Original Article

Oxidized High-Density Lipoprotein Induces the Proliferation and Migration of Vascular Smooth Muscle Cells by Promoting the Production of ROS

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***Aim*: As the major atheroprotective particle in plasma, high-density lipoprotein (HDL) is oxidized during atherosclerotic processes. Oxidized HDL (ox-HDL) may lose its cardioprotective properties and develop a proinflammatory and proatherogenic phenotype. The proliferation and migration of vascular smooth muscle cells (VSMCs) play a crucial role in atherogenesis. However, the influence of ox-HDL on VSMC proliferation and migration remains poorly understood.**

***Methods*: VSMCs were treated with native HDL (N-HDL) or ox-HDL at varying concentrations for different time intervals and used in several analyses. The degree of cell proliferation was assayed using CCK-8 kits. The level of cell migration was determined using a Transwell chamber and scratch-wound assay. The presence of intracellular reactive oxygen species (ROS) was detected based on ROS-mediated 2',7'-dichlorofluorescein fluorescence. The activation of NADPH oxidase was measured in terms of the Rac1 activity and $NADP^+$/NADPH ratio.**

***Results*: Compared to N-HDL, ox-HDL significantly promoted VSMC proliferation and migration in a dose-dependent manner. In addition, ox-HDL remarkably activated NADPH oxidase and enhanced ROS generation in the VSMCs. Diphenyleneiodonium chloride, an inhibitor of NADPH oxidase, and N-acetylcysteine, a ROS scavenger, efficiently inhibited the ROS production triggered by ox-HDL and subsequently blocked the proliferating and migrating effects of ox-HDL in the VSMCs.**

***Conclusions*: Ox-HDL significantly induces VSMC proliferation and migration by promoting NADPH oxidase activation and ROS production. Furthermore, the inhibition of NADPH oxidase and ROS generation blocks the proliferation and migration of VSMCs induced by ox-HDL. These proliferating and migrating effects of ox-HDL are closely related to its proinflammatory and proatherogenic roles.**

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Key words: Ox-HDL, ROS, VSMCs, Proliferation, Migration

Introduction

Atherosclerosis is a complex disease in which many factors contribute to the development of lesions[1]. Vascular smooth muscle cell (VSMC) proliferation and migration are the pivotal events of atherogenesis and play an essential role in atherosclerotic plaque progression[2]. Under the state of chronic inflammation observed in atherosclerosis, the VSMC phenotype shifts from a quiescent contractile state to an active synthetic state. Synthetic phenotypes of VSMCs proliferate and migrate from the medial layer of the vessel

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into the intima, resulting in the development of neointimal hyperplasia, which is implicated in coronary restenosis after angioplasty in patients with coronary heart disease (CHD)[2, 3]. The proliferative and migratory activities of VSMCs are regulated by many growth promoters and inflammatory factors, such as platelet-derived growth factor (PDGF), endothelin-1 (ET-1), angiotensin Ⅱ (Ang-Ⅱ) and oxidized low-density lipoprotein (ox-LDL)[4-7]. Therefore, the use of strategies aimed at inhibiting the proliferation and migration of VSMCs would prevent the development of atherosclerotic plaque and subsequent CHD.

High-density lipoprotein (HDL) is appropriately recognized as the major atheroprotective particle in plasma[8]. The protective effects of HDL are primarily attributed to its ability to promote reverse cholesterol transport (RCT) in addition to its antioxidant, anti-inflammatory and antithrombotic activities[9, 10]. HDL also exerts several beneficial effects on the vasculature, including preventing endothelial dysfunction and suppressing VSMC proliferation and migration[11, 12]. HDL inhibits VSMC migration via the sphingosine-1-phosphate (S1P)-2 receptor induced by PDGF[13]. Sun *et al*. also showed that endothelial lipase (EL) inactivation increases the amount of plasma HDL particles that can inhibit VSMC growth and migration induced by Ang-Ⅱ; however, EL overexpression decreases the HDL concentration, which increases the proliferation and migration of cultured VSMCs[14].

Meanwhile, increasing evidence has shown that HDL can lose its protective properties and even develop proinflammatory and proatherogenic phenotypes in the setting of systemic inflammation, including conditions such as atherosclerosis, diabetes mellitus and metabolic syndrome[15-17]. Dysfunctional HDL has become a key diagnostic and therapeutic target in cardiovascular disease[18]. The dysfunctional properties of HDL in systemic inflammation are associated with specific chemical modifications and structural changes, including the oxidation of phospholipids and apolipoproteins within HDL[19-21]. Several studies have shown that oxidized HDL (ox-HDL) loses its ability to stimulate cholesterol efflux from foam cells[22, 23]. In addition, ox-HDL can induce reactive oxygen species (ROS) production[24] and upregulate the expression of several proinflammatory and prothrombotic genes, including tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2) and plasminogen activator inhibitor-1 (PAI-1)[25-27], which elevates the risk of cardiovascular disease and accelerates the progression of atherosclerosis.

We also previously demonstrated that ox-HDL has an increased ability to induce the proliferation, migration and invasion of breast cancer cells, thereby promoting the progression of breast cancer[28]. Furthermore, hypochlorite-induced oxidative stress elevates the ability of HDL to promote breast cancer metastasis[29]. Meanwhile, oxidized HDL is dysfunctional in promoting endothelial repair both *in vitro* and in the reendothelialization of injured carotid arteries[30]. However, whether ox-HDL also promotes the proliferation and migration of VSMCs and aggravates the progression of atherosclerosis remains unknown. In the present study, we demonstrated that ox-HDL induces the proliferation and migration of VSMCs by activating NADPH oxidase and promoting the production of ROS, from which we conclude that ox-HDL is a key risk factor for restenosis of the coronary artery following percutaneous transluminal coronary angioplasty (PTCA) or percutaneous coronary intervention (PCI) in CHD patients.

Materials and Methods

Animals and Reagents

All procedures were approved by the Ethics Committee for the Use of Experimental Animals at Xiamen University, and all experiments followed the institution's instructions for animal care and use. Male Sprague-Dawley (SD) rats (120-160 g) were provided by the Experimental Animal Center of Xiamen University and used to isolate aortic smooth muscle cells. Dulbecco's modified Eagle's medium (DMEM), trypsin and fetal bovine serum (FBS) were purchased from Gibco Co. (Carlsbad, CA, USA). Diphenyleneiodonium chloride (DPI), N-acetyl-L-cysteine (NAC), 2', 7'-dichlorofluorescindiacetate (DCFH-DA) and crystal violet were purchased from Sigma (St. Louis, MO, USA). The Cell Counting Kit-8 (CCK-8) was purchased from Enzo Biochem, Inc. (Farmingdale, NY, USA). The Rac1/Cdc42 Activation Assay Kit and Transwell chamber with 8-μm pore polycarbonate membranes were obtained from Millipore Co. (Billerica, MA, USA). The NADP/NADPH Assay Kit was obtained from Abcam Co. (Cambridge, UK). The antibody to total Rac1 was purchased from SAB Biotech (College Park, MD, USA), and the antibody to β-actin was obtained from Beyotime (Haimen, China). All other chemicals and reagents were obtained from commercial sources and of analytical grade.

Cell Culture

Vascular smooth muscle cells (VSMCs) were isolated from the thoracic aortas of male SD rats as previously described[6]. The VSMCs were grown in DMEM medium supplemented with 10% FBS and antibiotics

(100 units/mL of penicillin, 100 μg/mL of streptomycin) at 37℃ and maintained in an atmosphere of 5% CO_2 and 95% air. Cells at passages 4-6 were used for further experiments. Generally, the cells were starved in serum-free DMEM for 24 hours before incubation with HDL or ox-HDL. The VSMCs were identified using immunofluorescence with a specific monoclonal anti-α-actin antibody.

HDL Isolation from Human Plasma

Fresh plasma was provided from healthy volunteers after overnight fasting. HDL (1.063-1.210 g/mL) was isolated via ultracentrifugation as previously described[31]. Briefly, the plasma density was adjusted to 1.3 g/mL with KBr, and saline (1.006 g/mL) was layered over the adjusted plasma to form a discontinuous NaCl/KBr density gradient. The sample with the gradient was centrifuged at 350,000 g for 3.5 hours at 4℃. HDL was collected, dialyzed with PBS, sterilized through a 0.22-μm filter and stored in the dark at 4℃ for use. The purity of HDL was evaluated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantified by measuring the apoA-I content using nephelometry.

HDL Oxidation

Ox-HDL was prepared by exposing the HDL to 5 μM of $CuSO_4$ for 24 hours at 37℃ *in vitro*[25]. The oxidation was terminated by adding ethylene diamine tetraacetic acid (EDTA) to a final concentration of 1 mg/mL, followed by dialyzing against PBS for 48 hours to remove the EDTA. The solution was then sterilized by passing it through a 0.22-μm filter. The extent of HDL oxidation was determined according to the level of thiobarbituric acid-reactive substances (TBARS), and the protein concentration was measured using the Bradford assay[22].

Cell Proliferation Assay

A CCK-8 cell viability assay was used to evaluate cell proliferation, following the manufacturer's instructions. Briefly, VSMCs were seeded at 3×10^3 cells/well in 96-well plates. After the cells reached 70-80% confluence, they were incubated with serum-free DMEM media for an additional 24 hours. The VSMCs were then treated with native HDL (N-HDL) or ox-HDL at varying concentrations for 24 hours to stimulate cell proliferation. Subsequently, 10 μL of CCK-8 reagent was added to each well, and the cells were further incubated for two hours at 37℃. The absorbance was read at 450 nm using a spectrophotometric plate reader.

Transwell Migration Assay

The degree of cell migration was determined using a 24-well Transwell plate containing polycarbonate 8-μm pore membrane filters. Serum-starved VSMCs mixed with N-HDL or ox-HDL at different concentrations were seeded in the upper wells (5×10^4 cells per 200 μL of serum-free DMEM with N-HDL or ox-HDL), while the lower wells were filled with 800 μL of DMEM containing 10% FBS. The cells were allowed to migrate across the porous filters for six hours at 37℃. After fixing with 4% paraformaldehyde in PBS and staining with crystal violet, the non-migrating cells on the upper surface of the filter were scraped, and the number of cells that had migrated to the lower side of the filter was counted in three random 100× fields per well using light microscopy (Nikon, Tokyo, Japan).

Scratch-Wound Assay

VSMCs were grown to 80% confluence in 6-well plates. The confluent monolayer was scratched gently with 200-μL pipette tips. The cells were rinsed twice with PBS buffer to remove cellular debris, and the linear wound was recorded. The VSMCs were then incubated with various doses of N-HDL or ox-HDL for 48 hours in DMEM media with 0.1% FBS. Following incubation, cell images were obtained, and the number of cells that had migrated into the wound space was manually counted in three random 100× fields per well using light microscopy (Nikon).

Measurement of Intracellular ROS

The intracellular ROS levels were detected using the oxidant-sensitive probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA)[4]. VSMCs were grown on glass coverslips in 35-mm dishes and cultured in serum-free DMEM for 24 hours. Subsequently, the cells were washed twice with PBS and incubated with 5 μmol/L of DCFH-DA for 30 minutes. The cells were then incubated with 50 μg/mL of N-HDL or ox-HDL for 0, 5, 15, 30, 60, 120 and 240 minutes. Thereafter, the relative DCF fluorescence intensity was detected at the different time points mentioned above using fluorescent microscopy (Nikon). The examination wavelength was 488 nm and the emission wavelength was 530 nm for DCF. The fluorescence intensity of the stained cells was determined using Image-Pro Plus/IOD.

Rac1 Activation Assay

An equal amount of proteins (500 mg) was used to assess the GTP-bound Rac1 levels using the p21-activated protein kinase p21 binding domain immobi-

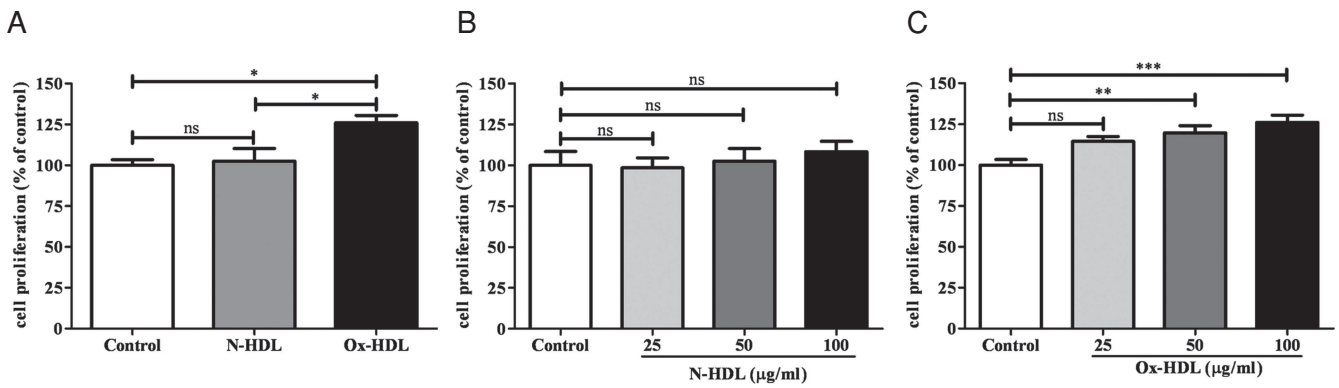

Fig. 1. Ox-HDL induces the proliferation of VSMCs.

A: VSMCs were incubated with PBS, N-HDL (50 μg/mL) or ox-HDL (50 μg/mL) for 24 hours, and the proliferation of the VSMCs was assayed using CCK-8 kits. B and C: VSMCs were incubated with PBS, 25, 50 and 100 μg/mL of N-HDL (B) or 25, 50 and 100 μg/mL of ox-HDL (C) for 24 hours, and the proliferation of the VSMCs was assayed using CCK-8 kits. The data are presented as the mean ± SEM of three separate experiments. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

lized onto glutathione-agarose beads following the manufacturer's instructions. The extent of Rac1 activation (GTP-Rac1) was expressed as the ratio of the density of the GTP-Rac1 band to that of the total amount of Rac1 in each sample.

Western Blot Analysis

Proteins from the Rac1 activation assay described above were separated via 12% SDS-PAGE and blotted onto a polyvinyldifluoride membrane. A mouse monoclonal antibody to GTP-Rac1 (1:1000), rabbit polyclonal antibody to total Rac1 (1:1000) and mouse monoclonal antibody to β-actin (1:1000) were used as the primary antibodies. The specific immunoreactive blots were detected with electrochemiluminescence and analyzed using the Quantity One 1-D Analysis Software program (Bio-Rad, Hercules, CA, USA).

Measurement of the NADPH Oxidase Activity

The activity of NADPH oxidase was measured using an $NADP^+$/NADPH assay protocol according to the manufacturer's instructions. Following the extraction of total $NADP^+$/NADPH from to 2×10^5 cells, the NADPH oxidase activity was expressed as the $NADP^+$/NADPH ratio measured by reading the absorbance at 450 nm on a microplate reader.

Statistical Analysis

Each experiment was repeated at least three times. The data are presented as the mean ± SEM. Differences were compared with two-tailed Student's t-test or a one-way analysis of variance (ANOVA) using the GraphPad Prism (5.0) software package. Values of $p<0.05$ were considered to be statistically significant ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

Results

Ox-HDL Induces the Proliferation of VSMCs

To investigate whether ox-HDL induces the proliferation of VSMCs, we incubated VSMCs with 50 μg/mL of native HDL (N-HDL) or ox-HDL for 24 hours and assayed the proliferation of VSMCs using the CCK-8 kits. As anticipated, ox-HDL significantly promoted the proliferation of VSMCs; however, N-HDL had no effect on VSMC proliferation (**Fig. 1A**). Even high doses of N-HDL had almost no proliferative effect on the VSMCs (**Fig. 1B**). To further identify whether the proliferative effects of ox-HDL were dose-dependent, we treated VSMCs with different doses of ox-HDL (25, 50 and 100 μg/mL) for 24 hours. We found that the proliferative function of ox-HDL was related to its concentration (**Fig. 1C**). Therefore, compared with N-HDL, ox-HDL has a much stronger effect on VSMC proliferation.

Ox-HDL Induces the Migration of VSMCs

To confirm whether ox-HDL also has an influence on the migration of VSMCs, we treated VSMCs with 50 μg/mL of N-HDL or ox-HDL for six hours and counted the number of VSMCs migrating through the Transwell membrane filters. Consistently, ox-HDL significantly facilitated the migration of VSMCs (**Fig. 2A** and **B**). However, 50 μg/mL of N-HDL did not promote VSMC migration, and instead slightly inhibited the migration of the VSMCs, although not significantly (**Fig. 2A** and **B**). Subsequently, we found

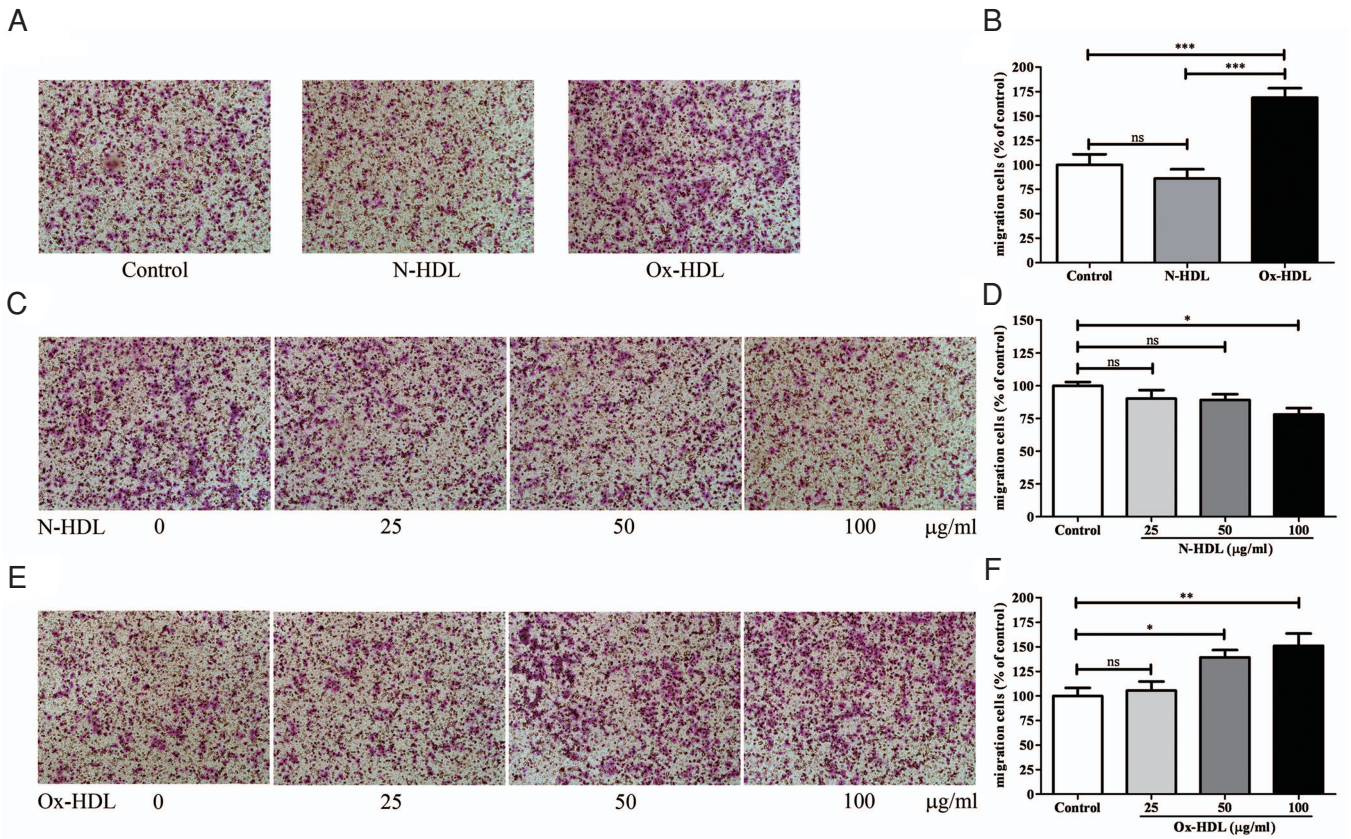

Fig. 2. Ox-HDL induces the migration of VSMCs.

A and B: VSMCs were incubated with PBS, N-HDL (50 μg/mL) or ox-HDL (50 μg/mL) for six hours, and the migration of the VSMCs was assayed using a Transwell membrane filter. C, D, E and F: VSMCs were incubated with PBS, 25, 50 and 100 μg/mL of N-HDL (C and D) or ox-HDL (E and F) for six hours, and the migration of the VSMCs was assayed using a Transwell membrane filter. The number of cells that had translocated to the lower layer was counted after staining with crystal violet (B, D and F). The data are presented as the mean ± SEM of three separate experiments. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

that 100 μg/mL of N-HDL inhibited the Transwell migration of the VSMCs, although low doses of N-HDL did not influence VSMC migration (**Fig. 2C** and **D**). However, the promigratory effects of ox-HDL were dose-dependent (**Fig. 2E** and **F**). Therefore, compared to N-HDL, ox-HDL significantly promotes the migration of VSMCs.

Ox-HDL Promotes Wound Healing in VSMCs

To further confirm the promigratory effects of ox-HDL, we evaluated the migration of VSMCs using a wound-healing assay and obtained results similar to those of the Transwell migration assay. We found that ox-HDL significantly promoted wound healing in the VSMCs (**Fig. 3A** and **B**); this effect was also dose-dependent (**Fig. 3E** and **F**). However, N-HDL had almost no effect on wound healing in the VSMCs (**Fig. 3A**), even at a dose of 100 μg/mL (**Fig. 3C** and **D**). Therefore, we concluded that ox-HDL promotes wound healing in VSMCs following scratch assays.

Ox-HDL Induces the Production of ROS in VSMCs

To investigate whether ROS were involved in the proliferation and migration of VSMCs induced by ox-HDL, we evaluated the production of ROS in VSMCs triggered by N-HDL and ox-HDL. We treated VSMCs with 50 μg/mL of N-HDL or ox-HDL for different time intervals and monitored the intracellular oxidation of DCFH-DA based on the fluorescence intensity. Our results showed that, compared with that observed in the control group, ox-HDL remarkably increased the production of intracellular ROS in the cultured VSMCs in a time-dependent manner, with the formation of ROS peaking at 15 minutes (**Fig. 4A** and **B**). However, N-HDL had no effect on ROS generation (**Fig. 4A** and **B**). These results led to the conclusion that ox-HDL is a strong inducer of ROS production in VSMCs.

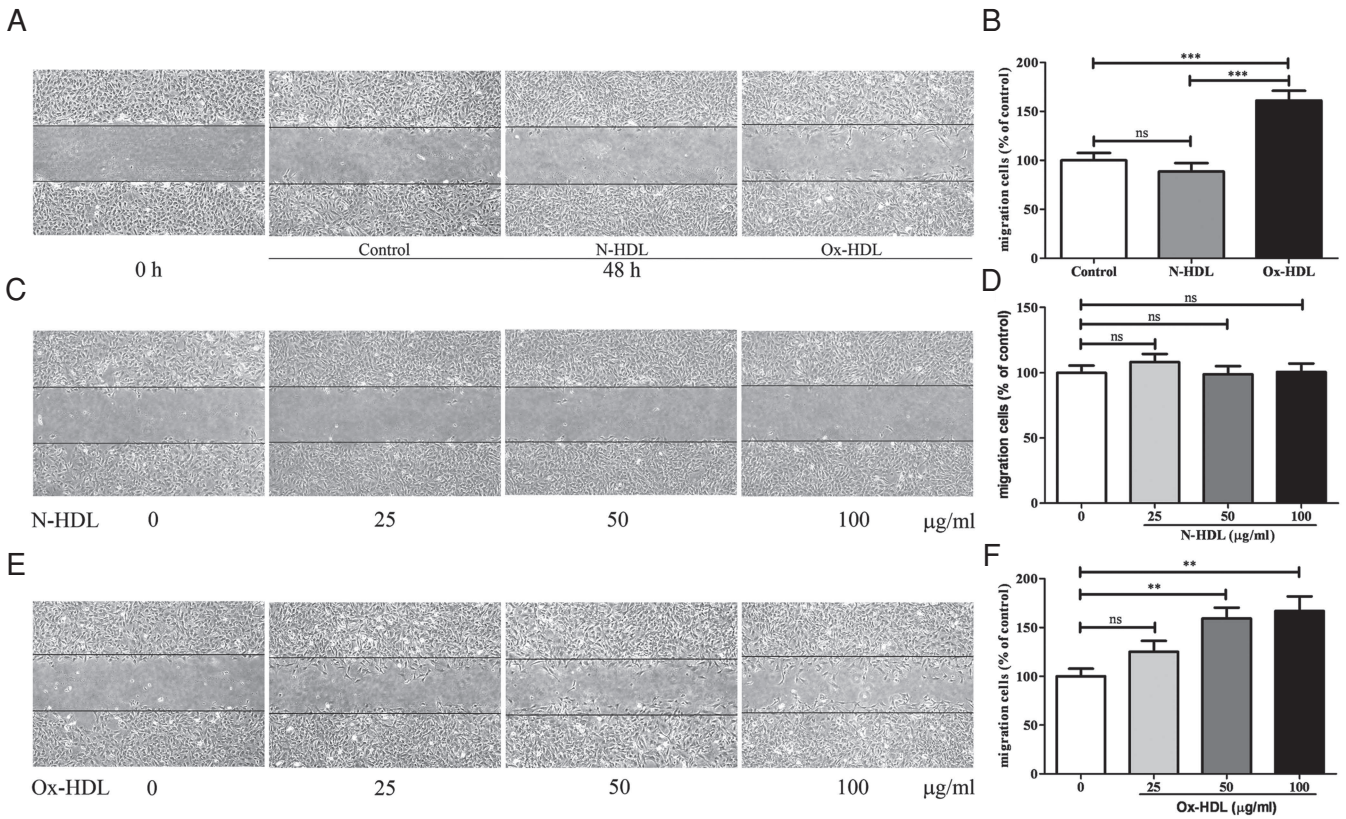

Fig. 3. Ox-HDL induces wound healing in VSMCs.

The confluent monolayer of the VSMCs was scratched and a linear wound was recorded. A and B: VSMCs were incubated with 50 μg/mL of N-HDL or ox-HDL for 48 hours. C, D, E and F: VSMCs were incubated with PBS, 25, 50 and 100 μg/mL of N-HDL (C and D) or 25, 50 and 100 μg/mL of ox-HDL (E and F) for 48 hours. Following incubation, cell images were obtained (×100), and the number of cells that had migrated into the wound space was manually counted in three fields per well using a light microscope (B, D and F). The data are presented as the mean ± SEM of three separate experiments. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

DPI Inhibits the Production of ROS and the Activation of NADPH Oxidase Induced by Ox-HDL in VSMCs

DPI, a specific inhibitor of NADPH oxidase, inhibits the activation of NADPH oxidase and the generation of intracellular ROS. To detect whether DPI inhibits NADPH oxidase activation and ROS production in VSMCs induced by ox-HDL, we preincubated VSMCs with 10 μmol/L of DPI for two hours, then treated the VSMCs with 50 μg/mL of ox-HDL for 15 minutes to assay the production of ROS and the activation of NADPH oxidase. The level of ROS production was assayed by monitoring the oxidation of DCFH-DA according to the fluorescence intensity. The NADPH oxidase activity was detected using a Rac1 pull-down assay and the intracellular $NADP^+$/NADPH ratio. Our results showed that DPI significantly inhibited the generation of ROS induced by ox-HDL in the VSMCs (**Fig. 5A** and **B**). Consistently, ox-HDL remarkably increased the activity of NADPH oxidase; however, DPI significantly inhibited the NADPH oxidase activity induced by ox-HDL in the VSMCs (**Fig. 5C**, **D** and **E**). These results led to the conclusion that the generation of ROS induced by ox-HDL in VSMCs is derived from the activation of NADPH oxidase triggered by ox-HDL and that DPI effectively inhibits the NADPH oxidase activity and ROS production induced by ox-HDL in VSMCs.

NAC Inhibits the Production of ROS Induced by Ox-HDL in VSMCs

NAC, as a ROS scavenger, can clear intracellular ROS. To detect whether NAC scavenges the intracellular ROS generated by ox-HDL, we preincubated VSMCs with 20 mmol/L of NAC for 30 minutes, then treated the VSMCs with 50 μg/mL of ox-HDL for 15 minutes. The level of intracellular ROS production was detected by monitoring the oxidation of

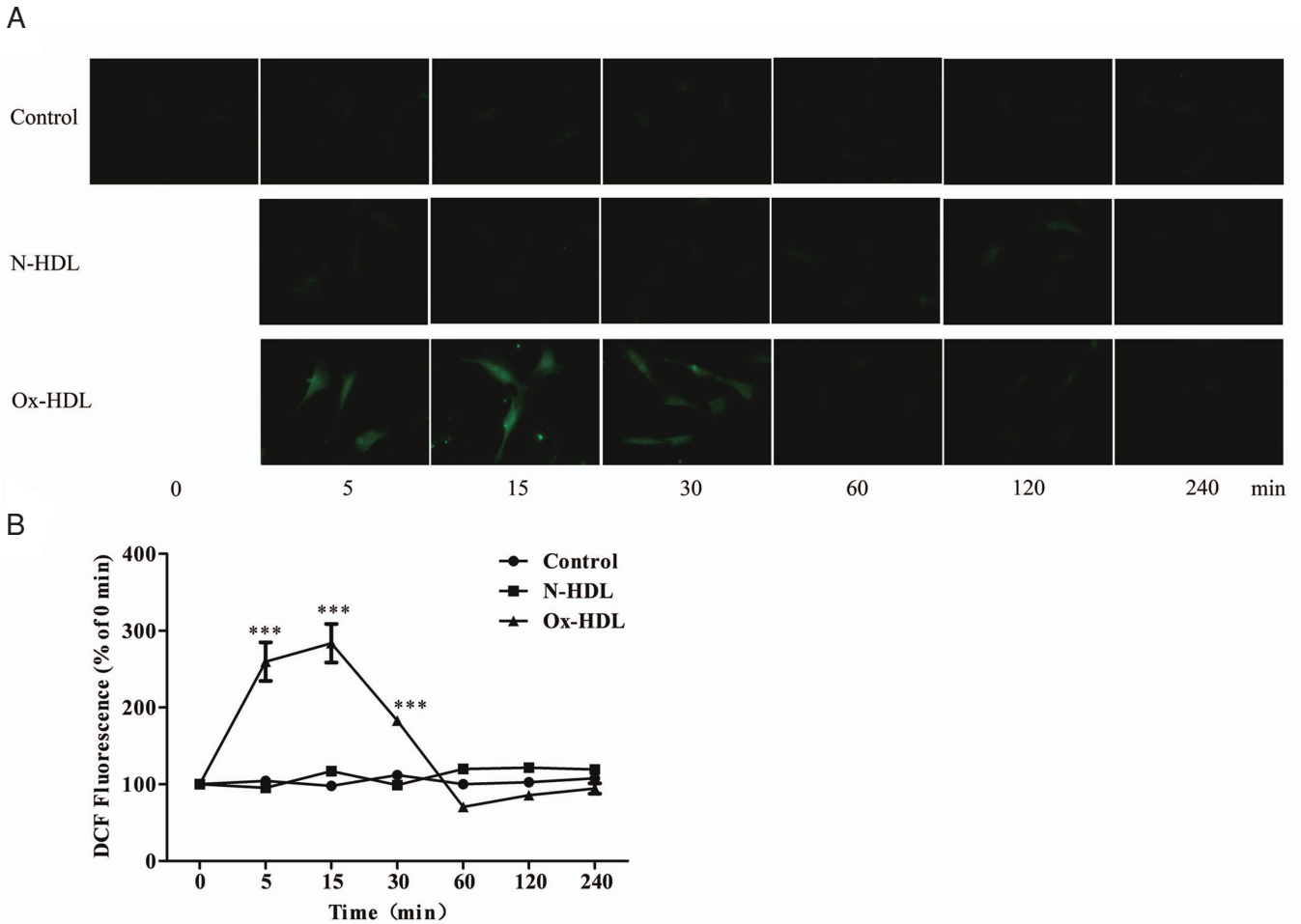

Fig. 4. Ox-HDL induces the generation of ROS in VSMCs.

VSMCs were incubated with 50 μg/mL of N-HDL or ox-HDL for 0, 5, 15, 30, 60, 120 and 240 minutes, and the intracellular ROS production was assayed by monitoring the intracellular oxidation of DCF according to the fluorescence intensity. A: Fluorescent images of cells were obtained at different time points using a fluorescence microscope (×400). B: The intensity of the DCF fluorescent images of the stained cells was quantified using Image-Pro Plus/IOD. The data are presented as the mean ± SEM of three separate experiments. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

DCFH-DA according to the fluorescence intensity, as described above. The results suggested that the increased ROS production induced by ox-HDL can be dramatically prevented by NAC, supporting the conclusion that the incubation of VSMCs with ox-HDL is associated with increased ROS formation (**Fig. 6A** and **B**). Furthermore, NAC hardly interfered with the basal ROS production in the VSMCs, even when mixed with N-HDL (**Fig. 6A** and **B**). Therefore, NAC effectively inhibits the production of ROS induced by ox-HDL in VSMCs.

NAC Inhibits the Proliferation of VSMCs Induced by Ox-HDL

To further investigate whether NAC inhibits the proliferation of VSMCs, we preincubated VSMCs with 20 mmol/L of NAC for 30 minutes, then incubated the VSMCs with 50 μg/mL of ox-HDL for 24 hours. Subsequently, the proliferation of VSMCs was assayed using the CCK-8 kits, as described above. We found that NAC significantly inhibited the proliferation of VSMCs induced by ox-HDL (**Fig. 7**). Meanwhile, NAC did not cause any changes in the proliferation of VSMCs treated with N-HDL (**Fig. 7**). Therefore, as a ROS scavenger, NAC effectively suppresses the proliferation of VSMCs triggered by ox-HDL.

NAC Inhibits the Migration of VSMCs Induced by Ox-HDL

To detect whether NAC also inhibits the migration of VSMCs induced by ox-HDL, we pretreated VSMCs with 20 mmol/L of NAC for 30 minutes, then

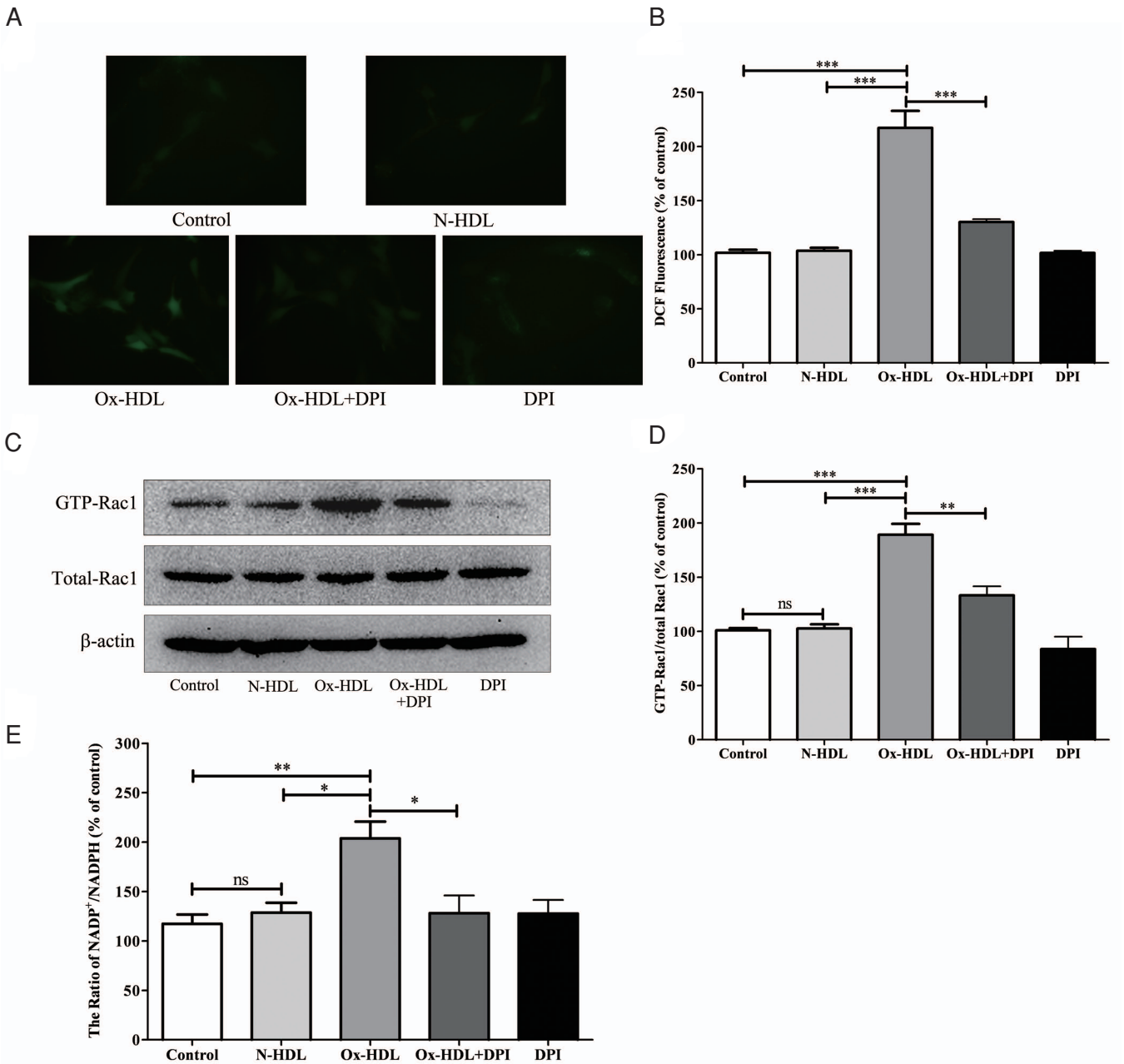

Fig. 5. DPI inhibits the production of ROS and activation of NADPH oxidase induced by ox-HDL in VSMCs.

VSMCs were preincubated with or without 10 μmol/L of DPI for two hours and subsequently treated with PBS, 50 μg/mL of N-HDL or 50 μg/mL of ox-HDL for 15 minutes. A: Fluorescent images of the cells were obtained using a fluorescence microscope (×400). B: The intensity of the DCF fluorescent images of the stained cells was quantified using Image-Pro Plus/IOD. C: GTP-Rac1 proteins were detected using the Rac1 pull-down assay and Western blotting. D: The extent of GTP-Rac1 was analyzed as the ratio of the density of the GTP-Rac1 band to that of the total amount of Rac1. E: The activation of NADPH oxidase was measured using $NADP^+$/NADPH ratio assay kits. The data are presented as the mean ± SEM of three separate experiments. $^*p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

applied the same procedures as those used in the Transwell migration and wound-healing assay experiments. Our results showed that, consistent with that observed for the inhibition of VSMC proliferation induced by ox-HDL, NAC significantly suppressed the migration of VSMCs triggered by ox-HDL, not only in the Transwell experiments (**Fig. 8A** and **B**), but also in the wound scratch assay (**Fig. 8C** and **D**). However, in both

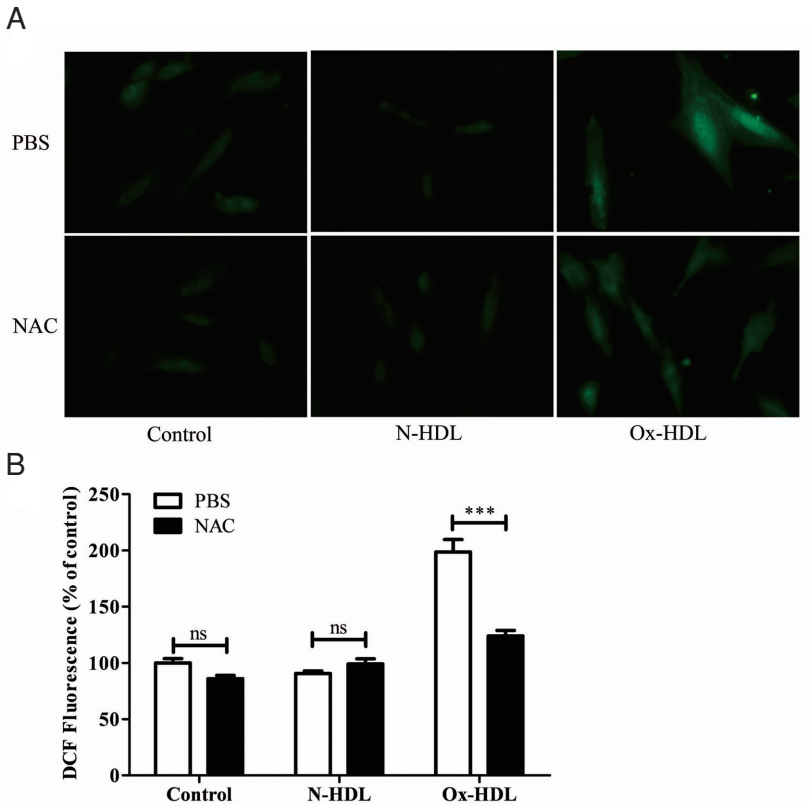

Fig. 6. NAC inhibits the production of ROS induced by ox-HDL in VSMCs.

VSMCs were preincubated with 20 mmol/L of NAC for 30 minutes and subsequently treated with 50 μg/mL of N-HDL or ox-HDL for 15 minutes. A: Fluorescent images of the cells were obtained using a fluorescence microscope (×400). B: The intensity of the DCF fluorescent images of the stained cells was quantified using Image-Pro Plus/IOD. The data are presented as the mean ± SEM of three separate experiments. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

the Transwell experiment (**Fig. 8A** and **B**) and the scratch assay (**Fig. 8C** and **D**), NAC had almost no effect on the VSMC migration induced by N-HDL. Therefore, NAC also effectively inhibits the migration of VSMCs induced by ox-HDL.

Discussion

Numerous studies have reported the presence of ox-HDL in atheromatous plaques as well as in plasma in animals and patients with CHD; however, the role of ox-HDL in the pathogenesis of atherosclerosis and restenosis following coronary angioplasty is not well established[20, 32]. In the present study, we demonstrated that ox-HDL, but not N-HDL, is a potent promoter of the proliferation and migration of VSMCs. The proproliferative and promigratory effects of ox-HDL were found to be positively correlated with intracellular NADPH oxidase activation and ROS production.

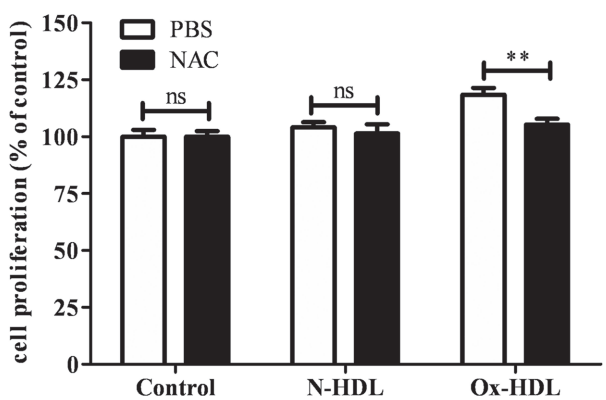

Fig. 7. NAC inhibits the proliferation of VSMCs induced by ox-HDL.

VSMCs were preincubated with 20 mmol/L of NAC for 30 minutes and subsequently incubated with 50 μg/mL of N-HDL or ox-HDL for 24 hours. The degree of proliferation of VSMCs was assayed using CCK-8 kits. The data are presented as the mean ± SEM of three separate experiments. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

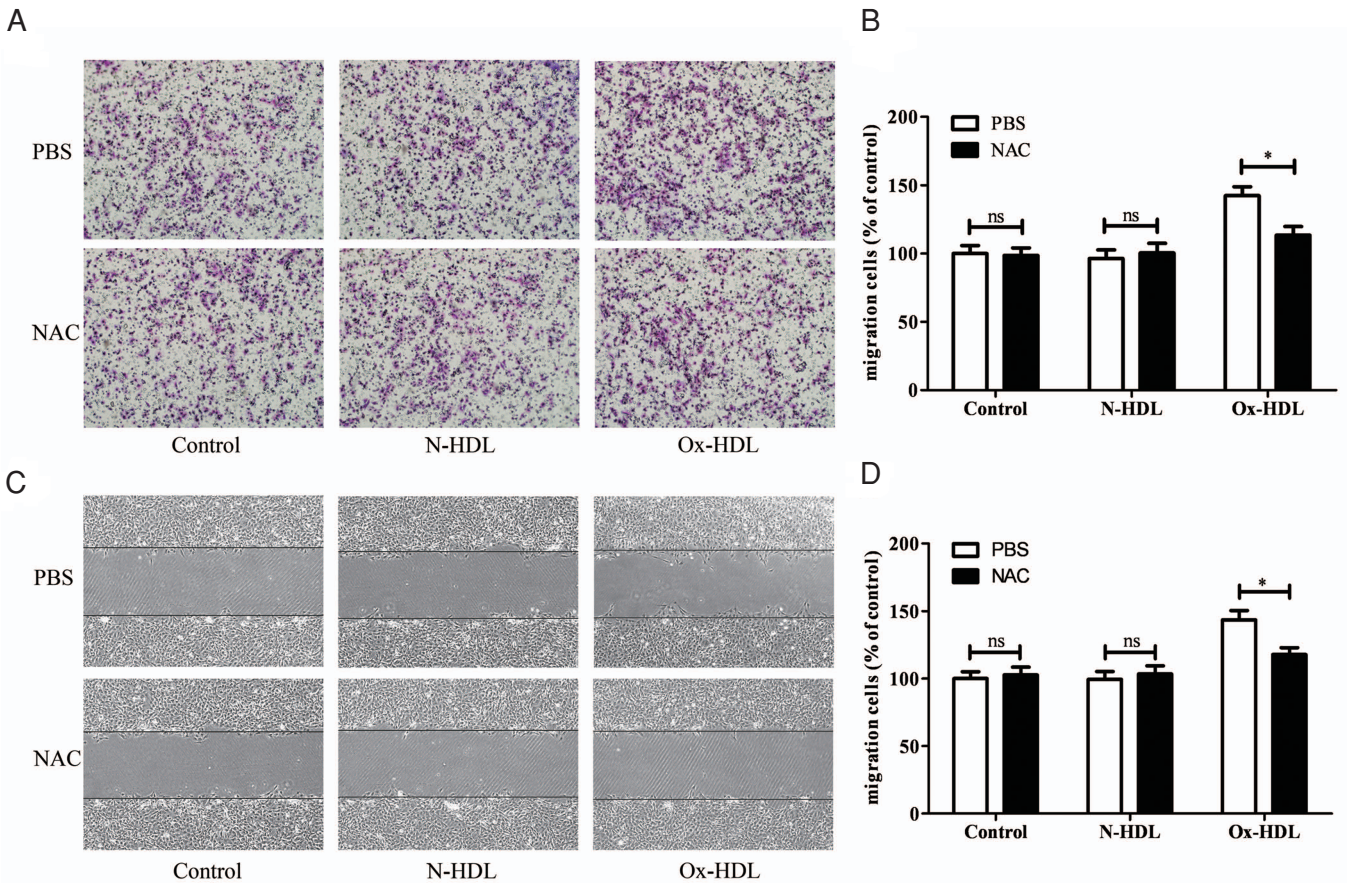

Fig. 8. NAC inhibits the migration of VSMCs induced by ox-HDL.

VSMCs were preincubated with 20 mmol/L of NAC for 30 minutes, then incubated with 50 μg/mL of N-HDL or ox-HDL for six hours for Transwell membrane migration (A and B) and for 48 hours for the scratch wound assay (C and D). The data are presented as the mean ± SEM of three separate experiments. $^{*}p<0.05$. $^{**}p<0.01$. $^{***}p<0.001$.

The synthetic phenotype of VSMC is capable of proliferating and migrating from the tunica media to the intima of the vessel, contributing to neointima genesis and extracellular matrix (ECM) deposition in the vessel wall and resulting in the narrowing of the vessel lumen after coronary angioplasty or stenting[3]. Although the proliferation and migration of VSMCs and presence of synthesized ECM proteins accelerate the repair of tissue and enhance the stabilization of atherosclerotic plaque, the activation of inflammatory and immune cells in the plaque may lead to intimal VSMC apoptosis, which makes the fibrous cap thinner and promotes plaque rupture[2, 3]. As a major atheroprotective element in plasma, HDL undergoes oxidative modification of its phospholipids and apolipoproteins during systemic inflammation and consequently loses its protective properties, even becoming a proinflammatory and proatherogenic factor[8, 16, 19]. In previous studies, we demonstrated that ox-HDL enhances the proliferation, migration and invasion of breast cancer cells, while hypochlorite-induced oxidative stress elevates the capability of HDL to promote breast cancer metastasis, which demonstrates that ox-HDL and oxidative stress play essential roles in the proliferation and migration of cells[28, 29]. In this study, our results showed that compared with N-HDL, ox-HDL significantly promoted the proliferation of VSMCs in a dose-dependent manner (**Fig. 1**). Accordingly, ox-HDL also facilitated the migration of VSMCs in the Transwell assay and wound-healing experiments in a dose-dependent manner (**Fig. 2** and **Fig. 3**). Taken together, these results indicate that ox-HDL promotes neointimal growth and aggravates narrowing of the vessel lumen in CHD patients. Tamama *et al.* proved that native HDL inhibits VSMC migration via the S1P2 receptor induced by PDGF[13]. In addition, increasing the plasma HDL content in endothelial lipase (EL) knockout mice more potently inhibits the

VSMC proliferation and migration induced by Ang-Ⅱ; however, decreasing the HDL concentration in EL transgenic mice enhances proliferation and migration in cultured VSMCs[14]. Our results are also consistent with the conclusion that N-HDL does not promote VSMC proliferation or migration (**Fig. 1**, **2** and **3**).

Several studies have clearly identified that intracellular ROS are involved in many pathophysiological processes in patients with cardiovascular disease, including the regulation of VSMC proliferation, migration, contraction, differentiation and apoptosis[33, 34]. Recent evidence suggests that many other proinflammatory factors aggravate the development of atherosclerosis and modulate the VSMC function through the production of ROS[35, 36]. Shimizu *et al*. and Wedgwood *et al*. showed that PDGF and ET-1 induce the generation of ROS and promote the proliferation and migration VSMCs[4, 5]. Similarly, Ang-Ⅱ and ox-LDL also promote the production of ROS in VSMCs, which enhances the growth and migration of VSMCs and aggravates the progression of atherosclerosis[6, 7]. In addition, intracellular ROS promote the activation of NF-kappa B, phosphorylation of Akt, ERK1/2 and p38 MAPK and proliferation and migration of VSMCs[4, 7, 37]. Meanwhile, Satoh *et al*. demonstrated that ROS promote the production of Cyclophilin A (CyPA), which stimulates VSMC proliferation and migration both *in vitro* and *in vivo*[38]. Therefore, the strong relationship between oxidant stress and vascular remodeling establishes a connection between ROS production and VSMC proliferation and migration. Matsunaga *et al*. reported that ox-HDL induces a significant dose-dependent increase in ROS production in human umbilical vein endothelial cells[24]. In the present study, we found that ox-HDL also promotes the generation of ROS in a time-dependent manner in VSMCs (**Fig. 4**). Robbesyn *et al*. indicated that HDL prevents the intracellular ROS increase triggered by ox-LDL in VSMCs[7]. Furthermore, Tolle *et al*. demonstrated that HDL inhibits NAD(P)H oxidase-dependent ROS generation in VSMCs via the S1P1 and S1P3 receptors[39]. Our study also found that native HDL does not induce ROS production in VSMCs (**Fig. 4**).

It is well recognized that CD36 is a receptor for ox-HDL[40]. Moreover, less ROS is produced in the vessel walls of CD36 knockout mice than in wild-type mice following chemically induced arterial injury[41]. These results suggest that CD36, as a receptor of ox-HDL, contributes to ROS generation in VSMCs. NADPH oxidase is the major source of intracellular ROS, and inhibiting the expression of NADPH oxidase suppresses the production of ROS in VSMCs[42]. We also found that DPI, a specific inhibitor of NADPH oxidase, significantly inhibits the activation of NADPH oxidase and suppresses the generation of ROS induced by ox-HDL in VSMCs (**Fig. 5**). Furthermore, Mahadevan *et al*. demonstrated that an enhanced intrinsic antioxidant capacity may promote VSMCs to resist migration against ox-LDL and Ang-Ⅱ[43]. N-acetylcysteine (NAC) is an effective ROS scavenger that inhibits PDGF- and ox-LDL-stimulated ROS production, ERK1/2 and p38 MAPK phosphorylation and NF-kappa B activation, which subsequently blocks the proliferative and hypertrophic pathways in VSMCs[4, 7]. In the present study, we found that NAC, as an antioxidant, also decreases the generation of intracellular ROS induced by ox-HDL (**Fig. 6**). Meanwhile, NAC almost completely blocks the proliferation and migration of VSMCs triggered by ox-HDL (**Fig. 7** and **8**).

In summary, HDL may be viewed as a shuttle that can be either anti-inflammatory or proinflammatory, depending on its quality[16]. In research aiming to retard the development of atherosclerotic diseases, dysfunctional HDL has become a key diagnostic and therapeutic target in patients with cardiovascular disease[18]. The major finding of this study is that ox-HDL facilitates the activation of NADPH oxidase and the generation of ROS in VSMCs, thereby promoting the proliferation and migration of VSMCs, which aggravates narrowing of the vessels following coronary angioplasty and stent placement. Consequently, we speculate that it may be possible to employ effective antioxidants to inhibit HDL oxidation and improve the quality of HDL in patients with atherosclerosis or systemic inflammation, which may constitute a novel therapeutic strategy for controlling cardiovascular diseases in the near future.

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Disclosures

None.

Abbreviations

HDL; high-density lipoprotein, N-HDL; native high-density lipoprotein, ox-HDL; oxidized high-density lipoprotein, ROS; reactive oxygen species, VSMCs; vascular smooth muscle cells, CHD; coronary heart disease.

References

1) Ross R: Atherosclerosis--an inflammatory disease. N Engl J Med, 1999; 340: 115-126
2) Lowe HC, Oesterle SN, Khachigian LM: Coronary instent restenosis: current status and future strategies. J Am Coll Cardiol, 2002; 39: 183-193
3) Rudijanto A: The role of vascular smooth muscle cells on the pathogenesis of atherosclerosis. Acta Med Indones, 2007; 39: 86-93
4) Shimizu H, Hirose Y, Nishijima F, Tsubakihara Y, Miyazaki H: ROS and PDFG-bera receptors are critically involved in indoxyl sulfate actions that promote vascular smooth muscle cell proliferation and migration. Am J Physiol Cell Physiol, 2009; 297: C389-396
5) Wedgwood S, Dettman RW, Black SM: ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. Am J Physiol Lung Cell Mol Physiol, 2001; 281: L1058-1067
6) Yaghini FA, Song CY, Lavrentyev EN, Ghafoor HUB, Fang XR, Estes AM, Campbell WB, Malik KU: Angiotensin II-induced Vascular Smooth Muscle Cell Migration and Growth Are Mediated by Cytochrome P450 1B1-dependent Superoxide Generation. Hypertension, 2010; 55: 1461-1467
7) Robbesyn F, Garcia V, Auge N, Vieira O, Frisach M-F, Salvayre R, Negre-Salvayre A: HDL counterbalance the proinflammatory effect of oxidized LDL by inhibiting intracellular reactive oxygen species rise, proteasome activation, and subsequent NF-kappaB activation in smooth muscle cells. FASEB J, 2003; 17: 743-745
8) Gotto AMJ, Brinton EA: Assessing low levels of high-density lipoprotein cholesterol as a risk factor in coronary heart disease: a working group report and update. J Am Coll Cardiol, 2004; 43: 717-724
9) Assmann G, Nofer JR: Atheroprotective effects of high-density lipoproteins. Annu Rev Med, 2003; 54: 321-341
10) Brewer HB: High-Density Lipoproteins: A New Potential Therapeutic Target for the Prevention of Cardiovascular Disease. Arterioscler Thromb Vasc Biol, 2004; 24: 387-391
11) Calabresi L, Gomaraschi M, Franceschini G: Endothelial protection by high-density lipoproteins: from bench to bedside. Arterioscler Thromb Vasc Biol, 2003; 23: 1724-1731
12) Mineo C, Shaul PW: Novel Biological Functions of High-Density Lipoprotein Cholesterol, Circ Res. 2012; 111: 1079-1090
13) Tamama K, Tomura H, Sato K, Malchinkhuu E, Damirin A, Kimura T, Kuwabara A, Murakami M, Okajima F: High-density lipoprotein inhibits migration of vascular smooth muscle cells through its sphingosine 1-phosphate component. Atherosclerosis, 2005; 178: 19-23
14) Sun L, Ishida T, Okada T, Yasuda T, Hara T, Toh R, Shinohara M, Yamashita T, Rikitake Y, Hirata K-i: Expression of Endothelial Lipase Correlates with the Size of Neointima in a Murine Model of Vascular Remodeling. J Atheroscler Thromb, 2012; 19: 1110-1127
15) Ansell BJ, Fonarow GC, Fogelman AM: High-density lipoprotein: is it always atheroprotective? Curr Atheroscler Rep, 2006; 8: 405-411
16) Ansell BJ, Fonarow GC, Fogelman AM: The paradox of dysfunctional high-density lipoprotein. Curr Opin Lipidol, 2007; 18: 427-434
17) Ansell BJ, Navab M, Hama S, Kamranpour N, Fonarow G, Hough G, Rahmani S, Mottahedeh R, Dave R, Reddy ST, Fogelman AM: Inflammatory/Antiinflammatory Properties of High-Density Lipoprotein Distinguish Patients From Control Subjects Better Than High-Density Lipoprotein Cholesterol Levels and Are Favorably Affected by Simvastatin Treatment. Circulation, 2003; 108: 2751-2756
18) Otocka-Kmiecik A, Mikhailidis DP, Nicholls SJ, Davidson M, Rysz J, Banach M: Dysfunctional HDL: a novel important diagnostic and therapeutic target in cardiovascular disease? Prog Lipid Res, 2012; 51: 314-324
19) Kontush A, Chapman MJ: Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. Pharmacol Rev, 2006; 58: 342-374
20) Zheng L, Nukuna B, Brennan ML, Sun M, Goormastic M, Settle M, Schmitt D, Fu X, Thomson L, Fox PL, Ischiropoulos H, Smith JD, Kinter M, Hazen SL: Apolipoprotein A-I is a selective target for myeloperoxidase-catalyzed oxidation and functional impairment in subjects with cardiovascular disease. J Clin Invest, 2004; 114: 529-541
21) Undurti A, Huang Y, Lupica JA, Smith JD, DiDonato JA, Hazen SL: Modification of high density lipoprotein by myeloperoxidase generates a pro-inflammatory particle. J Biol Chem, 2009; 284: 30825-30835
22) Nagano Y, Arai H, Kita T: High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. Proc Natl Acad Sci U S A, 1991; 88: 6457-6461
23) Rifici VA, Khachadurian AK: Oxidation of high density lipoproteins: characterization and effects on cholesterol efflux from J774 macrophages. Biochim Biophys Acta, 1996; 1299: 87-94
24) Matsunaga T, Hokari S, Koyama I, Harada T, Komoda T: NF-kappa B activation in endothelial cells treated with oxidized high-density lipoprotein. Biochem Biophys Res Commun, 2003; 303: 313-319
25) Soumyarani VS, Jayakumari N: Oxidatively modified high density lipoprotein promotes inflammatory response in human monocytes-macrophages by enhanced production of ROS, TNF-alpha, MMP-9, and MMP-2. Mol Cell Biochem, 2012; 366: 277-285
26) Callegari E, Norata GD, Inoue H, Catapano AL: Oxidized-HDL3 modulates the expression of Cox-2 in human endothelial cells. Int J Mol Med, 2006; 18: 209-213
27) Norata GD, Banfi C, Pirillo A, Tremoli E, Hamsten A, Catapano AL, Eriksson P: Oxidised-HDL3 induces the expression of PAI-1 in human endothelial cells. Role of p38MAPK activation and mRNA stabilization. Br J Haematol, 2004; 127: 97-104
28) Pan B, Ren H, Ma Y, Liu D, Yu B, Ji L, Pan L, Li J, Yang L, Lv X, Shen X, Chen B, Zhang Y, Willard B, He Y, Zheng L: High-density lipoprotein of patients with type 2 diabetes mellitus elevates the capability of promoting

migration and invasion of breast cancer cells. Int J Cancer, 2012; 131: 70-82
29) Pan B, Ren H, Lv X, Zhao Y, Yu B, He Y, Ma Y, Niu C, Kong J, Yu F, Sun W-b, Zhang Y, Willard B, Zheng L: Hypochlorite-induced oxidative stress elevates the capability of HDL in promoting breast cancer metastasis. J Transl Med, 2012; 10: 1-13
30) Pan B, Yu B, Ren H, Willard B, Pan L, Zu L, Shen X, Ma Y, Li X, Niu C, Kong J, Kang S, Chen E, Pennathur S, Zheng L: High-density lipoprotein nitration and chlorination catalyzed by myeloperoxidase impair its effect of promoting endothelial repair. Free Radic Biol Med, 2013; 60: 272-281. doi: 210.1016/j.freeradbiomed.2013.1002.1004
31) Liu D, Ji L, Tong X, Pan B, Han J-Y, Huang Y, Chen YE, Pennathur S, Zhang Y, Zheng L. Human apolipoprotein A-I induces cyclooxygenase-2 expression and prostaglandin I-2 release in endothelial cells through ATP-binding cassette transporter A1. Am J Physiol Cell Physiol. 2011; 301: C739-748
32) Nakajima T, Origuchi N, Matsunaga T, Kawai S-i, Hokari S, Nakamura H, Inoue I, Katayama S, Nagata A, Komoda T: Localization of oxidized HDL in atheromatous plaques and oxidized HDL binding sites on human aortic endothelial cells. Ann Clin Biochem, 2000; 37: 179-186
33) Lyle AN, Griendling KK: Modulation of Vascular Smooth Muscle Signaling by Reactive Oxygen Species. Physiology, 2006; 21: 269-280
34) Clempus RE, Griendling KK: Reactive oxygen species signaling in vascular smooth muscle cells. Cardiovasc Res, 2006; 71: 216-225
35) Niu X-L, Madamanchi NR, Vendrov AE, Tchivilev I, Rojas M, Madamanchi C, Brandes RP, Krause K-H, Humphries J, Smith A, Burnand KG, Runge MS: Nox Activator 1: a potential target for modulation of vascular reactive oxygen species in atherosclerotic arteries. Circulation, 2010; 121: 549-559
36) Wang J, Shi N, Chen S-Y: Manganese superoxide dismutase inhibits neointima formation through attenuation of migration and proliferation of vascular smooth muscle cells. Free Radic Biol Med, 2012; 52: 173-181
37) Nuria M, Nuria L, Verónica A, Daniel M, Antonio C, Julián N, Leocadio R-M, Carlos F S-F, Concepción P: Xanthine oxidase-derived extracellular superoxide anions stimulate activator protein 1 activity and hypertrophy in human vascular smooth muscle via c-Jun N-terminal kinase and p38 mitogen-activated protein kinases. J Hypertens, 2007; 25: 609-618
38) Satoh K, Nigro P, Berk BC: Oxidative stress and vascular smooth muscle cell growth: a mechanistic linkage by cyclophilin A. Antioxid Redox Signal, 2010; 12: 675-682
39) Tolle M, Pawlak A, Schuchardt M, Kawamura A, Tietge UJ, Lorkowski S, Keul P, Assmann G, Chun J, Levkau B, van der Giet M, Nofer J-R: HDL-Associated Lysosphingolipids Inhibit NAD(P)H Oxidase-Dependent Monocyte Chemoattractant Protein-1 Production. Arterioscler Thromb Vasc Biol, 2008; 28: 1542-1548
40) Thorne RF, Mhaidat NM, Ralston KJ, Burns GF: CD36 is a receptor for oxidized high density lipoprotein: implications for the development of atherosclerosis. FEBS Lett, 2007; 581: 1227-1232
41) Li W, Febbraio M, Reddy SP, Yu D-Y, Yamamoto M, Silverstein RL: CD36 participates in a signaling pathway that regulates ROS formation in murine VSMCs. J Clin Invest, 2010; 120: 3996-4006
42) Nagata D, Takeda R, Sata M, Satonaka H, Suzuki E, Nagano T, Hirata Y: AMP-activated protein kinase inhibits angiotensin Ⅱ-stimulated vascular smooth muscle cell proliferation. Circulation, 2004; 110: 444-451
43) Mahadevan VS, Campbell M, McKeown PP, Bayraktutan U: Internal mammary artery smooth muscle cells resist migration and possess high antioxidant capacity. Cardiovasc Res, 2006; 72: 60-68