Downregulation of Quinone Reductase 2 Attenuates Vascular Smooth Muscle Cells Proliferation and Neointimal Formation in Balloon Injured Rat Carotid Artery

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Key Words
Lentiviral vectors • Quinone reductase 2 • Vascular smooth muscle cells • Neointimal formation • Gene therapy

Abstract
Background/Aims: Quinone reductase 2 (NQO2) is a flavoprotein that catalyzes the metabolic reduction of quinines, but its biological mechanism in vascular smooth muscle cells (VSMCs) is unclear. The aim of this study was to evaluate the role of NQO2 on VSMCs proliferation and the neointimal formation in balloon injured rat carotid artery. Methods: Left common carotid arteries from Sprague–Dawley rats were injured by a balloon catheter, and the injured arteries were incubated with 50 µL solution of NQO2-siRNA-GFP lentiviral vectors, NC-siRNA-GFP lentiviral vectors or PBS for 1 h. The rats were euthanized for morphometric and immunohistochemical analysis, real-time PCR and western blot analysis at 2 weeks after balloon injury and gene transfer. The cultured rat VSMCs transduced with NQO2-siRNA-GFP or NC-siRNA-GFP lentiviral vectors were used for cell proliferation assay, real-time PCR and western blot analysis. In order to detect the vascular or intracellular ROS level, the lentiviral vectors without GFP were used to transfect the injured common carotid arteries and the cultured rat VSMCs. Results: Lentiviral vectors bearing NQO2 siRNA could reduce NQO2 protein level and suppress NQO2 mRNA expression in balloon injured artery walls and cultured rat VSMCs. Downregulation of NQO2 significantly suppressed VSMCs proliferation and intimal formation. NQO2 siRNA treatment could reduce vascular or intracellular ROS level and decrease the phosphorylation of the ERK1/2 in balloon injured artery walls and cultured rat VSMCs. Conclusion: Our study suggests that downregulation of NQO2 significantly suppresses VSMCs proliferation and progression of neointimal formation after vascular injury.

Introduction
Percutaneous coronary intervention (PCI) has been widely used for the treatment of coronary heart disease [1-2], but the long-term overall efficacy of PCI may be
compromised by the occurrence of restenosis [3]. Therefore, how to effectively prevent restenosis remains a crucial clinical problem that needs to be solved. In the past two decades, many clinical and experimental studies have demonstrated that proliferation of vascular smooth muscle cells (VSMCs) plays a major role in the pathogenesis of restenosis [4-6]. Consequently, suppression of VSMCs proliferation and the subsequent neointimal formation can be a useful therapeutic intervention for reducing the incidence of restenosis after PCI.

Many studies demonstrated that some natural polyphenols, such as quercetin, resveratrol, apigenin and some coumarin derivatives, could inhibit the proliferation of VSMCs [7-10]. Interestingly, all those compounds share a common structural feature in which two phenolate rings are spaced, and are potent inhibitors of quinone reductase 2 (NQO2) activity via special binding to the deep active-site cleft of NQO2 [11-12]. NQO2 is a flavoprotein that catalyzes the metabolic reduction of quinines [13], but its biological effect and mechanism on inhibition of VSMCs proliferation is unclear. In our previous studies, we found that resveratrol could bind to NQO2 and repress the mRNA and protein expressions of NQO2 in VSMCs [14]. However, the possible role of NQO2 in the development of intimal formation after vascular injury has not yet been determined.

There is mounting evidence showing that extracellular signal-regulated kinase (ERK1/2) involves in the regulation of VSMCs proliferation [15-16]. Diverse stimuli, such as angiotensin II, TNF-α and fetal bovine serum (FBS) could increase the activity of NAD(P)H oxidase, a major source of reactive oxygen species (ROS) in vascular tissues. The augmented ROS level can activate ERK1/2 and the subsequent proliferation of VSMCs [17-20]. Therefore, we hypothesized that downregulation of NQO2 can suppress intimal formation through reduction of ROS level and inhibition of ERK1/2 activation in injured carotid artery. To address this hypothesis, we used lentiviral vectors bearing NQO2 siRNA to infect the balloon injured artery wall and the cultured rat VSMCs, evaluated the effects of NQO2 silence on neointimal formation and VSMCs proliferation, and further studied its possible mechanism.

**Materials and Methods**

The ROS detection kit was purchased from Genmed Scientifics (Arlington, MA, USA). The rabbit monoclonal antibodies for ERK1/2 and phospho-ERK1/2 were obtained from Cell Signaling Technology (Beverly, MA, USA). NQO2 polyclonal antibody was purchased from Proteintech Group (Chicago, IL, USA). PCNA and GAPDH monoclonal antibodies were purchased from Bioworld Technology (Louis Park, MN, USA). Secondary antibodies and enhanced chemiluminescence (ECL) reagent were purchased from Pierce Chemical (Rockford, IL, USA). The bromodeoxyuridine (BrdU) Cell Proliferation Assay Kit, the specific short hairpin for NQO2 and negative control, the reagents for cDNA synthesis and polymerase chain reaction (PCR), and the SYBR Premix Ex Taq (perfect real-time) kit were obtained through companies previously described [14]. Other reagents were purchased from various commercial vendors.

**Rat carotid artery balloon injury model, cell culture and lentiviral transduction**

All experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee and followed the Guide for the Care and Use of Laboratory Animals of Nanjing Medical University, China.

The culture of rat VSMCs and the production process of lentiviral vectors as we previously described [14]. Rat VSMCs were seeded into 6-well culture plates 24 h prior to transfection at a density of 1x10⁵ cells/well. The cells were transduced with lentiviral vectors (5x10⁵ TU/mL, 20 μL per well) at a multiplicity of infection (MOI) of 100 in the presence of 5 μg/mL polybrene for 24 h. After 24 h incubation, rat VSMCs were cultured in fresh DMEM and were used for the experiments.

Sixty-four male Sprague–Dawley rats (350–400 g) were randomly divided into four equal groups (n=16). The rats were anaesthetized with 10% chloral hydrate (0.35 g/kg intraperitoneal) and subjected to balloon catheter injury of the left common carotid artery as reported previously [21]. Briefly, the left common, external and internal carotid arteries were exposed after intravenous injection of 200 U/kg of heparin sodium. A balloon angioplasty catheter (balloon diameter 1.5 mm, balloon length 20 mm, Medtronic) was introduced through the external carotid cut into the common carotid artery in balloon injured rats. The balloon was inflated by 2 atmospheric pressures and passed thrice in the common carotid artery. The balloon was removed and the blood flow of common and internal carotid arteries was temporarily interrupted by two microvascular clips. A 50 μL solution of NQO2-siRNA-GFP lentiviral vectors (5x10⁵ TU/mL), NC-siRNA-GFP lentiviral vectors (5x10⁴ TU/mL) or PBS was respectively infused into the injured common carotid artery and then the left external carotid artery was ligated. The blood flow through the common and internal carotid arteries was restored after 1 h. Sham operated rats only ligated the left external carotid artery. At two weeks after injury, rats were sacrificed and the left common carotid arteries were harvested for different experiments.

**Morphometric and immunohistochemical analysis**

Carotid artery segments were fixed in 4% paraformaldehyde solution, embedded in paraffin, cut into serial sections (5 μm thick) at equally spaced intervals, deparaffinized, and stained with hematoxylin/eosin for morphometric analysis.

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as we previously described [22]. The medial area was calculated by subtracting the area defined by the internal elastic lamina from the area defined by the external elastic lamina, and the intimal area was determined by subtracting the lumen area from the area defined by the internal elastic lamina. Finally, the intimal-to-medial area ratio was calculated.

For immunohistochemical analysis, the carotid sections were incubated with anti-PCNA overnight at 4°C, followed by incubation with an anti-rabbit secondary antibody for 1 h at room temperature. Diaminobenzidine was the chromogen, and the slides were counterstained with hematoxylin. PCNA was used to quantify the proliferative activity of cells. For calculating the percentage of PCNA-positive cells (number of PCNA-positive cell nuclei/ number of total cell nuclei x 100%), the normal cell nuclei number and PCNA-positive cell nuclei number were counted in 5 fields of every section under the light microscope.

**Cell proliferation assay**

The rat VSMCs (2x10⁵ cell/mL) were seeded into 96-well culture plates (100 µL/well). The bromodeoxyuridine (BrdU) reagent was diluted (1:500) and incorporated into the proliferating cells during the final 24 h incubation period. BrdU was detected using a monoclonal mouse antibody against BrdU (1:200). After the addition of the goat anti-mouse IgG peroxidase-conjugated secondary antibody, substrate, and stop solution, the amount of BrdU incorporated into the cells was measured as optical density (OD) using a spectrophotometer microplate reader at a wavelength of 450 nm.

**Detection of vascular and intracellular ROS level**

The vascular and intracellular ROS level was detected using an oxidation-sensitive fluorescent probe (DCFH-DA). The lentiviral vectors without GFP were used to transfect the injured common carotid arteries and the cultured rat VSMCs. The carotid arteries were isolated from the rats in each group and cut into serial sections. The tissues were incubated in ROS staining solution (DCFH-DA) at 37°C for 20min. After washing, the tissues were observed using a fluorescence microscope. To quantify the ROS level, the DCF fluorescence intensity in the tissues was detected using Synergy HT fluorescence microplate reader (BIO-TEK, USA) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

The culture medium of VSMCs was replaced with fresh medium not containing FBS and incubated for 24 h. Rat VSMCs were incubated for 1 h in culture medium containing 10 µmol/L DCFH-DA to establish a stable intracellular level for the probe and stimulated with 10% FBS for another 30 min. The cultured rat VSMCs were washed with PBS three times. The DCF fluorescence intensity of the cells was detected using the FACS/Aria flow cytometer (Becton Dickinson, USA). For each sample, 10,000 events were collected.

**Real-time quantitative polymerase chain reaction**

Total RNA was extracted from the carotid arteries and culture VSMCs with TRIzol reagent (Invitrogen) according to the instructions provided by the manufacturer. The total RNA (1 µg) was reverse transcribed (RT) using an oligo (dT) 18 primer and AMV reverse transcriptase. A real-time quantitative polymerase chain reaction (PCR) was performed using the ABI Prism 7300 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). The expression levels of NQO2 were analyzed by real-time PCR using a SYBR green dye. The primer sequences were as follows: rat GAPDH, 5'-TTCAACGGCACA GTC AAG G-3' (forward) and 5'- CTC AGC ACC AGC ATC ACC -3' (reverse); rat NQO2, 5'-TGG GAT AGA AGC CTATG AGC CTAC-3' (forward) and 5'-GGA TTG TTG CGA AAC CGG TGA AC-3' (reverse). Relative mRNA abundance was calculated using the 2⁴⁻ⁿᶜ⁻ method.

**Western blot analysis**

Rat common carotid arteries were homogenized with ice-cold lysis buffer containing PMSF. To the cultured rat VSMCs, the six-well plate was washed three times by PBS. The plate was placed on ice for 30 min, and added the lysis buffer containing PMSF. Then the cells were scraped to the side of the plate after schizolysis. The debris and lysate were moved to 1.5 mL centrifuge tubes, respectively. The tubes were run at 12000 rpm for 10 min at 4°C, and the supernatant was collected for protein analysis. Whole protein samples were boiled for 5 min in 5 x loading buffer and separated using 10% SDS-PAGE. Proteins were transferred to PVDF membranes at a current of 300 mA for 90 min. The membranes were incubated in a blocking solution of 5% fat-free milk in Tris-buffered saline plus Tween 20 (TBST) for 1 h. The GAPDH monoclonal antibody and NQO2 polyclonal antibody were diluted at 1:5000 and 1:1000, respectively. Rabbit polyclonal anti-ERK1/2 and phospho-ERK1/2 were diluted at 1:1000 and 1:2000, respectively. The blots were incubated overnight with primary antibodies at 4°C, and then the appropriate secondary antibodies were used. Specific immunoreactivity was detected by ECL.

**Statistical analysis**

Data were presented as the mean ± S.E.M, and statistical analysis was performed with one-way ANOVA. Results were considered as statistically significant at P < 0.05.

**Results**

**Lentiviral vectors containing NQO2 siRNA downregulate NQO2 expression**

NQO2 mRNA and protein were both detected in carotid arteries after 2 weeks of balloon injury. Balloon injury could significantly increase NQO2 mRNA expression 1.4-fold compared with Sham group without injury. NC siRNA had no inhibitory effect on the expression of NQO2 mRNA compared with PBS group (P>0.05). However, NQO2 siRNA treatment could decrease NQO2 mRNA expression by 16.5% compared with PBS group (P<0.05, Fig. 1A). Similarly, NQO2 siRNA treatment could reduce NQO2 protein level compared with PBS and NC siRNA group after balloon injury.
injury (0.937±0.019 vs. 1.165±0.031, P<0.05, Fig. 1B). There was no statistical difference between PBS and NC siRNA group.

The expression of NQO2 in lentiviral vectors-transduced VSMCs was evaluated by real-time quantitative PCR and western blotting. After 72 h transfection, real-time quantitative PCR showed that NQO2 siRNA treatment significantly suppressed the NQO2 mRNA expression level about 79.1% compared with Control group (P<0.01, Fig. 1C). NQO2 protein level decreased about 76.4% in NQO2 siRNA group compared with Control group after 14 days (P<0.01, Fig. 1D). There was no statistical difference between NC siRNA and Control group.

**Fig. 1. NQO2 siRNA treatment inhibited the expression of NQO2.** (A) NQO2 siRNA treatment inhibited NQO2 mRNA expression in balloon carotid arteries. (B) NQO2 protein expression level was inhibited by NQO2 siRNA treatment in balloon carotid arteries. (C) NQO2 mRNA expression levels in Control, NC siRNA, and NQO2 siRNA group using real-time quantitative PCR detection. (D) Western blot analysis of NQO2 expression levels in Control, NC siRNA, and NQO2 siRNA group. Values are expressed as mean ± S.E.M (n=3). #P<0.05 vs. Sham group; *P<0.05 vs. PBS and NC siRNA group; ΔP<0.01 compared with the Control group.

**Downregulation of NQO2 inhibits neointimal formation and cell proliferation**

Two weeks after balloon injury and lentiviral transfection, the degree of neointimal formation was evaluated morphologically and quantitatively (Fig. 2). Uninjured carotid arteries presented the endothelial monolayer. Neointimal hyperplasia dramatically developed after balloon injury within 2 weeks in PBS and NC siRNA group. NQO2 siRNA treatment significantly reduced the neointimal area compared with PBS and NC siRNA group (0.034±0.002 mm² vs. 0.133±0.003 mm² and 0.129±0.003 mm², P<0.05, Fig. 2A, B). No significant differences in the medial area were observed among all groups (Fig. 2C). NQO2 siRNA treatment also significantly reduced
the intima/media ratio compared with PBS and NC siRNA group (0.795±0.069 vs. 2.995±0.111 and 2.964±0.125, P<0.05, Fig. 2D).

Fig. 2. NQO2 siRNA treatment decreased neointimal growth after 2 weeks in balloon injured carotid arteries. (A) Arterial sections were stained with hematoxylin and eosin. Scale bar represents 25 µm. Black arrows indicate the elastic lamellae. Neointimal growth was determined by intima area (B), medial area (C) and ratio of intima to media (D). Values are expressed as mean ± S.E.M (n=10). #P<0.05 vs. Sham group; *P<0.05 vs. PBS and NC siRNA group.

Fig. 3. NQO2 siRNA treatment inhibited neointimal formation and cell proliferation. (A) Arterial sections were stained immunohistochemically for PCNA. Black arrows indicate PCNA labeled nuclei stained as dark brown. Scale bar represents 12.5 µm. (B) PCNA-positive nuclei were quantified as the percentage of total nuclei in the neointima. Values are expressed as mean ± S.E.M (n=10). #P<0.05 vs. PBS and NC siRNA group. (C) NQO2 siRNA treatment inhibited cultured rat VSMCs proliferation. Values are expressed as mean ± S.E.M (n=6). *P<0.01 vs. Control group; ΔP<0.01 vs. Normal and NC siRNA group incubated in the medium containing 10% FBS for 48 h.

Cell proliferation in the neointima after 2 weeks was examined by immunostaining for PCNA. No PCNA-positive cells were observed in the intima of Sham group.

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NQO2 siRNA treatment significantly reduced the percentage of PCNA-positive cells in the neointima compared with PBS and NC siRNA group (7.23±0.69 vs. 45.56±0.85% and 44.06±1.32%, P<0.05, Fig. 3A, B).

After incubation in the medium without FBS for 24 h, the rat VSMCs of Normal, NC siRNA and NQO2 siRNA group were incubated in the medium containing 10% FBS or not for another 48 h respectively. No statistical differences were observed when treated with the medium without FBS among three groups. However, NQO2 siRNA treatment significantly suppressed cell proliferation compared with Normal and NC siRNA group after incubation in the medium containing 10% FBS (162.2±4.9% vs. 290.4±4.3% and 291.3±5.0%, P<0.01, Fig. 3C). NC siRNA treatment had no inhibitory effect on cell proliferation.

**Downregulation of NQO2 inhibits ROS level in carotid artery and cultured rat VSMCs**

The effect of NQO2 downregulation on ROS level was examined after 2 weeks in balloon injured carotid arteries. The lentiviral vectors without GFP were used to transfect the injured common carotid arteries. Balloon injury could significantly increase the ROS level 1.26-fold compared with Sham group without injury. NC siRNA treatment had no inhibitory effect on the ROS level compared with PBS group (P>0.05). NQO2 siRNA treatment could inhibit the ROS level compared with PBS and NC siRNA group after balloon injury (1.017±0.052 vs. 1.261±0.025 and 1.222±0.025, P<0.05, Fig. 4A).

The effect of NQO2 downregulation on FBS-induced generation of ROS was examined in cultured rat VSMCs. The lentiviral vectors without GFP were used to transfect rat VSMCs. After incubation in the medium without FBS for 24 h, the rat VSMCs of Normal, NC siRNA and NQO2 siRNA group were incubated in the medium containing 10% FBS or not for 30 min respectively. No statistical differences were observed when treated with the medium without FBS among three groups. When treated with 10% FBS for 30 min, the ROS level in NQO2 siRNA group was significantly lower than in the Normal and NC siRNA group (1.812±0.083 vs. 3.210±0.124 and 3.183±0.109, P<0.01, Fig. 4B). NC siRNA treatment had no inhibitory effect on the ROS level.

**Downregulation of NQO2 inhibits activation of ERK1/2 in carotid artery and cultured rat VSMCs**

The effect of NQO2 downregulation on the activation of ERK1/2 was further examined in balloon-injured carotid arteries. Our data showed balloon injury induced the phosphorylation of ERK in carotid arteries. NC siRNA treatment had no inhibitory effect on the activation of ERK1/2 compared with PBS group (P>0.05). However, NQO2 siRNA treatment could inhibit the phosphorylation level of ERK compared with PBS and NC siRNA group after balloon injury (P<0.05, Fig. 5A).
The rat VSMCs were incubated in the medium without FBS for 24 h. After treatment with 10% FBS or not for 10 min, the rat VSMCs were harvested for western blot analysis. No statistical differences were observed when treated with the medium without FBS among three groups. Compared with the Normal and NC siRNA group, the phosphorylation of ERK significantly decreased in NQO2 siRNA group after incubation in the medium containing 10% FBS for 10 min (P<0.01, Fig. 5B). NC siRNA treatment had no inhibitory effect on the phosphorylation of ERK.

Discussion

In the present study, we have demonstrated that downregulation of NQO2 can suppress VSMCs proliferation and the subsequent intimal formation through reduction of ROS level and inhibition of ERK1/2 activation in vivo and vitro. These results suggest that NQO2 might play an important role in the development and progression of neointimal formation after vascular injury.

Important advances in the understanding of the atherosclerosis pathogenesis and postangioplasty restenosis have been made over the past two decades. Migration of VSMCs from the media to the intima and their subsequent proliferation play a major role in the process of atherosclerosis and postangioplasty restenosis [23-24]. However, postangioplasty restenosis is still a major problem in spite of the development of drug-eluting stents [25-26]. Thus, how to more effectively reduce the incidence of restenosis remains a crucial clinical problem that needs to be solved.

In recent years, gene therapy has been widely used to treat cardiovascular diseases [27-29], but choosing appropriate target genes is probably very important. Excessive production of ROS has been implicated to play an important role in restenosis after angioplasty or venous bypass grafting. The formation of ROS is balanced...
out by antioxidant defenses, and augmenting this defense by antioxidant therapies can provide potential means to treat cardiovascular disorders [27, 30-31]. NQO2 is a homologue of the well-characterized NAD(P)H:quinone oxidoreductase 1 (NQO1). Because of its sequence homology with NQO1, it has been suspected to detoxify quinones. However, in the past ten years, evidence begins to point to a versatile role of this enzyme [32]. Buryanovskyy L et al. found that the inhibition of NQO2 increased the concentration of the endogenous electrophiles and consequently upregulated the expression of some antioxidant enzymes [12]. Gong et al. found that NQO2 catalyzed the metabolic activation of vitamin K3, leading to cytotoxicity. NQO1 metabolically detoxified vitamin K3 and protected cells against oxidative stress and other adverse effects [33]. More interestingly, some antioxidants, such as quercetin, resveratrol and apigenin, are potent inhibitors of NQO2 activity via specifically binding to the deep active-site cleft of NQO2 [11-12]. These studies suggest that NQO2 may be involved in some adverse reactions. Thus, appropriate control over the protein level of NQO2 in VSMCs may be essential to prevent the development and progression of atherosclerosis and postangioplasty restenosis. In our studies, lentiviral vectors bearing NQO2 siRNA were chosen to infect the balloon injured artery walls and the cultured rat VSMCs. Our results showed that NQO2 was expressed in normal arterial walls and cultured VSMCs and upregulated significantly after balloon injury. Meanwhile, NQO2 siRNA treatment led to inhibitory effect on VSMCs proliferation and neointimal formation. NQO2 knockout mice have been shown to develop normally, indicating that NQO2 is not associated with mouse development [34]. Thus, NQO2 may be an appropriate intervention target for gene therapy of atherosclerosis and postangioplasty restenosis.

In this study, the underlying intracellular mechanisms of NQO2 silence inhibiting VSMCs proliferation and neointimal formation was further investigated. Increasing evidences have shown that ERK1/2 is a key enzyme involved in the regulation of cell proliferation [15, 16, 35, 36]. Our study found that the ROS level and the phosphorylation of ERK1/2 significantly decreased in cultured rat VSMCs and balloon injured arterial walls after NQO2 siRNA treatment. The decreased ROS level can inactivate the phosphorylation of ERK1/2 [17-20]. Thus, one possible mechanism is that the suppression of NQO2 attenuates the formation of ROS and subsequently decreases the phosphorylation of the ERK1/2. NQO2 is also required for the TNF-induced activation of MAP kinases in keratinocytes [37]. Accordingly, we presume that NQO2 may have a pivotal role in different cells.

Our study showed that lentiviral vectors transfection is an efficient method of cardiovascular gene therapy. Intravascular gene transfer to the vessel walls can be performed using catheters. But the short duration of the gene transfer during conventional angioplasty and the presence of blood components can decrease gene transfer efficiency. Our results also indicated that the transfer efficiency in vivo was weaker than that in vitro. Restricted local delivery via the intravascular route may not be possible due to leakage of the gene transfer solution into the systemic circulation. This could also result in the expression of the trans-gene in undesired locations [38]. Applied to the outside of the carotid walls may permit lentiviral vectors to act on specific localization of the arteries. Gene transfer vectors can be applied to the adventitial surface using a biodegradable adventitial collar, biodegradable gel or direct injection into the adventitia [39], but the long-term effects and safety still need further study.

In summary, our study elucidates the potential intracellular mechanism of NQO2 in the inhibition of rat VSMCs proliferation and neointimal formation. Through reducing NQO2 protein level and suppressing NQO2 mRNA expression, NQO2 siRNA treatment reduces ROS level in intracellular accumulation and decreases the phosphorylation of the ERK1/2 in balloon injured arterial walls. These findings show that NQO2 is the important target for gene therapy of atherosclerosis and postangioplasty restenosis.

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