventitia in group II LPS-treated rats (*Figure 1C,D*). Morphological alterations were limited to the media of carotid arteries, as no obvious morphological alterations were observed in the adventitia and intima of the carotid arteries in group II LPS-treated rats (*Figure 1C,D*). Importantly, the proliferation and disorganization of SM cells induced by LPS infusion into the adventitia was completely reversed by pretreatment with the Rho-kinase inhibitor fasudil in group III fasudil + LPS-treated rats (*Figure 1E,F*) or by treatment with the cholesterol-lowering agent atorvastatin in group IV atorvastatin + LPS-treated rats (*Figure 1G,H*). Quantitative measurement of intima and media thickness showed that LPS significantly increased medial thickness, which was completely blocked by fasudil or atorvastatin (*Table 1*). Real-time RT-PCR demonstrated that LPS and pretreatment with fasudil or atorvastatin had an obvious effect on TNF α or IL-6 mRNA levels (*Figure 1I,J,K,L*).

Adventitial LPS infusion had no obvious effect on the resting carotid blood flow rate when compared with the flow rate in the control rats (*Table 2*). Furthermore, fasudil and atorvastatin showed no apparent effect on resting carotid blood flow rate compared with the rate in the LPS-treated and control rats (*Table 2*). To evaluate the contractile responses of LPS-infused arteries, 1% serotonin was applied. Application of serotonin decreased carotid blood flow in control and LPS-treated rats, and the reduction induced by serotonin administration was significantly exacerbated in the LPS-treated rats compared with the control rats (*Table 3*), indicating that adventitial LPS infusion resulted in vascular hypersensitivity to serotonin. Fasudil completely blocked LPS-induced vessel hypersensitivity to serotonin (*Table 3*). Atorvastatin not only blocked the LPS-induced hypersensitivity, but it even increased carotid blood flow upon exposure to serotonin (*Table 3*).

Adventitial LPS infusion caused a significant increase in ROCK2 protein expression (*Figure 2A*). To assess ROCK2 activity, phosphorylation of its substrate MYPT1 was analyzed. LPS significantly increased levels of phosphorylated MYPT1 protein (pMYPT1) and phosphorylated MLC (pMLC) without obviously affecting their total protein expression levels (*Figure 2A*). Both fasudil and atorvastatin significantly blocked the LPS-induced increase in ROCK2, pMYPT1 and pMLC levels (*Figure 2A*). Fasudil showed a stronger suppressive effect than atorvastatin on the LPS-induced increase in ROCK2 levels (*Figure 2A*). Neither fasudil nor atorvastatin showed

obvious effects on total MYPT1 and MLC protein expression levels (*Figure 2A*). ROCK2 kinase activity in the pooled carotid arteries was also quantitatively measured. Consistent with increase in ROCK2 expression and phosphorylation of MYPT1, ROCK2 activity was significantly increased in LPS-treated group, which was suppressed by both fasudil and atorvastatin (*Figure 2B*). RT-PCR results indicated that neither LPS alone nor pretreatment with fasudil or atorvastatin had an obvious effect on ROCK2 or MYPT1 mRNA levels (*Figure 2C*). Western Blot results indicated that both LPS obviously inhibited the expressions of SERCA2 α and SERCA2 β , which could be restored by fasudil and atorvastatin (*Figure 2D,E*).

Adventitial LPS infusion significantly increased myocardin and SM α -actin protein expression (*Figure 3A*). LPS-induced increases in myocardin and SM α -actin levels were significantly suppressed by either fasudil or atorvastatin (*Figure 3A*). qPCR results indicated that LPS obviously increased mRNA expressions of SM α -actin and myocardin (*Figure 3B*), which could be restored by fasudil and atorvastatin.

Discussion

Many studies have suggested that the adventitia is activated during the development of atherosclerosis and hypertension, indicating a role for adventitia in providing cells and molecules with the capacity to influence neointima formation and vascular remodeling (17,20-23). The adventitia is also increasingly considered to be a highly active segment of vascular tissue that contributes to various disease-associated pathologies. Here, we demonstrated that transient LPS infusion to the adventitia of the rat common carotid artery induced vascular hypersensitivity to serotonin 5 days after surgery. Hypersensitivity to serotonin has been described in animal models of atherosclerosis and in patients with coronary atherosclerosis before the development of angiographically detectable atherosclerotic lesions (24-27). One of the most important steps for vascular SM contraction is phosphorylation of the MLC. The extent of MLC phosphorylation is ascribed to the balance between the activities of calcium/calmodulin-dependent MLC kinase and MLC phosphatase (MLCP). Rho-kinase, an effector of RhoA, has been shown to inhibit MLCP activity via the phosphorylation of its myosin-binding subunit, MYPT1, at Thr696. Thus, Rho-kinase plays a critical role in agonist-induced calcium

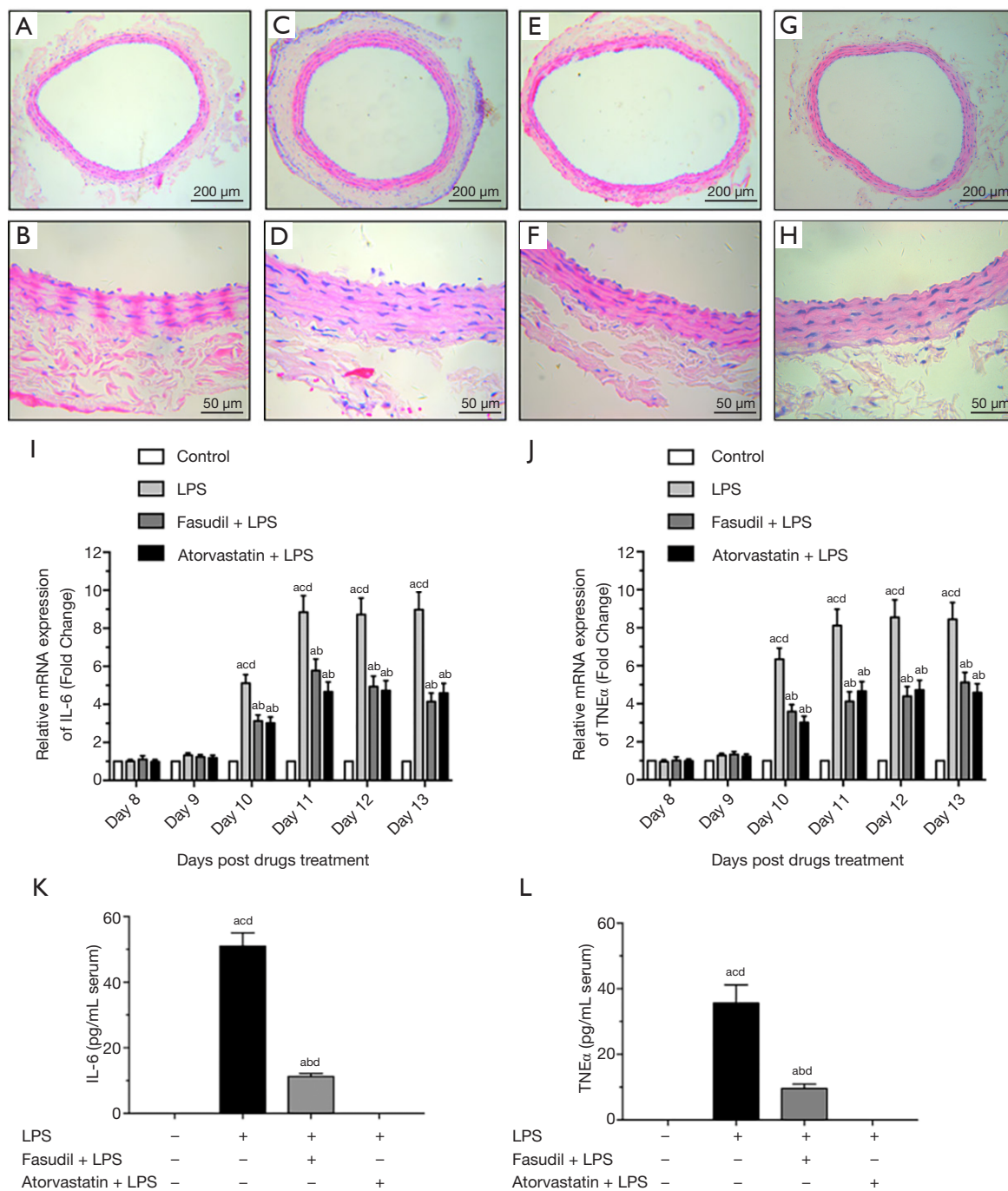


Figure 1 Representative histological images of rat carotid arteries. Representative photomicrographs showing the carotid artery morphology from group I (A,B) group II (C,D) group III (E,F) and group IV (G,H) rats; real-time PCR analysis of TNF α (I) and IL-6 (J) mRNA expression levels; the ratios of the expression these genes compared to control group levels (normalized to β -actin mRNA) are plotted for TNF α mRNA expression levels on the left and IL-6 mRNA expression levels on the right; (K) serum level of IL-6 and (L) TNF α . Data in graphs indicated the mean \pm SEM for RT-PCR analyses (n=6; ^a, P<0.05, vs. control; ^b, P<0.05, vs. LPS; ^c, P<0.05, vs. fasudil + LPS; ^d, P<0.05, vs. atorvastatin + LPS by one-way ANOVA with Bonferroni's post hoc test). LPS, lipopolysaccharide; PCR, polymerase chain reaction; TNF α , tumor necrosis factor α ; IL-6, interleukin-6; SEM, standard error of the mean; RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, analysis of variance. Dyeing method: hematoxylin and eosin stained.

Table 1 The thickness of intima and media

Group (n=6)	Intima thickness (μm)	Media thickness (μm)
Control (I)	3.12 \pm 0.68	30.91 \pm 1.56
LPS (II)	3.03 \pm 0.11	55.11 \pm 2.93 ^{†§¶}
Fasudil + LPS (III)	2.69 \pm 0.44	33.93 \pm 2.89 [‡]
Atorvastatin + LPS (IV)	2.51 \pm 0.62	38.47 \pm 2.96 ^{†‡}

[†], P<0.05, vs. control; [‡], P<0.05, vs. LPS; [§], P<0.05, vs. fasudil + LPS; [¶], P<0.05, vs. atorvastatin + LPS by one-way ANOVA with Bonferroni's post hoc test. Data shown indicate the mean \pm SD. LPS, lipopolysaccharide; ANOVA, analysis of variance; SD, standard deviation.

Table 2 Resting carotid blood flow rates

Group (n=6)	Resting carotid blood flow (mL/min) [†]
Control (I)	4.11 \pm 0.50
LPS (II)	4.02 \pm 0.48
Fasudil + LPS (III)	3.48 \pm 0.64
Atorvastatin + LPS (IV)	4.17 \pm 0.71

[†], data shown indicate the mean \pm SD. LPS, lipopolysaccharide; SD, standard deviation.

Table 3 Changes in carotid blood flow (% change) in response to serotonin

Group (n=6)	1% serotonin
Control (I)	-5.71 \pm 9.37
LPS (II)	-43.36 \pm 14.72 ^{†§¶}
Fasudil + LPS (III)	-3.36 \pm 6.82 [¶]
Atorvastatin + LPS (IV)	25.16 \pm 22.15 ^{†§}

[†], P<0.05, vs. control; [‡], P<0.05, vs. LPS; [§], P<0.05, vs. fasudil + LPS; [¶], P<0.05, vs. atorvastatin + LPS by one-way ANOVA with Bonferroni's post hoc test. Data shown indicate the mean \pm SD. LPS, lipopolysaccharide; ANOVA, analysis of variance; SD, standard deviation.

sensitization and hypercontraction of VSMCs. We found that transient LPS infusion led to a significant increase in ROCK2 protein expression and phosphorylated MYPT1 levels. Direct measurement of ROCK2 activity confirmed that LPS increased ROCK2 activity of carotid arteries, while both atorvastatin and fasudil significantly blocked the effect. Importantly, the Rho-kinase inhibitor fasudil restored the hypercontraction of carotid arteries induced by LPS, implying that the activation of the RhoA/Rho-kinase signaling pathway plays an important role in LPS-

induced alteration of vascular contractility. We also found that atorvastatin increased blood flow in the carotid artery, even after serotonin stimulation, indicating the potential for atorvastatin to combat coronary spasms. The mechanism by which atorvastatin prevents vascular spasm remains unclear. Statins are a class of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, and accumulating evidence supports the idea that they exert beneficial vascular effects that are independent of their cholesterol-lowering properties (28-31). Inhibition of ROCKs has recently been ascribed to the beneficial effects of statins on the cardiovascular system (28). We demonstrated that fasudil and atorvastatin had similar magnitudes of suppressive effects on LPS-induced phosphorylation of MYPT1 and activation of ROCK2, although atorvastatin showed weaker inhibitory effects on ROCK2 expression than fasudil. Atorvastatin might suppress ROCK2 activity without causing a profound reduction in its expression. Alternatively, atorvastatin might inhibit other kinase(s) responsible for the phosphorylation of MYPT1. The mechanism underlying the inhibition of MYPT1 phosphorylation and ROCK2 activation by atorvastatin will require further investigation in the future.

The arterial wall represents a highly plastic three-dimensional structure with a unique capacity to adapt to local changes during development and in some pathological vascular conditions (32-35). Throughout embryonic development VSMCs proliferate and mature into a differentiated contractile state. Postnatally, VSMCs remain in the differentiated state and express stereotypical markers, such as myocardin, SM-MHC, calponin, and SM22 α , and remain quiescent until injury or disease-associated pathologies, such as atherosclerosis, promote the phenotypic transition from a differentiated to a proliferative state. We demonstrated that LPS-induced transient adventitial inflammation resulted in the proliferation and disordered arrangement of VSMCs in the medial layer,

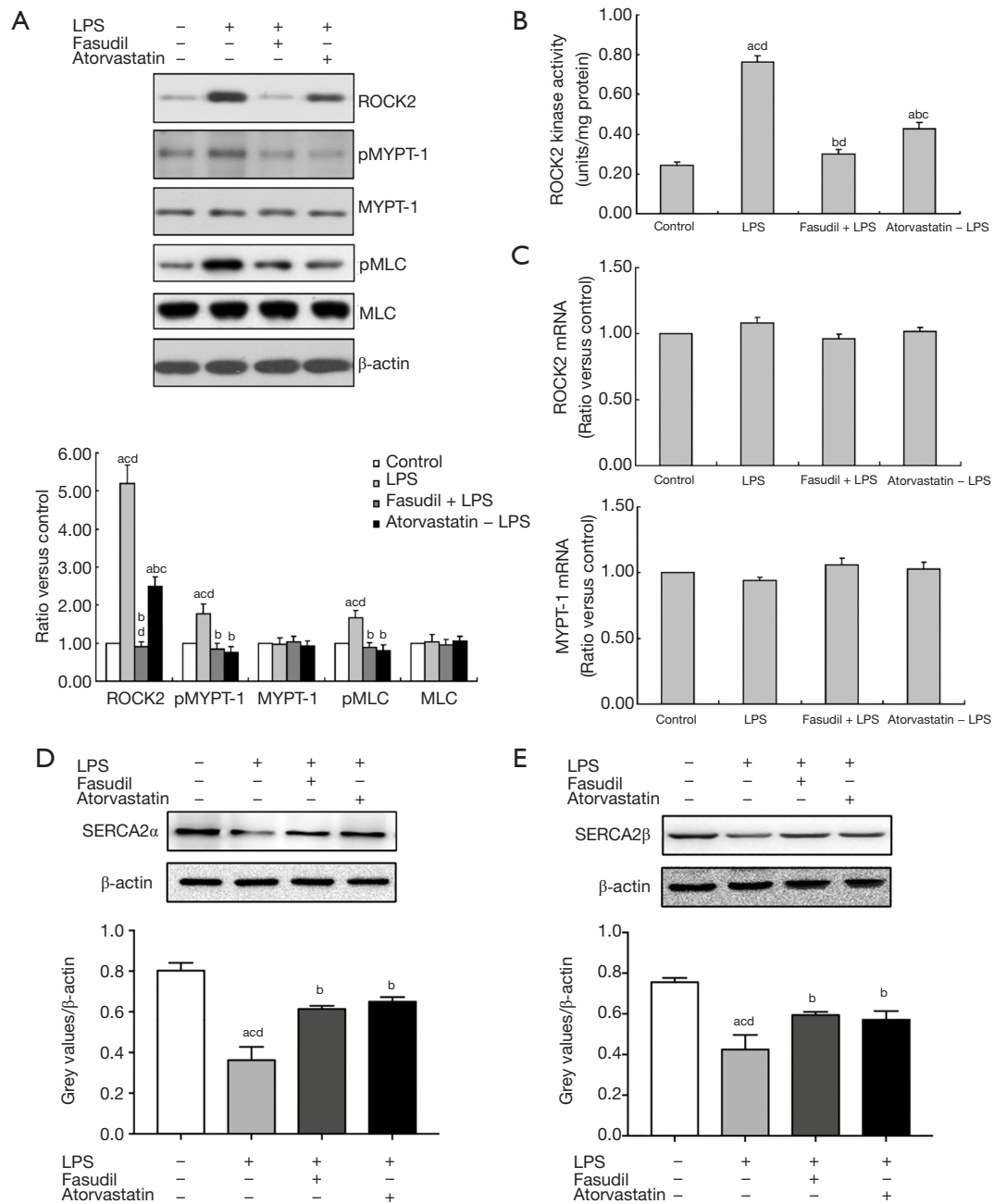


Figure 2 The effect of LPS treatment on ROCK2 expression and activity. (A) Western blot analysis of ROCK2, phosphorylated MYPT1, MYPT1, phosphorylated MLC and MLC proteins. The ratios of protein levels versus control protein levels (normalized to β -actin) are plotted on the left. A representative western blot images are shown on the right; (B) ROCK2 kinase activities of carotid arteries were quantitatively measured and normalized to total protein amounts; (C) RT-PCR analysis of ROCK2 and MYPT1 mRNA expression levels. The ratios of the expression these genes compared to control group levels (normalized to β -actin mRNA) are plotted for ROCK2 mRNA expression levels on the left and MYPT1 mRNA expression levels on the right; (D) expression analysis of SERCA2 α by western blot; (E) expression analysis of SERCA2 β by western blot. Data in graphs indicate the mean \pm SD for western blot analyses and the mean \pm SEM for ROCK2 kinase activity assay or RT-PCR analyses ($n=6$; ^a, $P<0.05$, *vs.* control; ^b, $P<0.05$, *vs.* LPS; ^c, $P<0.05$, *vs.* fasudil + LPS; ^d, $P<0.05$, *vs.* atorvastatin + LPS by one-way ANOVA with Bonferroni's post hoc test). LPS, lipopolysaccharide; ROCK2, Rho-associated coiled-coil-containing protein kinase 2; MYPT1, myosin phosphatase target subunit 1; MLC, myosin light chain; RT-PCR, reverse transcription-polymerase chain reaction; SERCA, sarco/endoplasmic reticulum Ca^{2+} -ATPase; SD, standard deviation; SEM, standard error of the mean; ANOVA, analysis of variance; pMYPT1, phosphorylated MYPT1; pMLC, phosphorylated MLC.

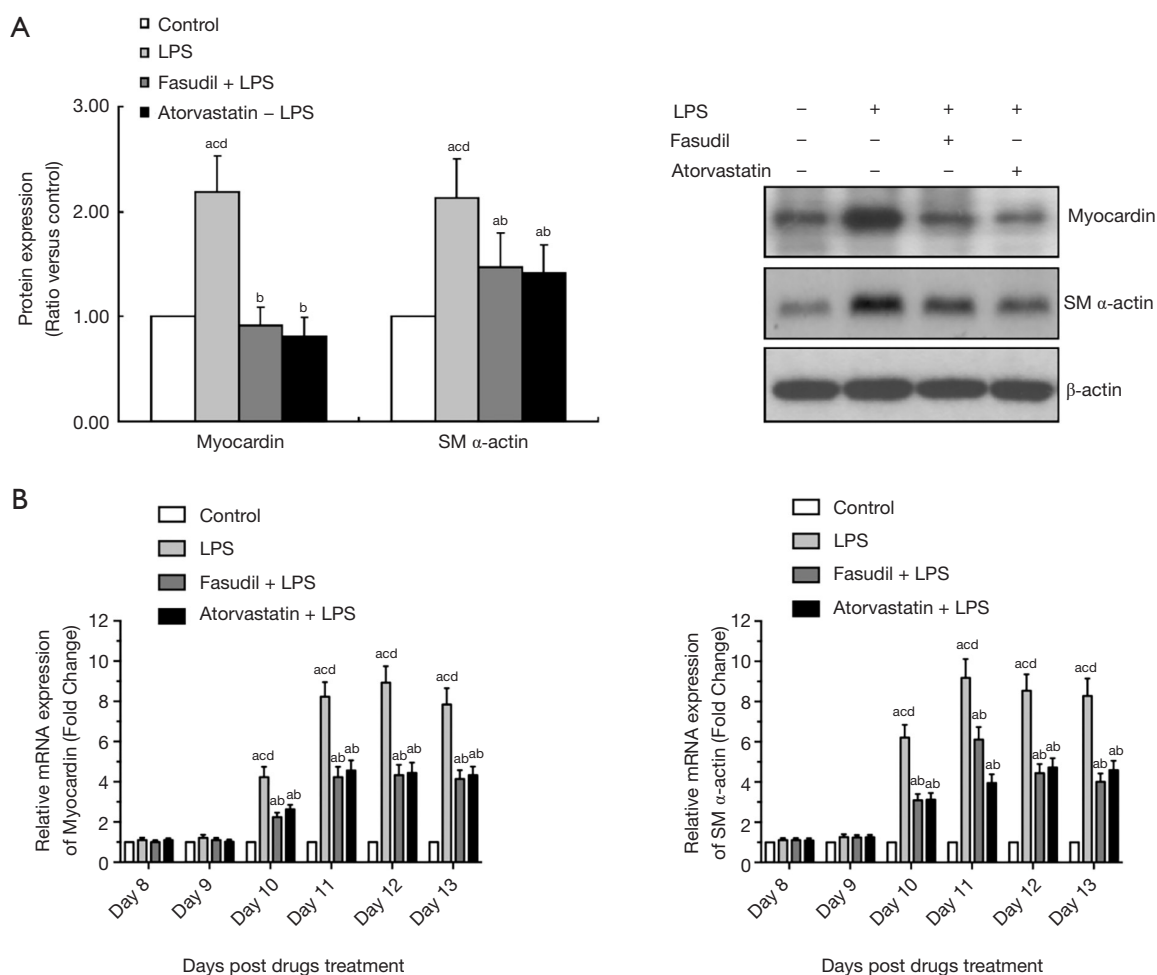


Figure 3 The effect of LPS treatment on myocardin and SM α -actin expression. (A) Western blot analysis of myocardin and SM α -actin proteins. The ratios of protein levels versus control protein levels (normalized to β -actin) are plotted on the left. A representative western blot is shown on the right; (B) RT-PCR analysis of myocardin and SM α -actin mRNA expression levels. The ratios of these genes compared to expression in control rats (normalized to β -actin mRNA) are plotted for myocardin mRNA expression levels on the left and SM α -actin mRNA expression levels on the right. Data in graphs indicate the mean \pm SD for western blot analyses and the mean \pm SEM for RT-PCR analyses (n=6; ^a, P<0.05, vs. control; ^b, P<0.05, vs. LPS; ^c, P<0.05, vs. fasudil + LPS; ^d, P<0.05, vs. atorvastatin + LPS by one-way ANOVA with Bonferroni's post hoc test). LPS, lipopolysaccharide; RT-PCR, reverse transcription-polymerase chain reaction; SD, standard deviation; SEM, standard error of the mean; ANOVA, analysis of variance.

whereas no obvious alterations in the intimal or adventitial layers were observed.

Inconsistency with our observations, it has been reported that LPS promotes proliferation and migration of cultured rat adventitial fibroblast cells (36), and LPS results in vascular hyporeactivity to noradrenalin in cultured rat carotid arteries (37). The current study investigated the influence of LPS on carotid arteries *in vivo*, while the previous experiments investigated the effect of LPS on cultured

adventitial fibroblast (36) or cultured fragments of vessels (37). The differences between *in vivo* and *in vitro* systems used in these studies might ascribe to the apparent different phenomena. Alternatively, the exposure period may also contribute to the difference. The previous studies examined the influence of LPS within 24 h, while the current study was performed at 5 days after LPS exposure. It should be noted that 5 days after LPS infusion might not allow sufficient time for intimal thickening to occur, because

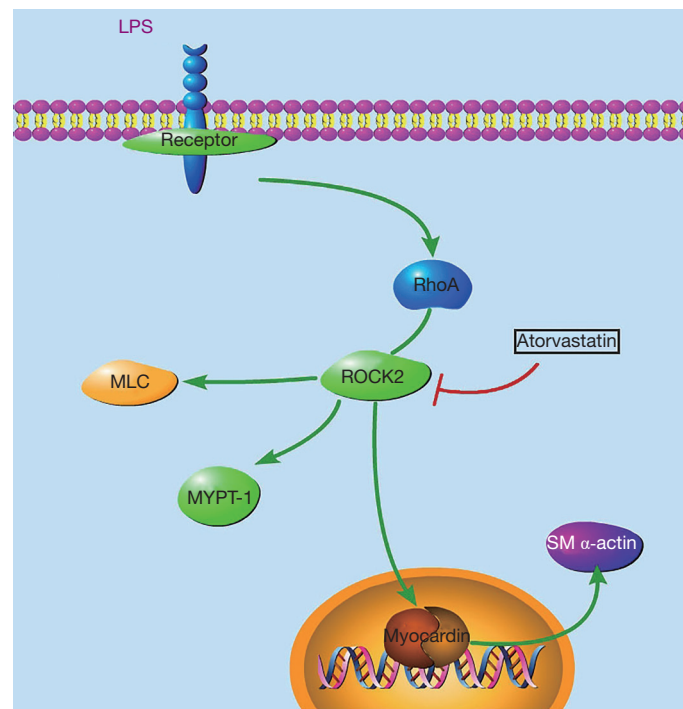


Figure 4 Scheme of Atorvastatin functions. LPS induces ROCK2 activation via RhoA, which activates MLC and MYPT-1. And the activated ROCK2 can regulate the expression of SM α -actin via co-transfer factor myocardin. LPS, lipopolysaccharide; ROCK2, Rho-associated coiled-coil-containing protein kinase 2; MYPT1, myosin phosphatase target subunit 1; MLC, myosin light chain.

this would be predicted to occur after the infiltration of inflammatory cells diminishes and tissue repair has occurred. Investigations at different time points upon LPS exposure are required in the future. myocardin has been shown to play a critical role during early SMC differentiation and to transactivate many SMC differentiation markers, such as SM α -actin, SM22, and SM-MHC (4,38,39). We demonstrated that transient LPS infusion caused protein levels of myocardin to increase, while myocardin mRNA expression levels were unaffected. Thus, LPS might induce myocardin expression at the post-transcriptional level. Consistent with the LPS-induced increase in myocardin protein expression levels, SM α -actin levels were also significantly increased.

In summary, we demonstrated that the transient addition of LPS to the adventitia caused vascular hypersensitivity and remodeling via the RhoA/Rho-kinase signaling pathway, accompanied by the dysregulation of some stereotypical VSMC genes. Additionally, we found that similar to the Rho-kinase inhibitor fasudil, the cholesterol-lowering agent atorvastatin showed beneficial effects on vascular dysfunction mediated by LPS via suppression of

RhoA/Rho-kinase signaling pathway (as shown in *Figure 4*). To our knowledge, the current study for the first time demonstrated that transient exposure of adventitia to LPS resulted in chronic alterations in vascular functions and VSMCs *in vivo*, reflecting the critical role of adventitia in the development of vascular diseases.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal

protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the China Medical University Animal Care and Use Committee (No. 2018157).

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