Biochemical and Biophysical Research Communications 407 (2011) 44–48

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Oxidized LDL at low concentration promotes *in-vitro* angiogenesis and activates nitric oxide synthase through PI3K/Akt/eNOS pathway in human coronary artery endothelial cells

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ARTICLE INFO

Article history:
Received 7 February 2011
Available online 23 February 2011

Keywords:
oxLDL
Coronary artery endothelial cell
Akt
eNOS
NO

ABSTRACT

It has long been considered that oxidized low-density lipoprotein (oxLDL) causes endothelial dysfunction and is remarkably related to the development of atherosclerosis. However, the effect of oxLDL at very low concentration (<10 μg/ml) on the endothelial cells remains speculative. Nitric oxide (NO) has a crucial role in the endothelial cell function. In this study, we investigated the effect of oxLDL at low concentration on NO production and proliferation, migration, tube formation of the human coronary artery endothelial cells (HCAEC). Results showed that oxLDL at 5 μg/ml enhanced HCAEC proliferation, migration and tube formation. These phenomena were accompanied by an increased intracellular NO production. L-NAME (a NOS inhibitor), LY294002 and wortmannin (PI3K inhibitors) could abolish oxLDL-induced angiogenic effects and prevent NO production in the HCAEC. The phosphorylation of Akt, PI3K and eNOS were up-regulated by oxLDL, which was attenuated by LY294002. Our results suggested that oxLDL at low concentration could promote *in-vitro* angiogenesis and activate nitric oxide synthesis through PI3K/Akt/eNOS pathway in HCAEC.

1. Introduction

Oxidized low-density lipoprotein (oxLDL) has long been implicated in the pathogenesis of atherosclerosis and as a risk marker for human cardiovascular disease [1–4]. Low-density lipoprotein (LDL) is oxidized at the vessel wall under oxidative stress, and the resulting oxLDL can cause endothelial dysfunction, which is considered as an early yet critical step in atherogenesis [5,6]. Endothelial cells exposed to oxLDL develop many signs of dysfunction or injury, such as impaired nitric oxide (NO) release [7], disrupted endothelial barrier [8], decreased migration [9], induction of apoptosis [10], and increased vessel stiffness accompanied by morphological changes [11].

Reports that demonstrate oxLDL impairing endothelial cell function usually employ a concentration of oxLDL ranging from 50 to 500 μg/ml [12–15]. However, these concentrations are relatively high. The effect of oxLDL at low concentration (<10 μg/ml) remains elusive but may be critical to the functional alteration of the endothelial cells in response to the early exposure of oxLDL. It has been reported that low concentration of oxLDL promotes endothelial cell capillary tube formation and proliferation by activation of NADPH oxidase-MAPKs-NF-kappaB pathway and regulation of $p27^{Kip1}$ [16,17].

NO release is an important mechanism for native endothelial cells to protect the vasculature against vascular diseases [18]. NO plays critical roles in endothelial cell proliferation, migration and tube formation [19,20]. NO is mainly produced by nitric oxide synthase (endothelial or type III, eNOS), and endothelial dysfunction is characterized by a loss of NO bioavailability. However, the effect of low-concentration oxLDL on the NO production is unknown. In this study, we identified the effects of low-concentration oxLDL on angiogenesis characterized by tube formation, cell proliferation and migration in cultured human coronary artery endothelial cells. In addition, we provide experimental evidence showing that the angiogenic effect is mediated through Akt/PI3K/eNOS pathway.

2. Materials and methods

2.1. oxLDL preparation

Human LDL was isolated from pooled human plasma from healthy volunteers by sequential ultracentrifugation method and then oxidized by 10 μM $CuSO_4$ for 16 h at 37 °C [21]. The oxidation reaction was terminated by the addition of 1 mM EDTA and cooling

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doi:10.1016/j.bbrc.2011.02.096

at 4 °C. OxLDL preparations were dialyzed extensively against PBS containing 0.5 mM EDTA. Protein concentration was measured by the Lowry method and adjusted to 2.5 mg/ml. Lipid peroxide content of native LDL (nLDL) and oxLDL was measured by thiobarbituric acid reactive substances (TBARS) assay. OxLDL was aliqouted and stored at −80 °C in the dark and used within 2 weeks, avoiding repeated freeze-and-defrost cycles. All procedures were performed under endotoxin-free conditions.

2.2. Cell culture

Human coronary artery endothelial cells (HCAEC) were obtained from Lonza and maintained at 37 °C under 5% CO_2 in EGM-2 MV (Lonza, EBM-2 basal medium supplemented with 5% FBS and endothelial growth supplements). Cells at passages 4–6 were used in this study. HCAEC were grown until confluence and thereafter they were plated for assays.

2.3. Tube formation assay

Tube formation was performed by *in-vitro* matrigel assay. Briefly, 50 μl of matrigel (BD Biosciences) was added to each well of 96-well plates and allowed to solidify for 30 min at 37 °C. HCAEC were starved in basal medium (without endothelial growth supplements), seeded (2×10^4 cells) on the matrigel per well and treated with various concentrations of oxLDL or nLDL, with or without N^G-nitro-L-arginine methyl ester (L-NAME, Sigma), LY294002 (Calbiochem) and wortmannin (Sigma). After incubating for 16 h, the effect of oxLDL on tubular morphogenesis was documented microscopically and photographed. The tube length per field was analyzed by Image-Pro software (Media Cybernetics, MD, USA).

2.4. Cell proliferation assay

Cells were seeded at 3×10^3 cells per well onto 96-well plates. After 16 h culture in the full-supplemented growth medium, the medium was changed to basal medium for 8 h. Cells were treated with oxLDL (0.1–40 μg/ml) for 3 days. Viable cells were determined by incubating with MTT in the culture medium for 3 h, followed by A_{490} measurement in a microplate reader. Native LDL (5 μg/ml) was added as a control. L-NAME, LY294002 and wortmannin, when used, were added 30 min prior to the addition of oxLDL.

2.5. Endothelial cell migration assay

Cell migration was assayed by a Transwell Boyden Chamber (Costar, polycarbonate filters with 8.0-mm pore size). The lower surface of the membrane was pre-coated with fibronectin (1 mg/ml, Sigma). HCAEC were starved in EBM-2 medium (supplemented with 0.5% BSA without FBS and endothelial growth supplements) for 16 h and pre-treated with various concentrations of oxLDL for 8 h. Cells were seeded onto the upper chamber at 5×10^4 cells and incubated at 37 °C. OxLDL were added in both upper and lower chambers. After 8 h, chambers were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet in 20% methanol for 30 min. Cells on the upper surface were removed with a cotton swap and cells migrated to the lower surface were photographed under the microscope. The number of migrated cells per field was counted. Data were presented as an average from three independent experiments.

2.6. Confocal microscopy on real-time NO production in endothelial cells

HCAEC were seeded on 0.5% gelatin-coated coverslip. The cells were exposed to oxLDL (5 μg/ml) for 16 h and thereafter real-time NO productions were measured as described [22]. Cells were first washed with normal physiological saline solution containing (in mM) NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10, and HEPES 5, pH ~7.4 and loaded with the NO indicator, DAF-FM diacetate (1 μM, Invitrogen) at room temperature for 10 min. Cells were then imaged with the Fluoview FV1000 laser scanning confocal system (Olympus America Inc., Melville, NY) coupled to the IX81 Olympus microscope with excitation and emission wavelengths of 495 nm and 515 nm respectively. Initial fluorescence intensity before ACh (10 μM) addition (time = 0 min) were compared and determined as the basal NO level. Real-time NO production was measured for 20 min by stimulating the cells with ACh and data was calculated as fold changes with respect to the initial intensity at time = 0 (i.e. the basal level before ACh stimulation). L-NAME (1 mM), when used, was added 2 h prior to the addition of oxLDL.

2.7. Western blot analysis

HCAEC were seeded in 100 mm culture plates and allowed to grow to 80% confluence. After exposure to various concentrations of oxLDL in basal medium for 16 h, total cellular protein was

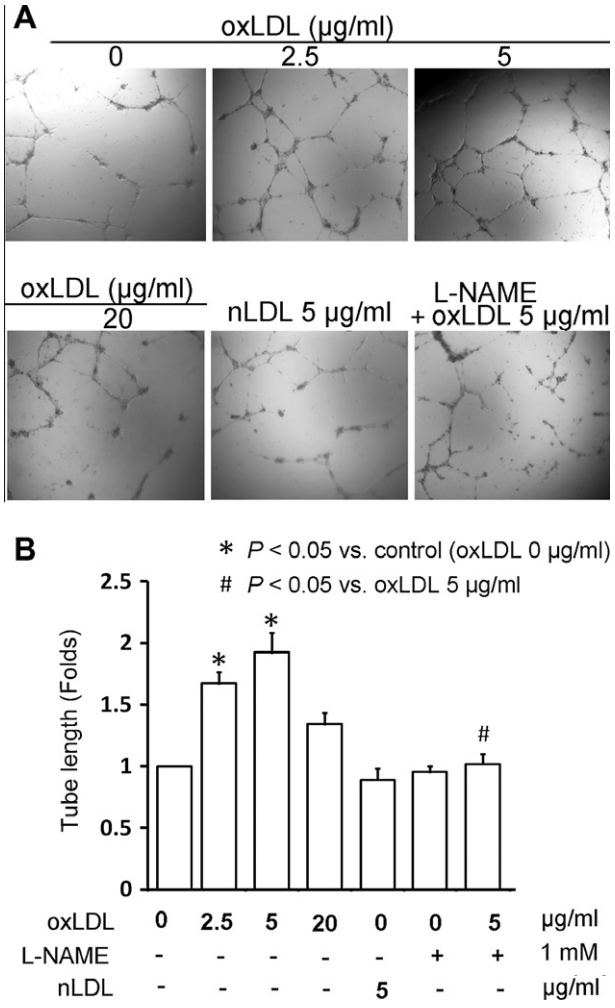

Fig. 1. Effects of low-concentration oxidized low-density lipoprotein (oxLDL) on tube formation capacity of cultured human coronary artery endothelial cells (HCAEC). (A) Representative light photomicrographs (40×) and (B) summarized data (Mean ± SEM) from three separate experiments. OxLDL (2.5, 5, and 20 μg/ml)-stimulated tube formation of HCAEC while native LDL (nLDL, 5 μg/ml) had no effect. OxLDL (5 μg/ml)-induced tube formation was suppressed in presence of L-NAME (1 mM).

extracted, separated by SDS–PAGE and transblotted onto PVDF membranes. Transblotted membranes were probed with primary antibodies followed by a colorimetric method for immunosignal detection. Primary antibodies used were as followed: phospho-Akt (Ser473), Akt1, phospho-phosphatidylinositol 3 kinase (PI3K) p55, PI3 kinase p85 (Cell Signaling); phospho-endothelial NO synthase (eNOS), eNOS (Abcam) and β-actin (Santa Cruz).

2.8. Statistical analysis

Results are mean ± SEM. Statistical analysis was performed by SPSS 13.0 (SPSS Inc., Illinois). The difference in mean values of various groups was analyzed by Scheffe's multiple comparisons in one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. OxLDL at low concentration enhances coronary artery endothelial cells tube formation, proliferation and migration

In-vitro matrigel assay showed that oxLDL (2.5 and 5 μg/ml) promoted HCAEC tube formation in the matrigel (Fig. 1). MTT assay showed that oxLDL (1, 2.5 and 5 μg/ml) enhanced cell proliferation (Fig. 2). Using the Boyden Chamber assay, oxLDL (2.5, 5, and 10 μg/ml) was shown to stimulate HCAEC migration (Fig. 3). The peak effects of oxLDL were found at 5 μg/ml. Native LDL (nLDL, 5 μg/ml) was used as the control, which was identified to exert no oxLDL-induced stimulatory effect on HCAEC.

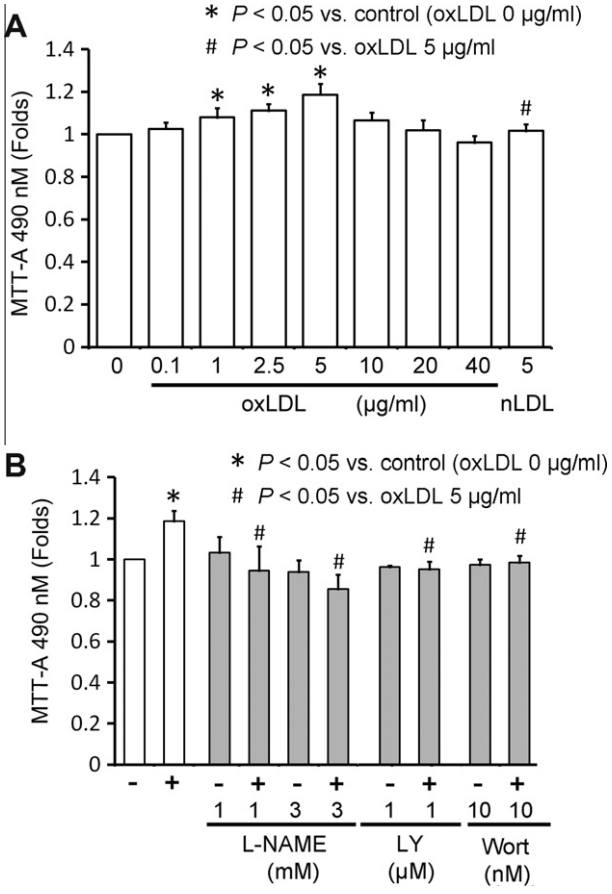

Fig. 2. Effects of oxLDL on HCAEC proliferation. (A) MTT results showed that oxLDL (1, 2.5, and 5 μg/ml) enhanced HCAEC proliferation while nLDL (5 μg/ml) showed no effect. (B) L-NAME, LY294002 and wortmannin attenuated oxLDL-induced cell proliferation.

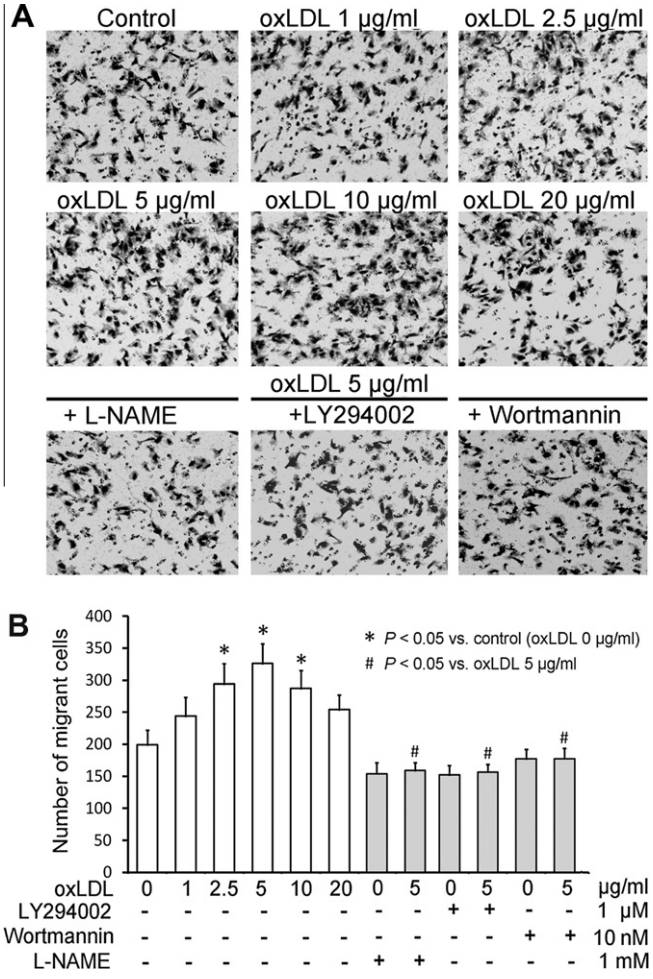

Fig. 3. Effects of oxLDL on HCAEC migration. (A) Representative photomicrographs (100×) and (B) summarized data (Mean ± SEM) from three separate experiments. HCAEC migration was enhanced by oxLDL (2.5, 5, and 10 μg/ml). Application of L-NAME, LY294002 and wortmannin abolished the effect of oxLDL.

3.2. Enhanced effect of low-concentration oxLDL on coronary artery endothelial cells is mediated by NO

NOS inhibitor, L-NAME, abolished the oxLDL-induced stimulatory effect on HCAEC. L-NAME prevented oxLDL (5 μg/ml)-induced tube formation (Fig. 1), cell proliferation (Fig. 2B) and cell migration (Fig. 3).

3.3. Low-concentration oxLDL increases intracellular NO levels in coronary artery endothelial cells

Confocal microscopic examination with the NO indicator DAF-FM showed that the basal intracellular NO level was elevated after 16 h-exposure to oxLDL (5 μg/ml; Fig. 4A and B). Addition of acetylcholine (ACh, 10 μM) to HCAEC further stimulated real-time NO production in cells pre-treated with oxLDL in comparison to the control (Fig. 4A and C). L-NAME attenuated the stimulatory effect of oxLDL on basal and stimulated NO production (Fig. 4C).

3.4. OxLDL up-regulates eNOS phosphorylation

OxLDL (1–20 μg/ml, 16 h) concentration-dependently up-regulated eNOS phosphorylation at Ser1177 (Fig. 4D), of which the peak phosphorylation was observed at 5 μg/ml. OxLDL treatment did not alter the eNOS protein expression level.

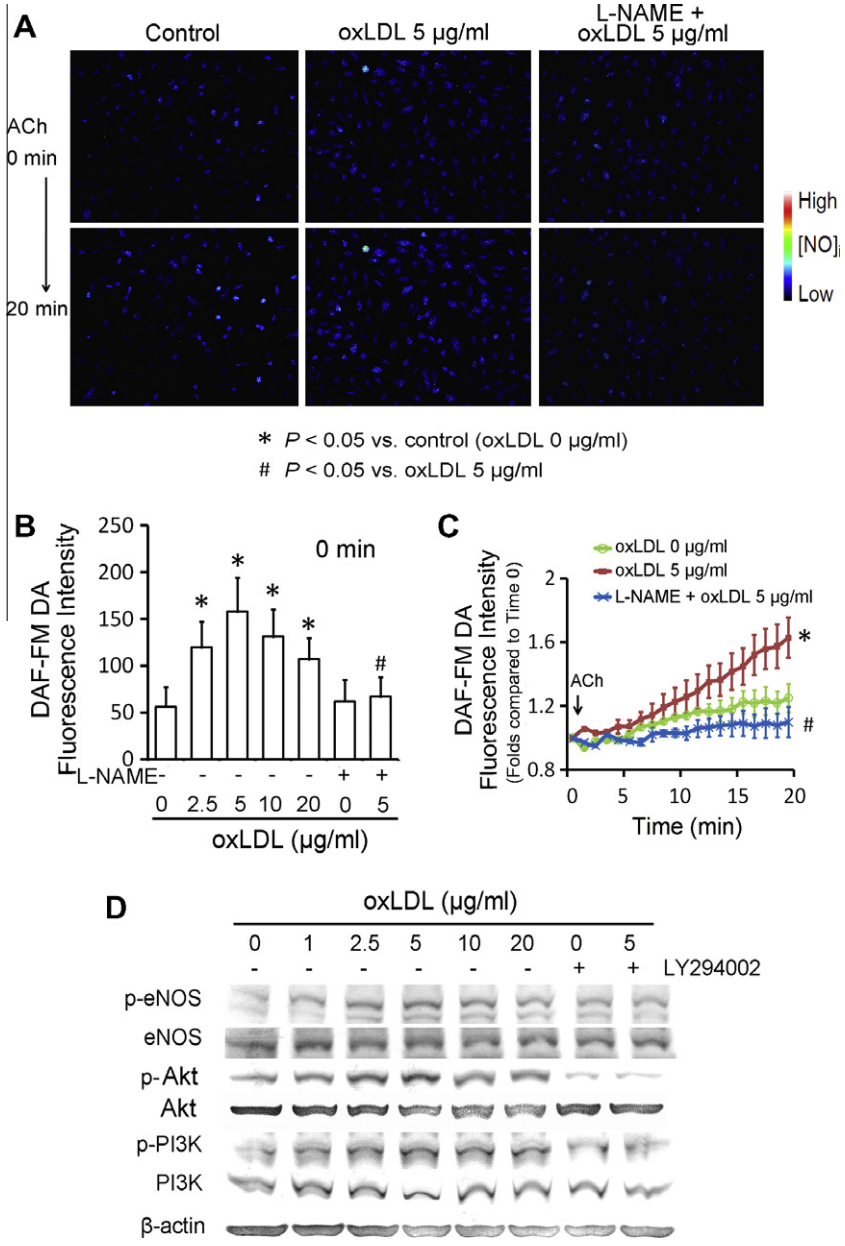

Fig. 4. *In-vitro* visualization of NO production and activation of Akt/PI3K pathway in oxLDL-treated HCAEC. (A) Representative confocal images showing the basal (0 min) and ACh (10 μM)-stimulated (20 min) NO levels of the HCAEC after 16 h-treatment of oxLDL (5 μg/ml). OxLDL-treated cells exhibited elevated (B) basal and (C) ACh-stimulated NO levels which were prevented by L-NAME. (D) Western blot showed that oxLDL (1–20 μg/ml) concentration-dependently up-regulated Akt, PI3K, and eNOS phosphorylation. LY294002 abolished the effect of oxLDL on Akt, PI3K, and eNOS.

3.5. OxLDL-induced eNOS phosphorylation is mediated through Akt/PI3K pathway

As shown in Fig. 4D, oxLDL treatment (1–20 μg/ml) increased the phosphorylation of Akt and PI3K without altering their protein expression levels. LY294002, a highly selective inhibitor of PI3K, prevented the oxLDL-induced phosphorylation of eNOS, Akt and PI3K. LY294002 and wortmannin, another PI3K inhibitor, abolished the oxLDL-stimulated cell proliferation and migration (Figs. 2B and 3).

4. Discussion

Our present results demonstrated that low concentration of oxLDL (5 μg/ml) stimulated cell proliferation, migration and tube formation in HCAEC, which were mediated through increased NO production, as evidenced by the abrogation of these angiogenic events in the presence of NOS inhibitor, L-NAME. Low concentration of oxLDL also promoted the activation of eNOS, Akt, and PI3K, as shown by their elevated phosphorylation levels in response to oxLDL. PI3K inhibitors, LY294002 and wortmannin, prevented the oxLDL-induced stimulatory effects on NO production, cell proliferation, migration and tube formation.

OxLDL has received paramount attention in the pathogenesis of atherosclerosis. It is believed that oxidation of LDL takes place in the arterial wall instead of in the circulation [23]. The concentration of circulating oxLDL in healthy subjects was shown at very low level [24–26], and it could be more than 10–100 folds at tissue level. Previous studies usually tended to employ high concentrations of oxLDL (50–500 μg/ml) in experimental settings and resulted in adverse effects on the endothelial cells [12–15]. In this study, we used a relatively low concentration of oxLDL (<10 μg/ml) to investigate its effect on HCAEC.

It is well known that angiogenesis commences by the migration of endothelial cells from the parent vessel, which then proliferate in response to the pro-angiogenic signals, resulting in the formation of nascent capillary sprouts and tubes. OxLDL at low concentrations (5 μg/ml) stimulated cell proliferation, migration and tube formation of HCAEC, suggesting that it may enhance the process of angiogenesis. Since angiogenesis is a favorable compensatory mechanism in ischaemic heart disease [27], the oxLDL-induced angiogenic effect may be beneficial. However, extensive angiogenesis is commonly found in atherosclerotic lesions, which are fragile and immature endothelial tubes with disorganized branching that are prone to rupture [28–30]. Such plaque angiogenesis may cause plaque expansion and plaque vulnerability, and enhance the risk of intravascular thrombosis [28–30]. Oxidized phospholipids, an oxLDL component, can stimulate angiogenesis via autocrine mechanisms, indicating that oxLDL may be able to stimulate angiogenesis within the plaque [31]. However, Bochkov et al. used a much higher concentration of oxidized phospholipids (100–300 μg/ml) to induce plaque angiogenesis in contrast to the present study (5 μg/ml) [31].

NO plays a crucial role in the local regulation of vascular homeostasis, including regulates systemic blood pressure, vascular remodeling and angiogenesis [32–34]. Endothelium-derived NO is mainly produced by eNOS and Akt/PI3K signaling pathway can activate NO synthesis in endothelial cells by phosphorylating eNOS [35]. Our present study provided direct evidence that oxLDL at low concentration enhanced NO production through eNOS phosphorylation via the Akt/PI3K pathway. PI3K and NOS inhibitors abolished the stimulatory effect of oxLDL on NO and abrogate its angiogenic effects in HCAEC. Altogether, the results presented herein suggest a novel mechanism of Akt/PI3K/eNOS-mediated angiogenic action of low-concentration oxLDL.

Acknowledgment

We gratefully acknowledge the support by the Li Ka Shing Institute of Health Sciences, Chinese University of Hong Kong.

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