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...
into the intima, resulting in the development of neo-
intimal hyperplasia, which is implicated in coronary
stenosis after angioplasty in patients with coronary
heart disease (CHD)\(^2\)\(^5\)\(^9\). 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-den-
sity 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 sup-
pressing VSMC proliferation and migration\(^11\)\(^12\). HDL
inhibits VSMC migration via the sphingosine-
1-phosphate (SIP)-2 receptor induced by PDGF\(^13\). Sun
et al. also showed that endothelial lipase (EL)
inactivation increases the amount of plasma HDL par-
ticles 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 condi-
tions such as atherosclerosis, diabetes mellitus and me-
tabolic syndrome\(^15\)\(^17\). Dysfunctional HDL has become
a key diagnostic and therapeutic target in cardiovascu-
lar disease\(^18\). The dysfunctional properties of HDL in
systemic inflammation are associated with specific
chemical modifications and structural changes, includ-
ing the oxidation of phospholipids and apolipoprote-
teins within HDL\(^19\)\(^21\). Several studies have shown that
oxidized HDL (ox-HDL) loses its ability to stimu-
late cholesterol efflux from foam cells\(^22\)\(^23\). In addi-
tion, ox-HDL can induce reactive oxygen species
(ROS) production\(^24\) and upregulate the expression of
several proinflammatory and proatherogenic genes,
including tumor necrosis factor-alpha (TNF-\(\alpha\)), cyclo-
oxxygenase-2 (COX-2) and plasminogen activator
inhibitor-1 (PAI-1)\(^25\)\(^27\), which elevates the risk of car-
diovascular 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\(^28\). Fur-
thermore, hypochlorite-induced oxidative stress elev-
ates the ability of HDL to promote breast cancer
metastasis\(^29\). Meanwhile, oxidized HDL is dysfunc-
tional in promoting endothelial repair both \textit{in vitro}
and in the reendothelialization of injured carotid arter-
ies\(^30\). 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 pro-
duction 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 inter-
vention (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). Diphenylenei-
donium chloride (DPI), N-acetyl-L-cysteine (NAC),
2’, 7’-dichlorofluorescin diacetate (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-\(\mu\)m pore polycarbonate
membranes were obtained from Millipore Co. (Biller-
ica, MA, USA). The NADP/NADPH Assay Kit was
obtained from Abcam Co. (Cambridge, UK). The anti-
body to total Rac1 was purchased from SAB Biotech
(College Park, MD, USA), and the antibody to \(\beta\)-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 iso-
lated from the thoracic aortas of male SD rats as previ-
ously described\(^30\). The VSMCs were grown in DMEM
medium supplemented with 10\% FBS and antibiotics
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 × 10⁴ 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°C. 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 50 μmol/L 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-
The Promotion Effects of Ox-HDL on the Proliferation and Migration of VSMCs

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 the 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...
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.
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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 scavengets 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 DCFH-DA according to the fluorescence intensity. Our results showed that NAC significantly inhibited the generation of ROS induced by ox-HDL in the VSMCs (Fig. 5F).
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 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 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 incubated...
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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 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

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**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.
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. 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. 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.

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.
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. 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. 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.

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. 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. 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. 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. Recent evidence suggests that many other proinflammatory factors aggravate the development of atherosclerosis and modulate the VSMC function through the production of ROS. 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. 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. 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. 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. 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. 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. Furthermore, Tolle et al. demonstrated that HDL inhibits NAD(P)H oxidase-dependent ROS generation in VSMCs via the S1P1 and S1P3 receptors. 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. Moreover, less ROS is produced in the vessel walls of CD36 knockout mice than in wild-type mice following chemically induced arterial injury. 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.

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. 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. 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. 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. 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.
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