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Original Article

Inhibition of NOX4 prevents the development of atherosclerosis in Ldlr$^{-/-}$ mice

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Abstract: Atherosclerosis is a progressive disease with endothelial cell inflammation. Recent studies showed that oxidative stress is an important factor in inducing endothelial cell inflammation. NOX4 is a major ROS-producing enzyme in endothelial cells, yet its role in the pathogenesis of atherosclerosis and plaque formation remains unclear. LDLr$^{-/-}$ mice were fed with high fat diet for 16 weeks to establish atherosclerosis model and treated with NOX4 siRNA. NOX4 expression was detected by western blot. H_2O_2 level was determined by the kit. Plaque formation was assessed by general oil red O staining and HE staining. Aorta endothelial cells were treated with oxLDL to mimic the atherosclerosis after transfected with NOX4 siRNA. ROS level and cell apoptosis were evaluated by flow cytometry and AV-PI staining. NOX4 expression was significantly increased in atherosclerotic mice with huge amount of tissue H_2O_2 production ($P < 0.01$). NOX4 siRNA obviously reduced the production of tissue H_2O_2 ($P < 0.01$), decreased atherosclerotic plaque area, and elevated endothelial integrity. In endothelial cells, NOX4 increased markedly in model group compared with normal control ($P < 0.01$), accompanied with massive production of ROS. Inhibition of NOX4 expression reduced ROS production and inhibited apoptosis ($P < 0.01$). In conclusion, a large amount of ROS produced by NOX4 is the initiating factor of atherosclerosis and inhibition of NOX4 level shows a protective effect on atherosclerosis, suggesting that NOX4 may serve as a key target for the treatment of atherosclerosis.

Keywords: Atherosclerosis, oxidative stress, NOX4

Introduction

Atherosclerosis (AS) is the main cause of coronary heart disease, cerebral infarction, and peripheral vascular disease [1]. It is a progressive, chronic arterial disorder in which part or entire bloodstream is blocked by the deposition of fat in the artery [2]. It is an important factor in the induction of myocardial infarction and cerebral infarction. However, the exact cause of AS has not been determined. Furthermore, it is also the high risk factor of diabetes mellitus, hypertension, and hyperlipidemia [3].

Chronic epithelial cell inflammation is a key mechanism of AS [4]. Chronic inflammation leads to stress and metabolic abnormalities in the early stage of AS, and is associated with excess cellular reactive oxygen species (ROS), endothelial cell adhesion disorder, and apoptosis in monocytes. The acute symptoms of patients with vascular disease are caused by AS plaque and unstable thrombosis which is induced by endothelial cell apoptosis. ROS is an important active product of oxidative stress. Physiologically, ROS is a normal metabolite of various redox reactions in cells and serves as an important signaling molecule involved in the inflammatory response. Stress increased ROS production, and induced cell proliferation and mutation, leading to the occurrence of oxidative stress.

The main sources of ROS production include NADPH oxidase, lipoxygenase, xanthine oxidase, and cyclooxygenase. Among them, NADPH oxidase is a key enzyme of oxidative stress, including NOX1-5, Duox1, and Duox1. NOX4 is mainly expressed in endothelial cells [5]. There are differences in the roles of different NOX subtypes in cardiovascular physiology and pathology. It was reported that NOX1 and NOX2 play a role in vascular diseases, such as atherosclerosis, in blood vessels and macro-

phages [6]. Endothelium-dependent diastolic function is significantly enhanced in patients with inherited NOX2 deficiency [6]. NOX5 promotes endothelium-dependent diastole in human coronary artery disease caused by oxidative stress [7]. A previous study reported that NOX4 was significantly upregulated in chronic inflammation models of atherosclerotic mice and endothelial cells [8]. There is also growing evidence that NOX4 plays an important role in the cardiovascular system. In addition, NOX4 has also been demonstrated to exert anti-atherosclerotic functions as shown by increased atherosclerosis formation in mice after deletion of NOX4 [8]. However, the role of NOX4 in the pathogenesis of AS has not been fully elucidated.

Therefore, this study aimed to investigate the role of NOX4 in AS plaque formation, endothelial cell oxidative stress, and apoptosis.

Materials and methods

Main materials and reagents

Total protein extraction kit was purchased from KGI Biotech Development Co., Ltd. (Nanjing, China); Western blot lysate and BCA protein assay kit were supplied by Beyotime (Suzhou, China); NOX4 antibody was purchased from Proteintech Ltd. (Wuhan, China); Horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) was purchased from ZSbio (Beijing, China); DMEM, fetal bovine serum, and penicillin-streptomycin were purchased from Gibco (New York, USA); SiNOX4, si control (NC), and lentivirus were designed and synthesized by Genepharma (Shanghai, China). Other reagents were purchased from sigma (New York, USA).

Main instruments

The bechtop was supplied by Boxun Industrial Co., Ltd. (Shanghai, China). Gel images were analyzed with UVP Multispectral Imaging System (California, USA). PS-9 semi-dry transfer electrophoresis equipment was purchased from Jimxin (Dalian, China). BD FACS Calibur flow cytometry was purchased from BD Biosciences (San Diego, CA, USA). Carbon dioxide incubator and Thermo-354 microplate reader were purchased from Thermo Fisher Scientific Inc. (New York, USA).

Experimental animals and model establishment

C57-derived LDLr$^{-/-}$ mice at 6-8 weeks old and weighted 18-22 g were purchased from Capital Medical University Experimental Animal Center (China). The mice were fed in clean animal houses with indoor temperature at 24°C, relative humidity of 60%, and 12 hour day night cycle. The mice were free to eat and drink with padding daily replacement to avoid infection. The animals were divided into three groups with 10 mice in each group, including normal control group, AS model group, and NOX4 knockdown model group. The mice in normal control group were given chow diet (CD). The other two groups received high cholesterol diet (HCD) (composed of casein, DL-Methionine, anhydrous milk fat, sucrose, corn starch, cholesterol and fiber) for 16 weeks. NOX4 siRNA in lentivirus (50 ng/kg/2 day) was injected to the mice through tail vein 12 hours before administration of high cholesterol.

Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of the First Hospital of Changsha, Changsha (Hunan, China).

Total protein extraction

The cells were washed by PBS for three times and treated with 100 mM PMSF on ice for 5-10 min. Then the cells were moved to a new Eppendorf tube and centrifuged at 12000 g and 4°C for 5 min.

Western blot

According to the reference [9], the extracted protein solution was quantified by BCA method and then denatured in boiling water for 5 min. The samples were separated by 10% SDS-PAGE and transferred to PVDF membrane at 300 mA for 1 hour. Next, the membrane was incubated in NOX4 antibody (1:1000) at 4°C overnight. After washed by PBS for three times, the membrane was incubated in secondary antibody (1:1000) at 37°C for 2 hour. At last, the membrane was treated by chemiluminescence to visualize the bands.

H_2O_2 detection

Tissue H_2O_2 detection kit was purchased from Nanjing Jiancheng Co., Ltd. (Nanjing, China). A

total of 100 mg fresh aorta tissue was rinsed into homogenate and centrifuged to obtain the supernatant. The supernatant was adopted to test H_2O_2 level according to the manual.

HE staining

The aortic valve was isolated and fixed in 4% paraformaldehyde for 24 hours and gradient dehydrated with 100%, 95%, 85%, and 75% ethanol. After hyalinized by turpentine, the tissue was embedded. Next, the sample was sliced at the thickness of 4 μm. Then the section was dewaxed, hydrated, and stained by hematoxylin. At last, the section was stained by eosin and sealed.

General oil red O staining

The aortic arch, thoracic aorta, and abdominal aorta were longitudinally incised and fixed with a needle. After rinsed with PBS, the tissue was fixed with 60% isopropanol for 5 min and stained with oil red O for 15 min. The injury area was calculated by the ratio of positive area and total surface area.

Cell culture and model construction

Human aortic endothelial cell line (HAECs) was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% carbon dioxide. The morphology and growth of the cells were observed by inverted phase contrast microscope. The cells in log phase were selected for experiment. The cells were divided into normal control group, model group, model + si Control group, and model + si NOX4 group. Endothelial cell atherosclerosis was induced by oxLDL (100 μg/mL) for 24 hours.

Cell transfection

NOX4 siRNA was synthetized by Genepharma [10, 11]. SiNOX4 sense: CCGCTTTGAAAGTGTCAGTCA; anti-sense: TCAGCTACCACATGGACAATC. The cells were passaged on the day before transfection to make the confluency at 30%-50% in a 24-well plate. A total of 1.25 μL of siRNA stock solution (20 μM) was pipetted into 100 μL of Opti-MEM medium as solution A, and 1 μL Lipofectamine 2000 or Lipofectamine™ RNAiMAX was dissolved in Opti-MEM medium as solution B. After 5 min, the solution A was mixed with solution B for 20 min before added to the cell culture plate. After 4 hours of incubation, the medium was changed to DMEM medium containing 10% fetal bovine serum. NOX4 expression was detected to calculate the transfection efficiency.

ROS level detection

CM-H2DCFDA was hydrolyzed into DCFH in cells. DCFH was oxidized by intracellular oxidant to DCF with high fluorescence intensity, which was used to detect ROS generation. After transfection, the cells were digested into single cell suspensions and incubated with CM-H2DCFDA probe for 30 min. Finally, the cells were washed with DMEM for three times to remove excess probes and tested on a BD FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA).

AV-PI staining

The cells were seeded on a 6-well plate. After lentivirus transfection, they were digested and resuspended into single cell suspension. Next, they were stained by AV and PI solutions on ice avoid of light for 10 min. At last, the sample was tested on a BD FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA). All experiments were repeated 3 times.

Statistical analysis

All data analyses were performed with SPSS 19.0 software. The measurement data were depicted as mean ± standard deviation and assessed by normality test and homogeneity test of variances. The data in normal distribution were compared by t test and one-way ANOVA. $P < 0.05$ was treated as statistical significance.

Results

NOX4 expression in AS mouse

As shown in **Figure 1**, NOX4 expression was significantly increased in AS model receiving HCD compared with normal control receiving CD, while NOX4 siRNA obviously reduced NOX4 level. It suggested successful inhibition of NOX4 levels at animal level.

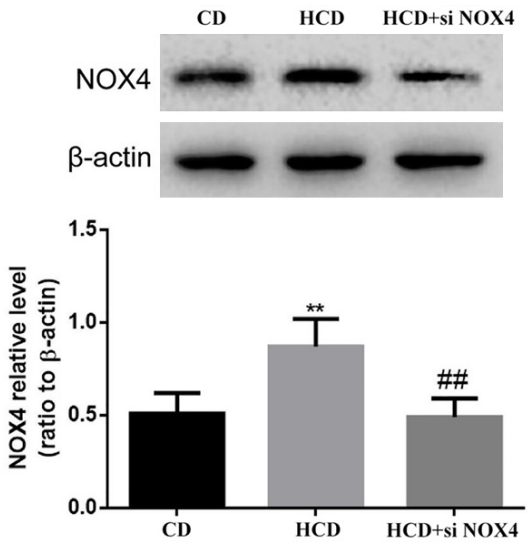

Figure 1. NOX4 expression in different groups. Total proteins were isolated from mice in different groups followed by analysis of NOX4 expression by western blot. The NOX4 expression was quantified as a ratio to β-actin. $^{**}P < 0.01$, compared with control; $^{\#\#}P < 0.01$, compared with model group. Chow diet: CD; high cholesterol diet: CHD.

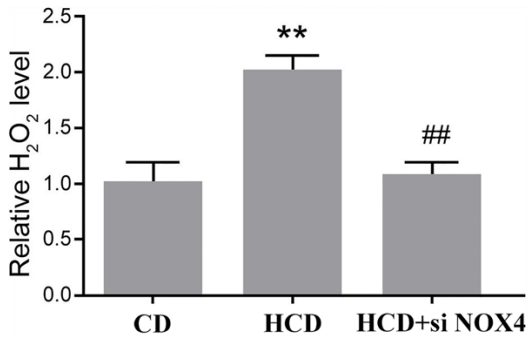

Figure 2. H_2O_2 level in the aorta of different groups. Fresh aorta tissue from mice in different groups was rinsed into homogenate and centrifuged to obtain the supernatant followed by measurement of H_2O_2 level using Tissue H_2O_2 detection kit. $^{**}P < 0.01$, compared with control; $^{\#\#}P < 0.01$, compared with model group. Chow diet: CD; high cholesterol diet: CHD.

H_2O_2 level in aorta

NOX4 was a crucial source of ROS in vivo, while H_2O_2 was one of the most important forms of ROS in tissues. The level of ROS in the tissue was shown in **Figure 2**. The level of H_2O_2 in the aorta of AS model group receiving HCD was significantly elevated almost twice as high as that in the normal group receiving CD. After NOX4 siRNA treatment, H_2O_2 production in the tissue was significantly suppressed compared with model group.

The relationship between NOX4 expression and aorta plaque formation

Oil red O staining and HE staining results of aorta in each group were shown in **Figure 3**. In **Figure 3A**, almost no oil red O-positive atheroma formed in the normal group. The atherosclerotic plaque formed in the model group was deeply stained with oil red, with large colored area and bright red color. SiNOX4 treatment significantly reduced the formation of atherosclerotic plaque, suggesting that inhibition of NOX4 expression could reduce plaque formation. As shown in **Figure 3B**, plaque area was significantly increased in the model group with enhanced inflammatory cell infiltration and weakened endothelial integrity as demonstrated by HE staining. Plaque area declined and endothelial integrity increased after administration of siNOX4. Our results indicated that reducing NOX4 expression inhibited plaque formation and improved endothelial morphology.

SiRNA transfection downregulated NOX4 level

As shown in **Figure 4**, the expression of NOX4 was significantly increased after oxLDL treatment, which was consistent with its expression level in AS model mice. However, NOX4 expression was significantly downregulated by NOX4 siRNA with a transfection efficiency of around 68%, confirming the successful knockdown of NOX4.

The influence of NOX4 on cell apoptosis

Cell apoptosis was detected by AV-PI staining (**Figure 5A**). After oxLDL treatment, the number of both early and late apoptotic cells markedly increased compared with the normal control. SiNOX4 apparently decreased the number of early and late apoptotic cells, indicating that inhibition of NOX4 level significantly prevented apoptosis.

Consistently, western blot analysis showed significantly increased the expression of pro-apoptotic molecule Bax after oxLDL treatment, which was significantly reduced after siNOX4 treatment (**Figure 5B**).

The impact of NOX4 on ROS level

As shown in **Figure 6**, intracellular ROS level increased significantly after oxLDL treatment compared with the normal control. However, siNOX4 intervention markedly reduced ROS level.

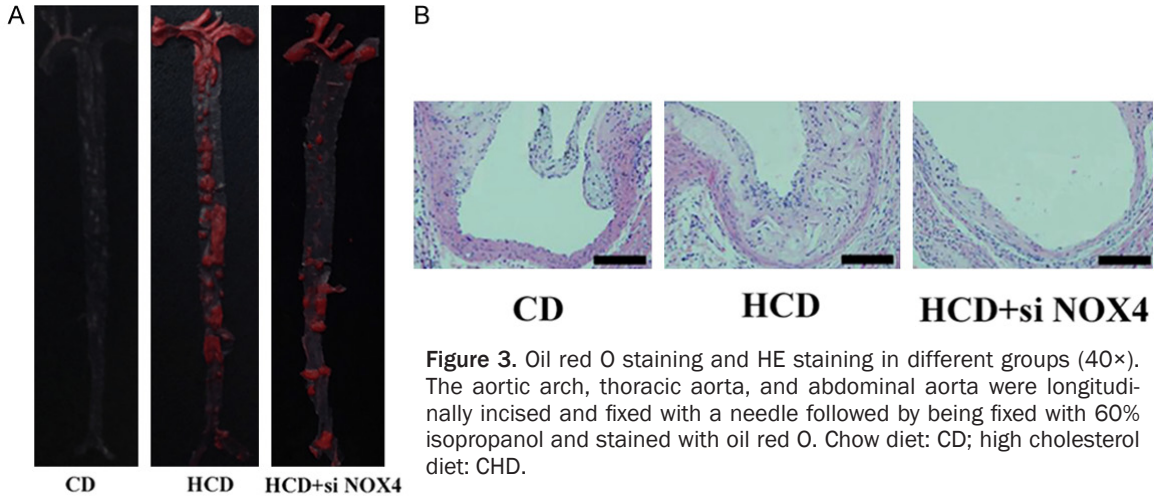

Figure 3. Oil red O staining and HE staining in different groups (40×). The aortic arch, thoracic aorta, and abdominal aorta were longitudinally incised and fixed with a needle followed by being fixed with 60% isopropanol and stained with oil red O. Chow diet: CD; high cholesterol diet: CHD.

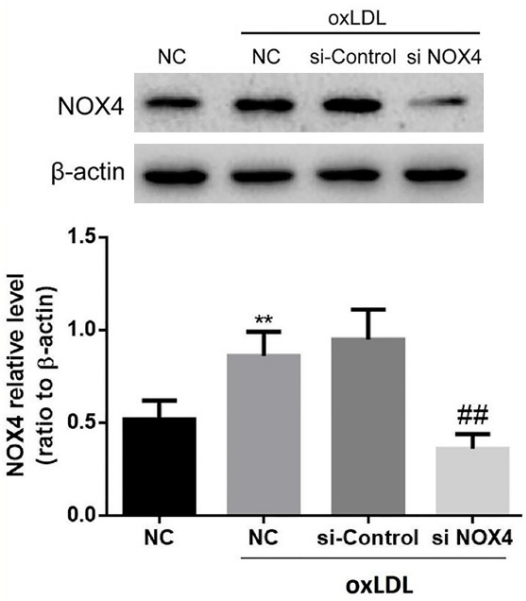

Figure 4. Cellular NOX4 expression after oxLDL treatment. Protein was isolated from cells after different treatment and NOX4 expression was measured by western blot. $^{**}P < 0.01$, compared with NC; $^{\#\#}P < 0.01$, compared with si-Control group.

Discussion

AS is an important cause of death around the world, while chronic inflammation is a common pathophysiological feature of AS. Chronic inflammation induces endothelial oxidative stress, apoptosis, and autophagy reduction, thus disturbing the normal function of endothelial cells and promoting the development of AS [12].

Oxidative stress plays a key role in the development of cardiovascular diseases, which can impair vascular endothelial function and increase the inflammatory reaction and lipid peroxidation [13]. ROS is a crucial product of oxidative stress and is widely involved in endothelial cell apoptosis and inflammatory response. Enzymes that affect intracellular ROS production include xanthine oxidase, nitric oxide synthase, mitochondrial electron transport, and NADPH oxidase, of which NADPH oxidase is an important source of intracellular ROS production. It is mainly divided into several subtypes, such as NOX1-5 and DUOX1-2, of which Nox4 is mainly expressed in vascular endothelial cells [14]. Nox4 and its regulatory subunit p22phox form heterodimer, which is the main cause of cell peroxidase production [15, 16].

In the AS plaque formation stage, NOX4 expression was significantly increased [17], which was consistent with our results. In addition, apocynin, an inhibitor of NADPH oxidase, was reported to have a vascular protective effect [18-20]. It was also reported that a significant elevation of NOX4 expression was detected both in hyperhomocysteine-induced oxidative stress and in oxLDL-induced endothelial oxidative stress [21]. Anti-oxidant drugs, such as potent antioxidant lipoic acid, traditional Chinese medicine antioxidant salvianolic acid B, and catalpol [21], can significantly inhibit the oxidative stress caused by NOX4 and play a protective effect. However, the role of other NOX subtypes in atherogenesis is controversial. It was shown that knockout the subunit of NOX2 complex presented anti-AS effect, whereas the others failed to exhibit similar influence [17]. Deletion of NOX1 reduced the adhesion of leukocytes and the expression of inflammatory markers. Non-

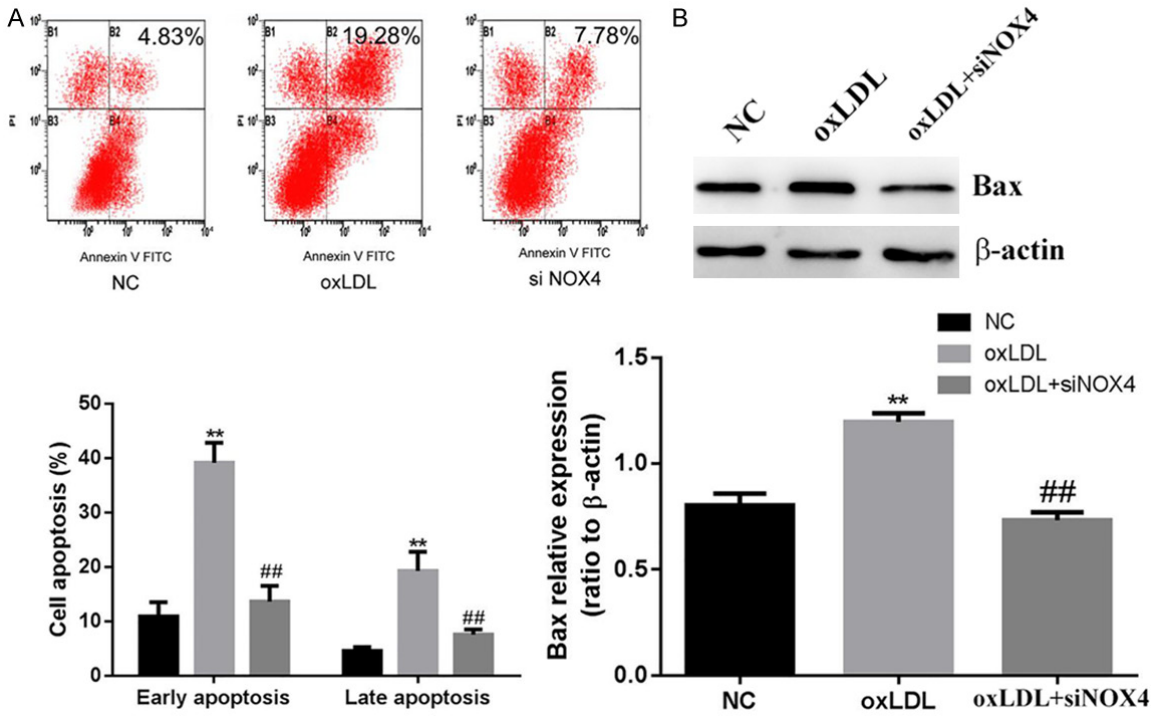

Figure 5. The influence of NOX4 on cell apoptosis. After transfection, cells were stained with AV and PI solutions on ice avoid of light followed by analysis of cell apoptosis by flow cytometry (A). Meanwhile, Bax expression after treatment was measured by western blot (B). $^{**}P < 0.01$, compared with NC; $^{\#\#}P < 0.01$, compared with oxLDL group.

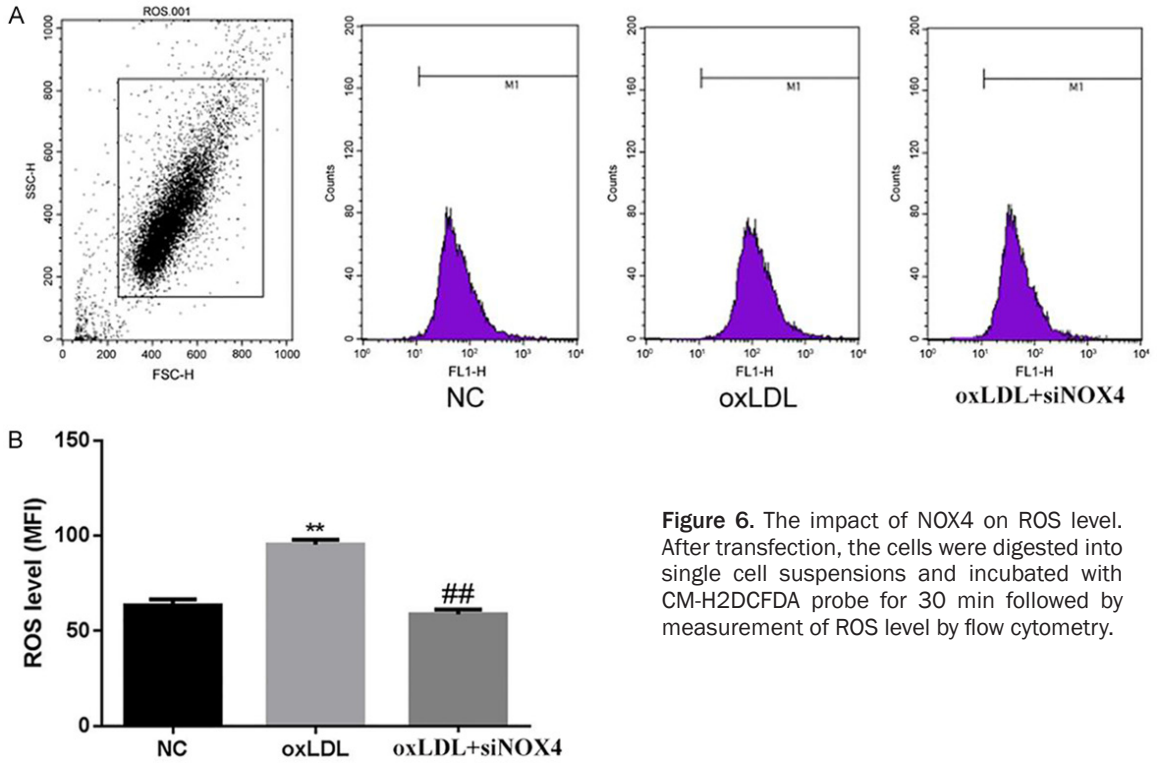

Figure 6. The impact of NOX4 on ROS level. After transfection, the cells were digested into single cell suspensions and incubated with CM-H2DCFDA probe for 30 min followed by measurement of ROS level by flow cytometry.

selective NOX1/NOX4 inhibitor GKT137831 slightly decreased the area of AS plaque in diabetic ApoE mice [22, 23], suggesting that reducing NOX4 may have a protective effect on AS.

Therefore, to further explore the role of NOX4 in AS, we first knockdown NOX4 level in HAECs which were treated with oxLDL and AS mice. NOX4 level was significantly upregulated in the animal and cell models, which was consistent with previous findings. After that, the level of NOX4 was knocked down by siRNA transfection. Compared with the specific knockout, the knockdown of NOX4 was less efficient but part of its physiological function was reserved. NOX4 level was obviously reduced in NOX4 knockdown AS mice and endothelial cells compared with the model group, demonstrating that specific knockdown was successful. Meanwhile, H_2O_2 and intracellular ROS levels were significantly inhibited, confirming the close relationship between oxidative stress and NOX4 overexpression in AS mice and endothelial cells. In the oxLDL-induced endothelial cell inflammation model, the expression of NOX4 was significantly increased. NOX4 siRNA transfection markedly suppressed cell apoptosis, indicating that NOX4 inhibition has a protective effect against endothelial injury in AS.

NOX4 plays an important role in maintaining normal redox levels and regulating ROS production under physiological conditions. In the present study, through knockdown of Nox4, we evaluated the role of Nox4 in the pathogenesis of AS and found that inhibition of Nox4 expression prevented AS development, possibly through reducing ROS production and decreasing endothelial cell apoptosis. Further investigation is required to elucidate the influence of NOX4 on formation period and stable phase of AS plaque.

Conclusion

Knockdown of NOX4 level shows a protective impact on AS, possibly through reducing ROS generation and endothelial cell apoptosis, suggesting that NOX4 may be considered as a key target for AS treatment.

Disclosure of conflict of interest

None.

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