Inhibition of reactive oxygen species generation attenuates TLR4-mediated proinflammatory and proliferative phenotype of vascular smooth muscle cells

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Reactive oxygen species (ROS) are associated with inflammation and vasculature dysfunction. This study aimed to investigate the potential role of the ROS on vascular Toll-like receptor 4 (TLR4)-mediated proinflammatory and proliferative phenotype of vascular smooth muscle cells (VSMCs). A wire-induced carotid injury model was used in male TLR4-deficient (TLR4^{-/-}) and wild-type C57BL/6J mice to induce neointima formation. In the presence or absence of the ROS scavenger apocynin for 14 days, increased TLR4 and proinflammatory cytokines were observed in wire injury-induced carotid neointima and in platelet-derived growth factor-BB (PDGF-BB)-stimulated VSMCs. The TLR4^{-/-} protected the injured carotid from neointimal formation and impaired the cellular proliferation and migration in response to PDGF-BB. Apocynin attenuated intimal hyperplasia. Pre-treatment with apocynin significantly inhibited intracellular ROS generation, accompanied by a significant suppression of TLR4 and proinflammatory cytokines expression, and VSMC proliferation and migration. However, the results were not obvious in TLR4^{-/-} condition. These findings highlight the importance of ROS inhibition in TLR4-mediated proinflammatory and proliferative phenotype of VSMCs, and suggest ROS as an essential therapeutic target for TLR4-associated vascular inflammation and vascular diseases.

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Vascular smooth muscle cell (VSMC) proliferation and migration has been accepted as a common event in the pathophysiology of many vascular diseases, including atherosclerosis, atherogenesis and intimal hyperplasia.¹ The quiescent VSMCs in adult vessels can be activated and regain the capability of proliferation and migration in response to injury, inflammation and stretch, and ultimately contribute to the formation of neointima.² Suppression of VSMC proliferation and migration represents an important target point for therapeutic intervention to attenuate vascular diseases and prevent restenosis after vascular reconstructive procedures.

Reactive oxygen species (ROS) are involved in many pathophysiological processes of VSMCs, such as growth, proliferation, migration and differentiation.^{3–5} The main sources of ROS are NADPH oxidase, xanthine oxidase, lipoxygenase, mitochondria and the uncoupling nitric oxide synthase.⁶ ROS derived from VSMCs are involved in growth signaling pathways and have an important role in the pathogenesis of several vascular diseases associated with the

proliferation and migration of VSMCs, such as atherosclerosis and neointima growth after angioplasty or vascular stenting.^{7–10} Studies have shown that intimal VSMCs in hyperplastic and atherosclerotic lesions exhibit high levels of ROS.^{11,12} However, despite intensive research efforts, the precise mechanisms underlying the effect of ROS remain to be fully elucidated.

An inflammatory response is widely accepted as an essential event in VSMC activation and intimal hyperplasia that occurs early in atherosclerosis.^{13,14} Many studies have shown that ROS overproduction is associated with intimal hyperplasia and atherosclerosis.¹⁵ In addition, ROS participate in initiating acute inflammatory responses and ultimately lead to vasculature dysfunction. Inhibition of the inflammatory response by ROS scavenger has been increasingly examined in recent studies.¹³ Toll-like receptor 4 (TLR4) is well known to have a critical role in initiating inflammation through increasing the production of proinflammatory factors, including interleukin (IL)-1, IL-6, interferon γ and tumor

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necrosis factor α (TNF- α),^{16,17} and may make a fundamentally significant contribution to the crucial pathophysiological relationship between inflammation and vascular disorders.¹⁸ TLR4 is also reported to be involved in the modulation of VSMC proliferation, migration and neointimal formation.^{17,19} Studies have shown that TLR4 is expressed in the endothelial cells and upregulated by ROS.^{20,21} However, it remains unknown whether inhibition of ROS could ameliorate TLR4-mediated inflammation, VSMC proliferation and migration, intimal hyperplasia, and atherosclerosis.

In this study, we have investigated the essential role of ROS on vascular TLR4 expression and neointimal formation. In addition, we have tested the hypothesis that ROS promote VSMC proliferation and migration by enhancing TLR4-mediated inflammation, and eventually lead to intimal hyperplasia after wire injury, and inhibition of ROS generation could restrain this progress.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Carlsbad, CA, USA). Apocynin, recombinant murine platelet-derived growth factor-BB (PDGF-BB), methyl thiazolyl diphenyl-tetrazolium (MTT) and dimethyl sulfoxide were obtained from Sigma-Aldrich (St Louis, MO, USA). Antibodies targeting TLR4, GAPDH and smooth muscle α -actin (α -SMA) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, USA).

Animals

Male TLR4-deficient mice (C57BL/6J background) and wild-type mice (WT, C57BL/6J) were obtained at 8–10 weeks of age from the Jackson Laboratory (Bar Harbor, ME, USA). A wire-induced carotid injury model was used in this study to induce intimal hyperplasia as described previously.²² Mice were briefly anesthetized with an intraperitoneal injection of 60 μ g/g body weight sodium pentobarbital, and repeated intraperitoneal injections (12 μ g/g body weight) were given as needed to maintain anesthesia. Mice displayed full sedation, no response to pain stimuli, muscle relaxation, even breathing and pink oral mucosae, and were well anesthetized and ready for surgery.

To determine the effects of ROS scavenger on intimal hyperplasia, apocynin²³ was administered orally (200 mg/kg/day by gavage) for 14 days starting from the day of wire injury. All the animals had *ad libitum* access to food and water. Mice were killed at 14 days after wire injury, blood serum was collected and carotid injured arteries were perfusion fixed in situ with 4% formaldehyde solution (pH 7.4) and then harvested. The arterial segments were embedded in paraffin and cut at eight to ten sites (600 μ m apart). Sections of 6 μ m thickness were prepared for morphometric and immunohistochemical analysis.

Ethics Statement

The experimental protocol used in the study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Animal Research Committee of the Third Military Medical University.

Histopathology Analysis

Morphometric analysis was performed on hematoxylin–eosin-stained slides. The area of each vascular layer was measured by tracing the external elastic lamina, internal elastic lamina and vessel lumen. The intimal area was determined by subtracting the lumen area from the area within the internal elastic lamina. Media area was considered as the area between the external elastic lamina and internal elastic lamina. The intimal hyperplasia was assessed by calculating the ratio of intima to media area.

For immunohistochemistry, sections were deparaffinized and rehydrated by serial immersion in xylene, alcohol and water. Thereafter, 3% hydrogen peroxide was used to block endogenous peroxidase. Nonspecific binding was blocked with 0.5% blocking solution. α -SMA was used as a protein marker for VSMCs and was applied at a dilution of 1:100 for 1 h at room temperature, followed by amplification with secondary antibodies, stained with DAB and counterstained with hematoxylin then visualized.

Cell Culture

VSMCs were isolated from the thoracic aorta of TLR4^{-/-} and C57BL/6J mice using an explant technique and cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in air supplemented with 5% CO₂ at 37 °C. Cultures were confirmed to consist primarily (>95%) of VSMCs both morphologically by their classical 'hill and valley' appearance, and immunohistochemically by α -SMA immunoreactivity. Cells in the second to sixth passages were used for experiments.

ROS generation was pharmacologically manipulated respectively by PDGF-BB and apocynin. Before exposure to 20 ng/ml PDGF-BB, VSMCs were pre-treated for 30 min with apocynin (100 μ mol/l).^{24–26} Untreated cells were served as controls. Cells and their supernatants and lysates were collected for ROS detection, enzyme-linked immunosorbent assay (ELISA) and western blot, respectively.

Measurement of ROS

Intracellular ROS were determined by oxidative conversion of cell permeable DCFH-DA to fluorescent dichlorofluorescein (DCF) as previously described.²⁷ Briefly, VSMCs with 70% confluency were washed with serum-free DMEM and incubated in the dark with DCFH-DA 10 μ mol/l for 20 min at 37 °C. Cells were then trypsinized, resuspended in PBS and subjected to flow cytometry analysis (Calibur, Becton Dickinson). The mean of DCF fluorescence intensity was obtained from 10 000 events. Cells were excited at 488 nm,

and DCF fluorescence was read on FL1 (530 ± 15) in log scale.

ELISA

IL-1 β , IL-6 and TNF- α were measured both in the serum of mice and in the supernatants of VSMCs by ELISAs according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Western Blot Analysis

Western blot analysis was used to determine the levels of TLR4. Protein samples were obtained either from homogenized arteries or cultured cells, and the protein concentration was determined. Protein samples (30 μ g) were subjected to SDS-PAGE, transferred to a nitrocellulose membrane. The membranes were probed with rabbit anti-TLR4 antibody at 1:1000, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG. The proteins were detected by enhanced chemiluminescence (NEN, MA, USA) and quantified using a Gel Doc 2000 Imager (Bio-Rad, CA, USA). Western blot quantification was performed by densitometry and normalized to GAPDH.

MTT Assay

Cell proliferation was determined by MTT assay. Briefly, VSMCs were plated on a 96-well plate at 3×10^4 cells per well, cells were incubated with PDGF-BB (20 ng/ml) for 24 h in the presence or absence of pre-treatment with apocynin (100 μ mol/l) for 1 h. After incubation, a 20 μ l aliquot of 5 mg/ml MTT dye was added, and the cells were incubated for 4 h at 37°C. After the 4-h incubation, 150 μ l of DMSO was added to the cells and mixed thoroughly. The cells were agitated on a plate shaker for 5 min and absorbances were measured with a microplate reader (Bio-Rad) at 570 nm.

BrdU Assay

DNA synthesis was measured by BrdU Cell Proliferation ELISA (Roche Diagnostic, Mannheim, Germany). VSMCs were plated on a 96-well plate at 2×10^3 cells per well, cells were incubated with PDGF-BB (20 ng/ml) for 24 h in the presence or absence of pre-treatment with apocynin (100 μ mol/l) for 1 h. BrdU (10 mM) was added and incubated for 8 h. At the end of the incubation period, the medium was removed, cells were fixed, denatured and probed with anti-BrdU antibody as suggested by the manufacturer's manual. The optical densities were measured at 490 nm.

Cell Migration Assay

Cell migration was examined using modified Boyden chambers as described previously.¹⁷ Briefly, VSMCs (5.0×10^4 cells/ml) were plated in the upper chamber with or without pharmacological intervention (apocynin, 100 μ mol/l). DMEM containing 0.4% FBS and PDGF-BB (20 ng/ml) was added to the lower chamber. The cells were then allowed to migrate for 24 h at 37°C. Thereafter, cells on the underside of the filters

were fixed with methanol and stained with hematoxylin, and then counted from five high-power ($\times 400$) fields per well. The average was used as the migratory cell number.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) or SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as mean $\pm$ s.e.m. of at least three independent experiments. Multiple-group statistical analyses were performed by one-way ANOVA followed by least significant difference *post hoc* testing. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Intimal Hyperplasia in Wire-Injured Arteries

Wire was used to induce carotid injury in male TLR4^{-/-} and WT mice. After 14 days, the intima/media ratio was increased in injured carotid arteries compared with arteries from sham-operated WT mice. In contrast, TLR4^{-/-} mice displayed very low intima/media ratio in response to carotid injury. Apocynin treatment effectively suppressed neointimal formation induced by wire injury in WT mice, but exerted no obvious effect on that in TLR4^{-/-} mice (Figure 1a).

In agreement with our previous study,¹⁷ VSMCs, identified by α -SMA-positive immunostaining, were found to make a major contribution to the formation of neointima (Figure 1b).

Intracellular ROS Generation in VSMCs

We examined the VSMC ROS generation in response to PDGF-BB. Intracellular release of ROS from VSMCs was measured in cells treated for 60 min with a single dose of PDGF-BB (20 ng/ml), which is known to generate intracellular ROS.²⁴ ROS concentration was measured by flow cytometry using DCFH-DA.²⁷ Compared with untreated VSMCs, treatment with PDGF-BB caused a significant increase in intracellular ROS generation. Pre-treatment of VSMCs with apocynin (100 μ mol/l) for 30 min strikingly suppressed PDGF-BB-induced ROS generation (Figure 2).

TLR4 Expression is Dependent on the Generation of ROS

To determine the associations between ROS generation and TLR4 expression, we examined the effects of ROS on TLR4 expression. It was shown that wire injury increased the expression of TLR4 in vascular tissues taken from WT mice. Moreover, apocynin treatment significantly impeded the upregulation of TLR4 induced by wire injury (Figure 3a).

We also detected the potential impact of ROS on TLR4 expression in WT VSMCs. As shown in Figure 3b, exposure of VSMCs to PDGF-BB showed a significant increase in TLR4 expression in WT VSMCs, and this could be effectively inhibited by apocynin. These data suggest an important role of ROS in TLR4 expression, the upregulation of TLR4 in response to vascular injury or PDGF challenge is dependent on ROS generation.

Figure 1 Intimal hyperplasia in carotid arteries. (a) Hematoxylin–eosin staining on cross-sections from representative injured carotid arteries are presented. Carotid wire injury-induced intimal hyperplasia with increased intima/media ratio in WT mice. TLR4^{-/-} mice displayed a lower intima/media ratio in response to carotid injury. Apo inhibited the wire injury-induced intimal hyperplasia in WT mice but not in TLR4^{-/-} mice. (n = 3–4 mice per group. *P < 0.05 vs WT + sham; #P < 0.05 vs WT + In and △P < 0.05 vs TLR4^{-/-} + sham.) (b) Accumulation of VSMCs in the neointima identified by α-SMA immunostaining. Apo, apocynin; In, carotid wire injury; sham, sham operation; TLR4^{-/-}, TLR4-deficient; WT, wild-type.

Inhibition of ROS Generation Attenuates TLR4-Mediated Inflammation

To determine whether carotid injury was associated with systemic inflammation, we measured serum levels of proinflammatory cytokines (IL-1 β , IL-6 and TNF- α) in wire injured and sham-operated mice. As shown in Figure 4a, cytokines expression was elevated at day 14 in sera from injured mice compared with sham-operated mice. Injury-induced cytokines expression was much higher in WT mice compared with that in TLR4^{-/-} mice. In addition, the elevated expression of proinflammatory cytokines in WT

mice was significantly abrogated by apocynin, whereas apocynin did not show any effect on that in TLR4^{-/-} mice.

Cultured VSMCs in basal conditions displayed very low levels of proinflammatory cytokines. PDGF-BB increased the expression of cytokines especially in cultured WT VSMCs, which were largely retarded by apocynin (Figure 4b). These results appear most consistent with the interpretation that at least part of the suppression of atherosclerosis observed in mice/VSMCs with TLR4 deficiency may be mediated by a general reduction in circulating and supernatant levels of proatherogenic inflammatory molecules.

Inhibition of Intracellular ROS Generation Attenuates TLR4-Mediated VSMC Proliferation and Migration

ROS generation in WT and TLR4^{-/-} VSMCs was pharmacologically manipulated respectively by PDGF-BB and apocynin, and VSMC proliferation and migration were thereafter detected. Cell proliferation was determined by MTT assay (Figure 5a) and BrdU assay (Figure 5b). Cell migration was measured by modified Boyden chambers (Figure 5c). As shown in Figures 5a–c, both WT and TLR4^{-/-} VSMCs cultured in basal conditions displayed low levels of proliferation and migration. VSMCs from WT mice showed a significant increase in proliferation and migration in response to PDGF-BB, whereas VSMCs from TLR4^{-/-} mice failed to show this increase. However, treatment with apocynin significantly attenuated PDGF-BB-stimulated VSMC proliferation and migration, and this effect was more striking than TLR4^{-/-} VSMCs. These data suggested that ROS exerts an essential role in the TLR4-mediated VSMC proliferation and migration.

DISCUSSION

In this study, we have explored the association between ROS generation and TLR4-mediated proinflammatory and proliferative phenotype of VSMCs. VSMCs exposed to PDGF-BB showed significant increase in ROS generation,

Figure 2 Intracellular ROS generation in VSMCs. VSMCs were treated with PDGF-BB (20 ng/ml) in the presence or absence of Apo (100 μ mol/l) for 60 min. Treatment with PDGF-BB had a significant effect on the basal intracellular ROS generation both in WT and TLR4^{-/-} VSMCs, and pre-treatment with Apo led to suppression of PDGF-BB-induced ROS generation. The intracellular ROS generation was measured by flow cytometry analysis. ($n=3-4$ cell lines. * $P<0.05$ vs control and # $P<0.05$ vs PDGF). PDGF-BB, platelet-derived growth factor-BB; ROS, reactive oxygen species; TLR4^{-/-}, TLR4-deficient; VSMC, vascular smooth muscle cell; WT, wild-type.

Figure 3 TLR4 expression is dependent on ROS generation. Expressions of TLR4 protein was detected by western blot. (a) Carotid wire injury increased TLR4 protein expression in WT mice and Apo pre-treatment blocked the expression of TLR4 protein. ($n=3-4$ mice per group. * $P<0.05$ vs sham and # $P<0.05$ vs In). (b) ROS overproduction with PDGF-BB upregulated TLR4 protein expression whereas ROS inhibition with Apo significantly abrogated this increase in WT VSMCs. ($n=3-4$ cell lines. * $P<0.05$ vs control and # $P<0.05$ vs PDGF). In, carotid wire injury; PDGF, platelet-derived growth factor; ROS, reactive oxygen species; sham, sham operation; TLR4, Toll-like receptor 4; VSMC, vascular smooth muscle cell; WT, wild-type.

Figure 4 Inhibition of ROS attenuates TLR4-mediated inflammation. Expressions of proinflammatory cytokines (IL-1 β , IL-6 and TNF- α) were detected by ELISA. (a) Carotid wire injury elevated the serum levels of cytokines in WT mice but not in TLR4^{-/-} mice. Apo pre-treatment blocked the expression of cytokines induced by wire injury in WT mice but not in TLR4^{-/-} mice. ($n = 3-4$ mice per group. * $P < 0.05$ vs WT + sham and # $P < 0.05$ vs WT + In). (b) PDGF-BB upregulated cytokines whereas Apo significantly abrogated this increase in WT VSMCs, but both of them exerted no obvious effect on that in TLR4^{-/-} VSMCs. ($n = 3-4$ cell lines. * $P < 0.05$ vs WT control and # $P < 0.05$ vs WT PDGF). In, carotid wire injury; IL, interleukin; PDGF-BB, platelet-derived growth factor-BB; ROS, reactive oxygen species; sham, sham operation; TNF- α , tumor necrosis factor α ; TLR4^{-/-}, TLR4-deficient; VSMC, vascular smooth muscle cell; WT, wild-type.

Figure 5 Impact of ROS on VSMC proliferation and migration. Both cultured WT and TLR4^{-/-} VSMCs in basal conditions displayed low levels of proliferation (a, b) and migration (c), ROS overproduction with PDGF-BB accelerated whereas ROS inhibition with Apo attenuated the VSMC proliferation (a, b) and migration (c). (a) MTT assay; (b) BrdU assay; (c) modified Boyden chambers. ($n = 3-4$ cell lines. * $P < 0.05$ vs WT control and # $P < 0.05$ vs WT PDGF). MTT, methyl thiazolyl diphenyl-tetrazolium; OD, optical density; PDGF-BB, platelet-derived growth factor-BB; ROS, reactive oxygen species; TLR4^{-/-}, TLR4-deficient; VSMC, vascular smooth muscle cell; WT, wild-type.

and this could be effectively suppressed by apocynin. Inhibition of ROS attenuated both TLR4 and proinflammatory cytokines expression in carotid injured mice and VSMCs, and accompanied by a significant inhibition of VSMC proliferation and migration, and neointimal formation. Our studies have provided evidence that ROS may be a key component in TLR4-mediated vascular inflammation and intimal hyperplasia. Inhibiting the primary source of ROS could attenuate TLR4-mediated proinflammatory and proliferative phenotype of VSMCs, and reduce detrimental arterial remodeling and inflammatory reaction such as that in the early stages of atherosclerosis.

Intimal hyperplasia is the common pathological lesion of proliferative vascular diseases, including atherosclerosis, hypertension and restenosis after vascular reconstructive procedures.²⁸ Intimal hyperplasia is initiated by endothelial injury or loss, inflammation and stretch. Thereafter, increased VSMC proliferation occurs within 1–3 days, followed by migration of VSMCs into the intima, which eventually contributes to neointimal formation.²⁹

An increase in ROS generation is an essential step for development of intimal hyperplasia, atherosclerosis and other vascular pathologies.²⁷ ROS accumulation has been demonstrated to have an important role in neointima formation through inducing the proliferation and migration of VSMCs.^{7–9} ROS derived from VSMCs are involved in growth signaling pathways and have an important role in the pathogenesis of several vascular diseases associated with the proliferation and migration of VSMCs. West *et al*¹¹ and Yokoyama *et al*¹² found that intimal VSMCs in hyperplastic and atherosclerotic lesions express high levels of ROS. Consistently, our study found that PDGF-BB, a established stimulant that promotes ROS generation and VSMC viability,¹⁰ significantly increased ROS generation in VSMCs.

Therefore, inhibition of ROS-stimulated VSMC proliferation, migration and intimal hyperplasia appears to be an important strategy for the prevention of vascular diseases.³⁰ In support of this notion, our study showed that pretreatment of VSMCs with apocynin strikingly suppressed PDGF-BB-induced ROS generation. Furthermore, *in vivo* study showed that apocynin protected wire-injured carotid arteries from intimal hyperplasia. Intensive research efforts have been dedicated to potential mechanisms responsible for the effect of ROS in intimal hyperplasia. It has reported that ROS could induce the VSMC proliferation and migration by enhancing the inflammatory response of ERK1/2 and NF-kappa B pathway, and increasing the expression of mitochondrial biogenesis-related genes.^{31,32} These data further demonstrate the critical role of ROS in VSMC proliferation and migration, and in the neointima formation induced by mechanic injury.

It is widely accepted that inflammation is a major force in the pathophysiology of intimal hyperplasia after vascular injury. TLR4 and its associated proinflammatory cytokines have been shown to promote VSMC proliferation and

migration and be closely associated with intimal hyperplasia. Recently, particular attention has been given to the link between TLR4-mediated inflammation and ROS. Inflammatory cytokines stimulate and prime the oxidase and thus contribute to systemic oxidative stress. ROS are reported to be essential in innate immunity,³³ and related to inflammation, intimal hyperplasia, restenosis, arteriosclerosis and vascular remodeling.^{34,35} ROS were considered to be second messengers in the inflammatory response.³⁶ ROS scavenger can reduce ROS generation and attenuate the abnormal production proinflammatory cytokines.¹³ In this study, ROS overproduction increased VSMC proliferation and migration, promoted intimal hyperplasia, and elevated TLR4 and proinflammatory cytokines (IL-1 β , IL-6 and TNF- α) expression in WT mice/VSMCs, which were attenuated significantly by apocynin. In contrast, TLR4^{-/-} inhibited carotid wire injury-induced intimal hyperplasia, VSMC proliferation and migration, and proinflammatory cytokines expression. All the previous results suggested that ROS have a key role in the TLR4-mediated proinflammatory and proliferative phenotype of VSMCs, and inhibition of ROS could attenuate VSMC proliferation and migration, inflammation, and intimal hyperplasia via a TLR4-mediated signaling pathway.

There is increasing evidence showing that TLR4 can transduce proinflammatory signals produced by ROS, and ROS appear to modulate inflammatory responses through the TLR4-dependent mechanisms.³⁷ However, the precise mechanisms by which ROS induced VSMC proinflammatory and proliferative phenotype through TLR4 are not well understood currently. Although ROS may directly affect TLR4 dimerization, an essential step required for initiation of intracellular signaling pathways, it is also possible that ROS may affect TLR4-related signaling pathway through indirect mechanisms. Powers *et al*³⁸ have shown that ROS could increase membrane lipid raft localization of TLR4 during hemorrhagic shock. Of note, Cavassani *et al*³⁹ demonstrated that TLR3 may also be implicated in ROS-dependent inflammation as shown in hyperoxia or intestinal ischemia. Nevertheless, the actual mechanism through which ROS may affect TLR4 signaling pathway, including the extracellular domain of TLR4, remains an important question for future investigation. In this study, we have shown that ROS could regulate the expression of TLR4, and thus affect downstream signaling pathways including the proinflammatory and proliferative phenotype of VSMCs.

CONCLUSIONS

In conclusion, our study provides the first evidence that inhibition of ROS could attenuate VSMC proliferation, migration and inflammation by suppressing TLR4 expression, and thus attenuate neointima or vascular remodeling following carotid injury. These findings advance our understanding of the pathophysiology function of ROS in vascular disease, and suggest ROS as a perspective target for the prevention and treatment of vascular diseases.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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