Original Article

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Protective effect of oleanolic acid on oxidized-low density lipoprotein induced endothelial cell apoptosis

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Summary **Oleanolic acid (3β-hydroxyolean-12-en-28-oic acid, OA) is a naturally-occurring triterpenoid with various promising pharmacological properties. The present study was conducted to determine the protective effects of OA against oxidized low-density lipoprotein (ox-LDL) induced endothelial cell apoptosis and the possible underlying mechanisms. Our results showed that ox-LDL significantly decreased cell viability and induced apoptosis in human umbilical vein endothelial cells (HUVECs). OA in the co-treatment showed a protective effect against ox-LDL induced loss in cell viability and an increase in apoptosis, which was associated with the modulating effect of OA on ox-LDL induced hypoxia-inducible factor 1α (HIF-1α) expression. Moreover, our results showed that the modulating effect of OA against ox-LDL induced HIF-1α expression was obtained via inhibition of lipoprotein receptor 1 (LOX-1)/reactive oxygen species (ROS) signaling. Collectively, we suggested that the protective effect of OA against ox-LDL induced HUVEC apoptosis might, at least in part, be obtained via inhibition of the LOX-1/ROS/HIF-1α signaling pathway.**

Keywords: Hypoxia-inducible factor 1α (HIF-1α), lipoprotein receptor 1 (LOX-1), reactive oxygen species (ROS)

1. Introduction

Dysfunction of vascular endothelial cells (EC) is now believed to play an important role in the pathogenesis of atherosclerosis (AS) (*1*). A number of studies have showed that EC apoptosis functions as an initiating step for AS by inducing atherosclerotic lesion formation and plaque shedding (*2,3*). Oxidized low-density lipoprotein (ox-LDL), an essential atherosclerotic risk factor, has been found to play a crucial role in multiple functional alternations occurring during the pathogenesis of AS, including enhancing EC apoptosis (*4,5*). Therefore, inhibition of ox-LDL induced EC apoptosis may have therapeutic significance in the prevention and treatment of AS.

The mechanism of ox-LDL induced apoptosis was thought to be related to ox-LDL elicited reactive oxygen species (ROS) release through lipoprotein receptor (LOX), which in turn up-regulates the transcription factor hypoxia-inducible factor 1 (HIF-1) (*6,7*). In recent years, oleanolic acid (3β-hydroxyolean-12-en-28-oic acid), a naturally-occurring triterpenoid with widespread distribution in many fruits and plants like apple, grape, date, pomegranate and olive oil, is attracting much attention for its various pharmacological properties, such as hepatoprotective, vasorelaxant, antiproliferative, anti-inflammatory and apoptosis-inducing effects in various cancerous tissues (*8,9*). Notably, a synthetic triterpenoid analog of oleanolic acid has been shown to have a potent antioxidative effect by inducing NADH-quinone oxidoreductase and heme oxygenase 1 (*10*). However, the effect of OA against ox-LDL induced apoptosis in EC remains largely unknown.

In this study, we used human umbilical vein endothelial cells (HUVECs) to investigate the effects of OA on ox-LDL induced cytotoxicity in HUVECs and the relevant underlying mechanisms.

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2. Materials and Methods

2.1. *Cell culture*

HUVEC cells were purchased from ATCC (Manassas, VA, USA) and cultured in F-12K medium (ATCC, VA, USA) containing 10% fetal bovine serum (FBS, Gibco BRL Co.Ltd., Grand Island, NY, USA), 1% antibiotic-antimycotic solution (Gibco BRL Co.Ltd., Grand Island, NY, USA), 0.1 mg/mL heparin(Sigma. St. Lious, MO, USA), and 0.03 mg/mL endothelial cell growth supplement (Sigma. St. Lious, MO, USA) under an atmosphere of 5% CO_2 at 37°C. The cells were subcultured and split 1:4 every 4 days.

2.2. *Cytotoxicity assay*

A viability assay was performed using a trypan blue exclusion method described previously (*11*). Briefly, HUVEC (2×10^5 cells) were cultured in 24-well tissue culture plates (Corning-Costar, Cambridge, MA, USA). After drug administration, cells were trypsinized with 0.1% trypsin-EDTA (Gibco-BRL, Grand Island, NY, USA). Following centrifugation and washing, HUVECs were suspended in PBS and stained with trypan blue dye (Sigma, St Louis, MO, USA). Fractions of dead cells with a blue signal were visualized and counted using a reverse phase microscope (Nikon, Tokyo, Japan).

2.3. *Analysis of apoptotic cells*

Apoptotic cells were determined according to the method of Tai *et al*. (*12*). After drug treatment, HUVECs were harvested and fixed in cold 80% ethanol. Following a process of centrifugation and washing, fixed cells were stained with propidium iodide (Sigma. St. Lious, MO, USA) and analyzed using a FACS flow cytometer (Beckman Coulter Inc., Brea, CA, USA) on the basis of a 560-nm dichromic mirror and a 600-nm bandpass filter.

2.4. *Immunoblotting*

HUVEC cells in logarithmic growth phase were used and plated in 60 mm dishes at a density of 2×10^5 cells/well and were cultured overnight at 37°C under 5% CO_2. The cells were treated, harvested and lysed as described previously. Briefly, the cells were washed twice with ice-cold PBS and lysed in lysis buffer (25 mmol/L Tris/HCl pH 7.5, 25 mmol/L NaCl, 0.5 mmol/L EGTA, 10 mmol/L NaF, 20 mmol/L h-glycerophosphate, 1 mmol/L Na_3VO_4, 1 mmol/L PMSF, and 10 mg/mL aprotinin) (Sigma. St. Lious, MO, USA) at 4°C. After sonication and centrifugation at 15,000 rpm, the supernatant was used for immunoblotting. The lysate (20 μg protein per lane) was separated on 12% SDS-polyacrylamide gel (Sigma. St. Lious, MO, USA), electroblotted onto nitrocellulose membrane (Millipore, Billerica, MA, USA) and incubated with specific primary antibodies (Cell Signaling Technology, Inc., Boston, MA. USA). The antibodies were probed with a horseradish peroxidase-linked secondary antibody (Cell Signaling Technology, Inc, Boston, MA. USA) using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Shinjuku-ku, Tokyo). Densitometric analysis was performed using an image scanner and analyzing software. The rabbit polyclonal antibody to β-actin (Cell Signaling Technology, Inc, Boston, MA. USA) was used as a gel loading control.

2.5. *Silencing HIF-1α with shRNA and overexpression of HIF-1α*

For silencing HIF-1α, the HIF-1α-targeting shRNA (Sense sequence: GATCCCGCACAGTTACAGTA TTCCATCAAGAGTGGAATACTGTGCTTTTTT; Antisense sequence: CTAGAAAAAAGCACAGTT ACAGTATTCCACTCTTGATGGAATACTGTAAC TGTGCGG) was inserted into pGE-1 plasmid (pGE-1 PredigestedCloning Kit, Agilent Technologies Inc., Shanghai, China) after cutting with restriction enzymes BamH I and Xba I. Scrambled shRNA was used as a negative control. Transfection of the cells with shRNA was performed following the standard protocol. Briefly, the cells were plated at a density of 5×10^3 cells/well in a 6-well culture plate and incubated to allow 70-80% confluence (about 24 h). The cells were then starved in serum-free culture for 1 h. The transfection mixture containing HIF-1α-targeting shRNA and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was incubated for 20 min at room temperature. The cells were then incubated with the above mixture for 5 h at 37°C in a humidified atmosphere containing 5% CO_2. Subsequently, the cells were washed with PBS and maintained in DMEM containing 10% FBS for 48 h. The expression of HIF-1α was detected by Western blot analysis.

Based on the human HIF-1α sequence, primers (Sense primer: GCGGATCCAACGTCGAAAAGAAA AGTCTCG; Antisense primer: GCTCTAGAAAGTTTG TGCAGTATTGTAGCC) were designed to obtain the HIF-1α sequence. Then the PCR product was cut with restriction enzymes BamH I and Xba I before cloning into pEGFP-N1 vector (Takara Biomedical Technology Co., Ltd., Beijing, China). For HIF-1α overexrepression, the HUVECs were grown to sub-confluent densities and transfected with pEGFP-N1 empty or pEGFP-N1-HIF-1α vector (Takara Biomedical Technology Co., Ltd., Beijing, China) constructs using the transfection reagent TransIT-LT-1 and following manufacturer's instructions (Mirus Bio Corp., Madison, WI, USA). Overexpression of HIF-1α was confirmed by Western blots.

2.6. *LOX-1 knockdown and LOX-1 overexpression*

LOX-1 knockdown was performed using an RNA interference (RNAi) synthesized based on a previously published sequence (Sense: CCCTTCAGGTACCTGTGCATATATA) following a siRNA transfection protocol provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA) (*13*). Briefly, after culturing HUVECs in antibiotic free Dulbecco's modified Eagle's medium at 37°C in a humidified atmosphere of 5% CO_2 for 24 h, the siRNA duplex solution was added to HUVECs. After transfection for 24 h, the medium was replaced with normal Dulbecco's modified Eagle's medium (Sigma, St. Lious, MO, USA), and HUVECs were treated with OA, ox-LDL, or a combination of OA and ox-LDL.

To generate LOX-1 overexpression vectors, CDS region of LOX-1-coding sequences were obtained by RT-PCR and cloned into pEGFP-N1 vector (Takara Biomedical Technology Co., Ltd., Beijing, China). The resulting plasmid was named pEGFP-N1- LOX-1. HUVECs were transfected with pEGFP-N1-LOX-1 vector to induce excessive LOX-1 expression or pEGFP-N1 vector to generate stable clones expressing LOX-1 constitutively as control.

2.7. *Quantitative RT-PCR (qRT-PCR)*

Total RNA was extract from cells using RNA simple Total RNA Kit (TIANGEN Co., Beijing, China) and 3 μg of RNA was converted into cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). For detection of LOX-1 mRNA the primers used were: upstream 5'-CTGCCAGCCTGAAGTCCATT-3' and downstream 5'-TCTGTCTGTCTGTCCGTAAGTG-3', with an amplified fragment of 340 bp. The synthesis of the primers used for HIF-1α was based on the published sequence (*14*). For each PCR reaction, a master mix that including SYBR GREEN mastermix (Solarbio Co., Beijing, China), forward primer, reverse primer, and 10 ng template cDNA was prepared. The PCR conditions were 5 min at 95°C followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Data were analyzed using the comparative ΔCt method (ABPrism software, Applied Biosystems, Foster City, CA) using GAPDH as an internal normalization control.

2.8. *Detection of intracellular ROS detection*

Intracellular ROS level was measured by oxidation-sensitive fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) (Invitrogen, Carlsbad, CA, USA) method. Briefly, after washing once with PBS, treated cells were incubated with 20 μM DCF-DA in serum-free DMEM at 37°C for 30 min before analysis.

2.9. *Statistical analysis*

All statistical analysis was performed using PASW Statistical software (version 18.0 for Windows). Values are presented as the mean ± S.D. Statistical comparisons were performed by one-way ANOVA. Tukey's post hoc test was used for multiple group comparisons and Student's *t*-test was used for single comparisons. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Effects of OA on ox-LDL induced cytotoxicity in HUVECs*

We first examined the cytotoxicity of OA (Melone Biotech, Ltd., Dalian, China, Purity ≥ 98%) on HUVECs. Our results showed that incremental doses of OA treatment for 24 h did not affect HUVECs cell viability until 25 μM ($p < 0.05$ *vs*. control) (Figure 1A). Meanwhile, ox-LDL treatment for 24 h resulted in a dose-dependent decrease in cell viability, 100 μg/mL ox-LDL led to 49% reduction (Figure 1B). Therefore 100 μg/mL ox-LDL was chosen for the following experiments. Then the effect of ox-LDL and OA co-treatment was examined. We found OA at 1, 5, and 10 μM lowered the ox-LDL-caused decrease in viability of HUVECs by 5%, 12%, and 29%, respectively (Figure 1C).

3.2. *Effect of OA on ox-LDL induced apoptosis in HUVECs*

Apoptosis of HUVECs was analyzed by flow cytometry. As shown in Figure 2A, treatment of HUVECs with 100 μg/mL ox-LDL for 24 h increased the apoptotic cell population up to 31% while OA at 1, 5, and 10 μM significantly lowered the ox-LDL-induced apoptosis of HUVECs by 5%, 12%, and 21%, respectively (Figure 2A). In addition, activation of caspase-3 and PARP was also examined in HUVECs following treatments, which also showed OA treatment at 10 μM significantly attenuated the ox-LDL-induced apoptosis (Figure 2B).

3.3. *Effect of OA on ox-LDL induced HIF-1α*

It has been shown that HIF-1α is involved in regulatory pathways that leads to the activation of several transcription factors and to the release of cytokines and growth factors from endothelial cells (*6*). Therefore, we postulated that the protective effect of OA on ox-LDL induced apoptosis in HUVECs may be through modulating the HIF-1α related pathway. To confirm our postulation, the expression of HIF-1α was examined by Western blot. As shown in Figure 3A, ox-LDL treatment resulted in a dose-dependent increase of HIF-1α expression in HUVECs, while co-treatment

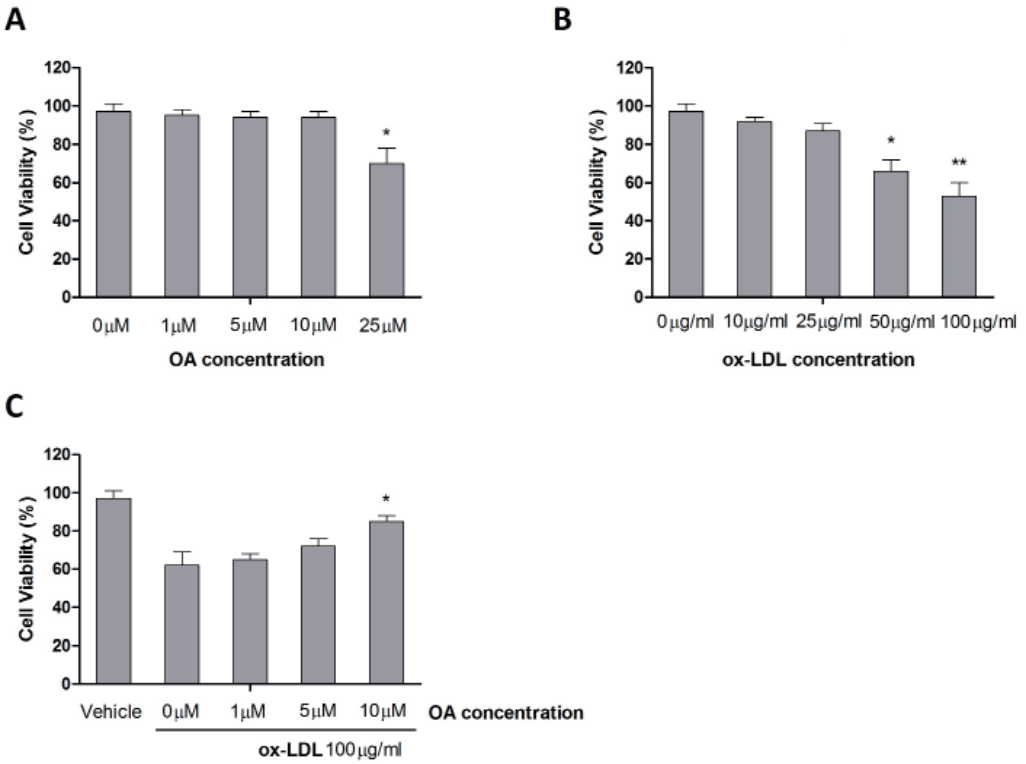

Figure 1. OA suppresses ox-LDL-induced loss of cell viability in HUVECs. (A), Effect of OA on cell viability of HUVECs. **(B)**, Effect of ox-LDL on cell viability of HUVECs. **(C)**, Suppressive effect of OA on ox-LDL-induced loss of cell viability in HUVECs. * $p < 0.05$ *vs*. control, ** $p < 0.01$ *vs*. control.

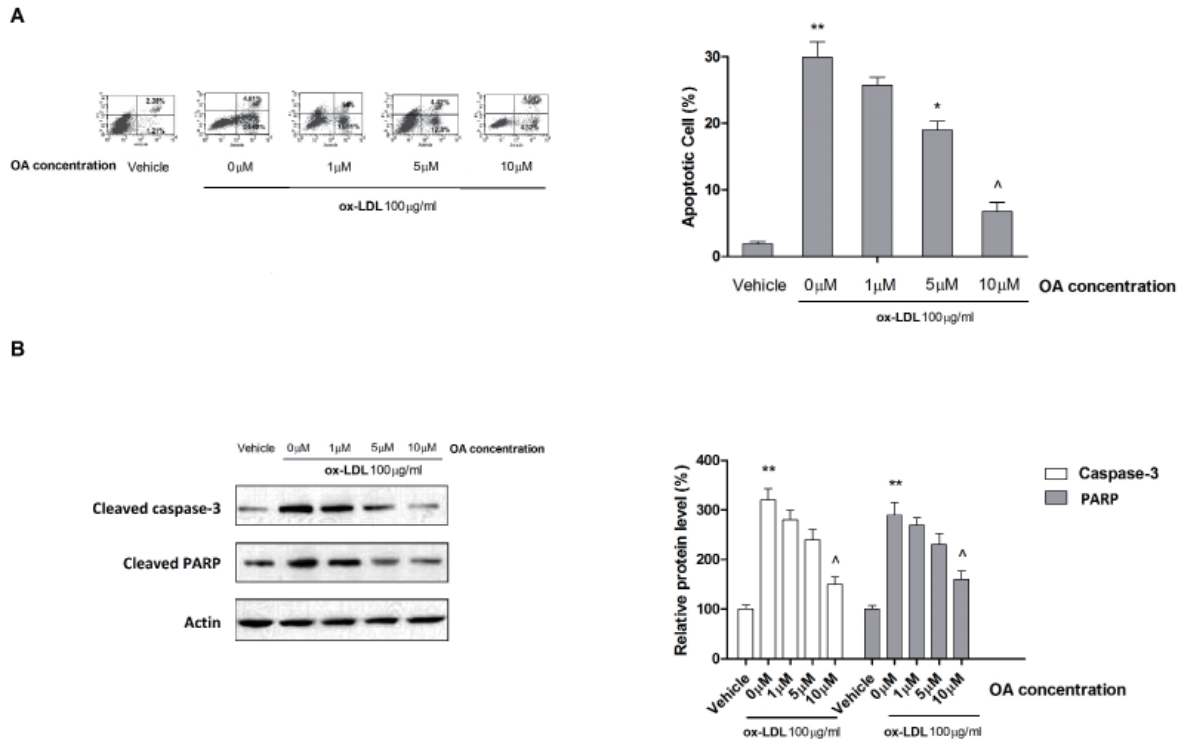

Figure 2. OA protects ox-LDL-induced apoptosis in HUVECs. (A), Suppressive effect of OA on ox-LDL-induced apoptosis in HUVECs. **(B)**, Suppressive effect of OA on ox-LDL-induced activation of caspase-3 and PARP in HUVECs. * $p < 0.05$ *vs*. control, ** $p < 0.01$ *vs*. control, ^ $p < 0.05$ *vs*. ox-LDL.

with OA caused a dose-dependent decrease of HIF-1α expression, suggesting that OA attenuates ox-LDL induced enhancement in HIF-1α expression (Figure 3B). To further demonstrate the association between HIF-1α expression and ox-LDL-induced apoptosis, the expression of HIF-1α was manipulated with siRNA and

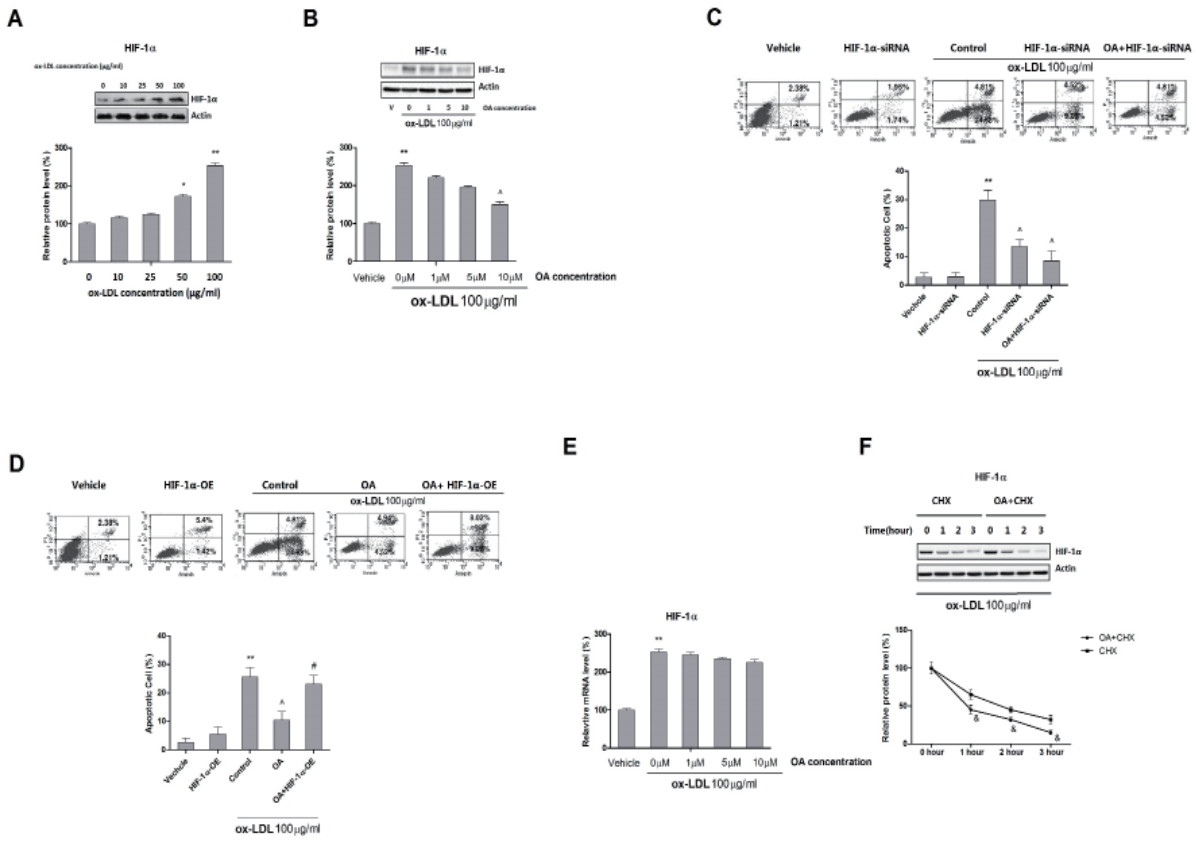

Figure 3. Protection of OA against ox-LDL-induced apoptosis is mediated by downregulation of HIF-1α. (A), Effect of ox-LDL on HIF-1α expression in HUVECs. **(B)**, Effect of OA on ox-LDL-induced HIF-1α expression. **(C)**, Effect of HIF-1α knockdown on ox-LDL-induced apoptosis and the protection of OA against ox-LDL-induced apoptosis. **(D)**, Effect of HIF-1α overexpression on ox-LDL-induced apoptosis and the protection of OA against ox-LDL-induced apoptosis. **(E)**, Effect of OA on ox-LDL-induced HIF-1α mRNA expression. **(F)**, Effect of OA on HIF-1α degradation. * $p < 0.05$ *vs*. control, ** $p < 0.01$ *vs*. control, ^ $p < 0.05$ *vs*. ox-LDL, # $p < 0.05$ *vs*. ox-LDL+OA, & $p < 0.05$ *vs*. CHX.

overexpressing plasmid. As shown in Figure 3C, siRNA targeting HIF-1α alone did not cause significant change in apoptotic cells while it significantly attenuated ox-LDL-induced HUVEC apoptosis and co-treatment with OA and HIF-1α siRNA synergistically reduced ox-LDL caused cell apoptosis. In comparison, overexpression of HIF-1α alone in HUVECs slightly affected cell apoptosis but almost completely attenuated OA-involved protection against ox-LDL induced apoptotic insults (Figure 3D).

We further examined the expression level of HIF-1α mRNA during co-treatment and found that, contrary to the protein expression pattern, which was suppressed in a dose-dependent manner by OA, the expression of HIF-1α mRNA remained unchanged even with the highest concentration of OA (Figure 3E). We then examined the involvement of the proteasomal degradation of HIF-1α in HUVECs treated by using cycloheximide (CHX, a translational inhibitor). Following incubation with ox-LDL for 24 hours, HUVECs cells were challenged with CHX alone or in combination with OA for 0, 1, 2, and 3 h. As shown in Figure 3F, our results revealed that HIF-1α protein degradation rate was significantly increased in cells treated with both OA and CHX, which indicated that OA interfered with the stabilization of HIF-1α in HUVECs. Collectively, our results showed that OA modulated ox-LDL induced HIF-1α expression by both suppressing translation and promoting degradation.

3.4. *Involvement of ROS in the modulating effect of OA on ox-LDL-induced HIF-1α*

Recent evidence has showed that HIF-1α can be induced by free radicals, especially ROS, and a number of studies have established that ROS generation was closely associated with ox-LDL induced endothelial cell apoptosis (*15-17*). Based on these previous studies, we explored the effect of OA on ox-LDL-induced ROS generation and the association between ROS and HIF-1α expression. Our results showed that OA alone did not cause significant change in ROS level in HUVECs while ox-LDL treatment resulted in ROS generation in a dose-dependent manner (Figure 4A). When ox-LDL (100 μg/mL) and OA (10 μM) were used in combination, OA was able to significantly attenuate ox-LDL induced ROS generation (Figure 4B). To further demonstrate the association between ROS generation and HIF-1α, ROS inducer ($CoCl_2$) and inhibitor NAC were employed. We found that NAC, serving as a positive control, was able to significantly downregulate

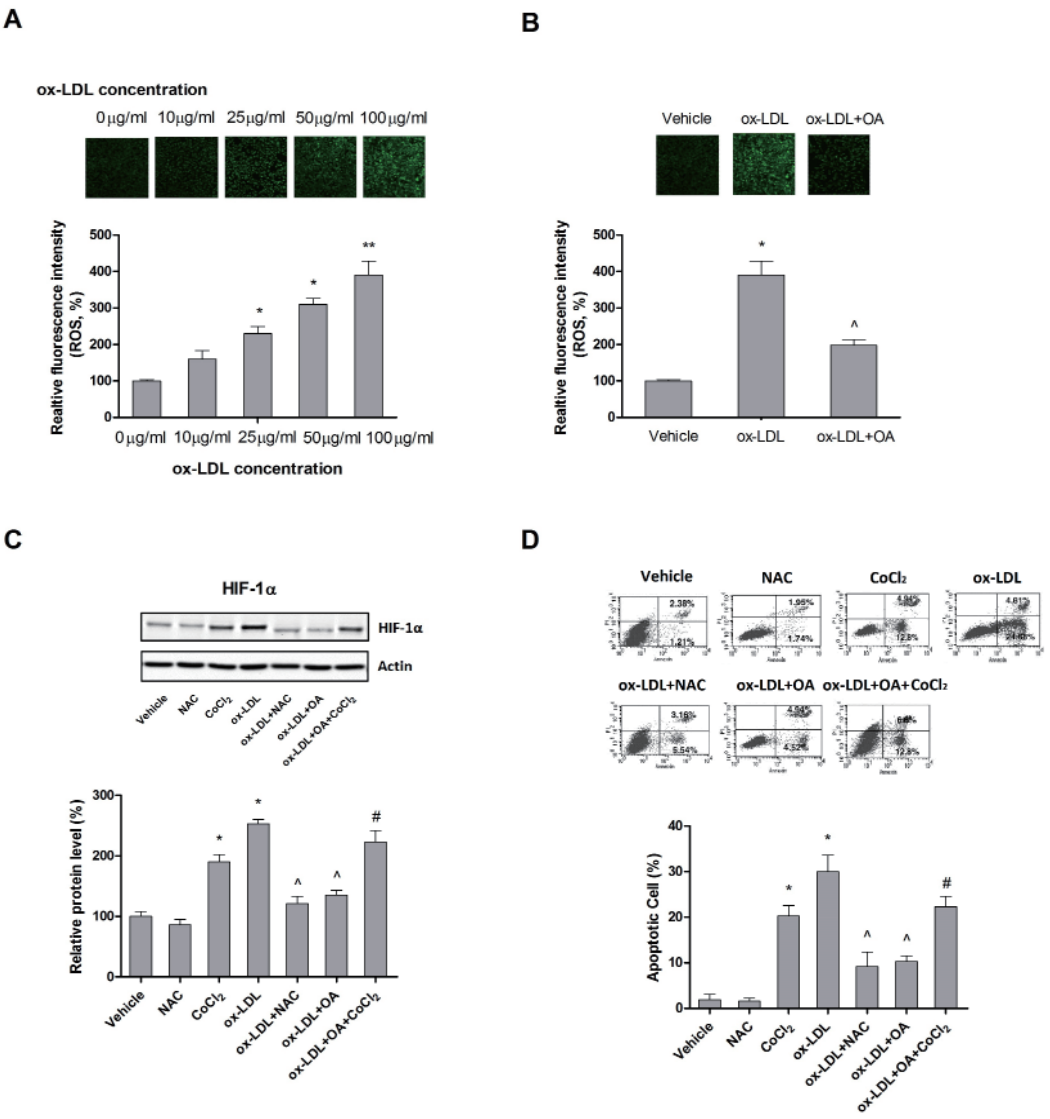

Figure 4. ox-LDL enhances HIF-1α expression by accumulating intracellular ROS. (A), Effect of ox-LDL on intracellular ROS in HUVECs. **(B)**, Effect of OA on ox-LDL-induced ROS accumulation. **(C)**, Effect of ROS inducer ($CoCl_2$) and inhibitor (NAC) on ox-LDL-induced HIF-1α expression and the attenuating role of OA in ox-LDL-induced HIF-1α expression. **(D)**, Effect of ROS inducer ($CoCl_2$) and inhibitor (NAC) on ox-LDL-induced apoptosis and the protection of OA against ox-LDL-induced apoptosis. $^{*}p < 0.05$ *vs*. control, $^{**}p < 0.01$ *vs*. control, $^{\wedge}p < 0.05$ *vs*. ox-LDL, $^{\#}p < 0.05$ *vs*. ox-LDL+OA.

ox-LDL induced HIF-1α expression. In contrast, $CoCl_2$ was able to significantly elevate the level of HIF-1α and the combination of OA and $CoCl_2$ significantly abolished the attenuating effect of OA on ox-LDL-induced HIF-1α expression. Moreover, the effect of both NAC and $CoCl_2$ on ox-LDL-induced apoptosis was also examined. Our results showed that NAC, similar to OA, significantly attenuated ox-LDL-induced HUVEC apoptosis while $CoCl_2$ significantly abrogated the protective effect of OA against ox-LDL-induced HUVEC apoptosis.

3.5. *Modulation of OA on LOX-1 mRNA and protein expression*

LOX-1 is expressed in endothelial cells and activation of LOX-1 promotes intracellular ROS accumulation (*18,19*). Given the fact that OA can modulate ox-LDL-induced intracellular ROS generation, we investigated the effect of OA on ox-LDL-induced LOX-1 expression. As shown in Figure 5A, after administration of ox-LDL, the expression of Lox-1 mRNA was enhanced by approximately 3-fold whereas OA decreased ox-LDL-induced Lox-1 mRNA production in a dose-dependent manner. Similar results were obtained when the effect of OA on LOX-1 protein level was analyzed (Figure 5B). The involvement of LOX-1 in the modulation of ROS and protective effect of OA against ox-LDL-induced apoptosis was further investigated by LOX-1 targeting siRNA and LOX-1 overexpressing plasmid. In the HUVECs with LOX-1 knockdown, siRNA targeting LOX-1 by itself did not affect apoptosis or ROS accumulation in HUVECs (Figure 5C and 5D). However, LOX-1 siRNA significantly decreased the

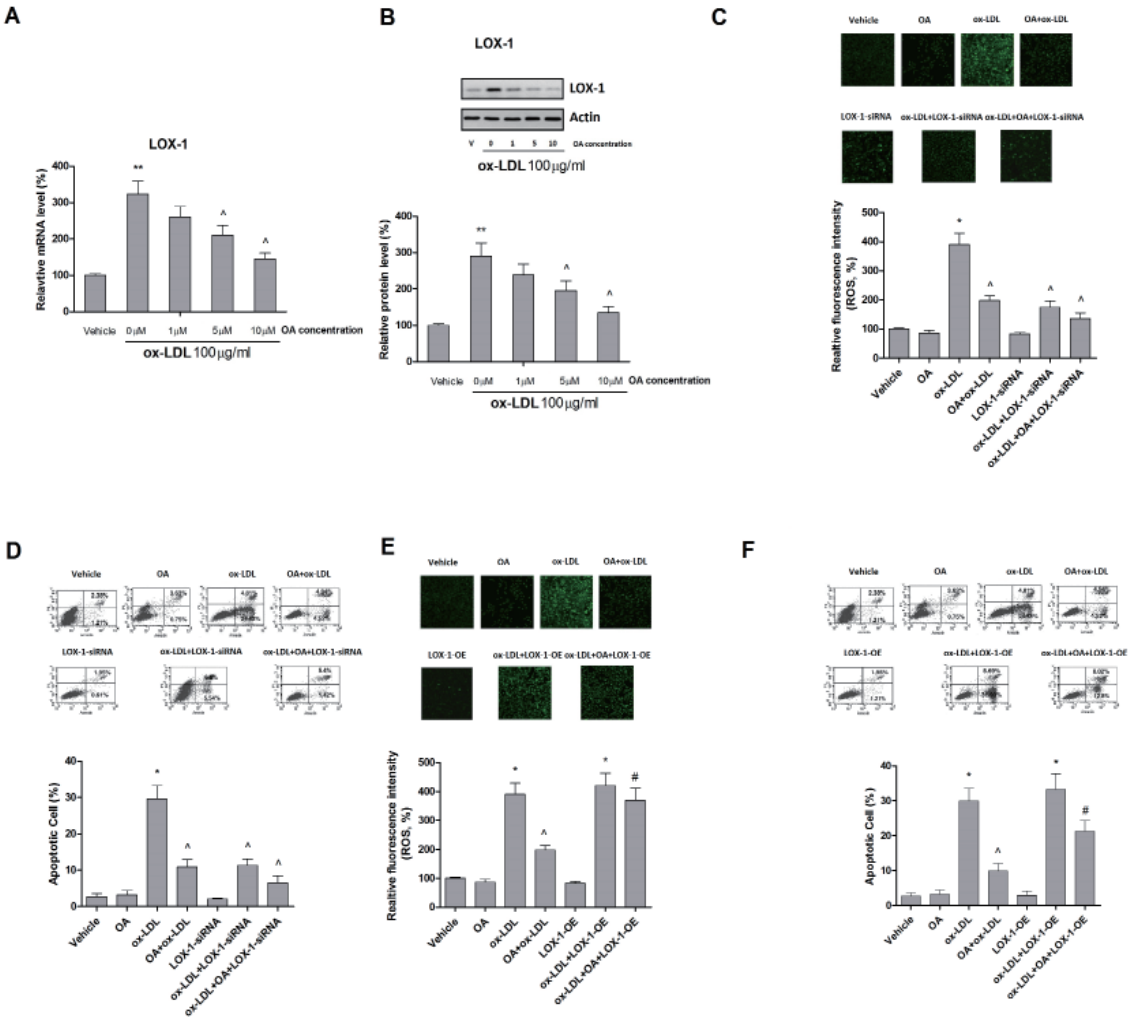

Figure 5. OA attenuates ox-LDL-induced ROS generation by modulating LOX-1 expression. (A), Effect of OA on ox-LDL-induced LOX-1 mRNA expression. **(B)**, Effect of OA on ox-LDL-induced LOX-1 protein expression. **(C)**, Effect of LOX-1 knockdown on ox-LDL-induced ROS generation and the protection of OA against ox-LDL-induced ROS generation. **(D)**, Effect of LOX-1 knockdown on ox-LDL-induced apoptosis and the protection of OA against ox-LDL-induced apoptosis. **(E)**, Effect of LOX-1 overexpression on ox-LDL-induced ROS generation and the protection of OA against ox-LDL-induced ROS generation. **(F)**, Effect of LOX-1 overexpression on ox-LDL-induced apoptosis and the protection of OA against ox-LDL-induced apoptosis. * $p < 0.05$ *vs*. control, ** $p < 0.01$ *vs*. control, ^ $p < 0.05$ *vs*. ox-LDL, # $p < 0.05$ *vs*. ox-LDL+OA.

capability of ox-LDL to induce apoptosis as well as ROS accumulation in HUVECs. Cotreatment with OA and LOX-1 siRNA synergistically reduced oxLDL-caused cell apoptosis and ROS generation by 64.7%. Although overexpression of LOX-1 alone in HUVECs did not affect cell apoptosis or ROS generation, enhanced expression of LOX-1 markedly potentiated the capability of ox-LDL to induce ROS accumulation and apoptosis. However, overexpression of LOX-1 significantly attenuated OA involved protection against oxLDL-induced apoptosis and ROS generation in HUVECs (Figures 5E and 5F). Taken together, our results showed that the protective effect of OA against ox-LDL induced HUVEC apoptosis might, at least in part, be obtained *via* inhibition of LOX-1/ROS/HIF-1α signaling.

4. Discussion

A growing body of evidence suggests that ox-LDL induced endothelial cell apoptosis plays key roles in the pathogenesis of atherosclerosis by promoting a reduction of vascular integrity, deposition of lipids, invasion of vascular smooth muscle cells, migration of monocytes, and formation of atherosclerotic plaque (*20,21*). Therefore, suppressing ox-LDL-induced endothelial cell apoptosis may provide a new therapeutic option for prevention and treatment of atherogenesis. In this study, we employed human umbilical vein endothelial cells (HUVECs) which have high relevancy to actual human cardiovascular system cells as an *in vitro* model to investigate the protective effects of OA protected against ox-LDL induced apoptosis in endothelial cells. Our results demonstrated that (*i*) OA protected HUVECs from ox-LDL-induced loss in cell viability and apoptosis in a concentration-dependent manner; (*ii*) the modulating effect of OA on HIF-1α participated in the anti-apoptotic effects of OA; (*iii*) the anti- apoptosis effects of OA were obtained

via suppressing the signaling cascade of LOX-1/ROS/HIF-1α. To our knowledge, this is the first study that elucidates the role of HIF-1α in ox-LDL induced apoptosis in endothelial cells.

The involvement of HIF-1α in the pathogenesis of atherosclerosis has been shown in a variety of cell types (*6*). A few studies have shown that HIF-1α played an essential role in the function of macrophages when challenged by ox-LDL and promoted the transformation of macrophages to foam cells (*22,23*). In leucocytes, which function as a key factor in the development and progression of atherosclerotic lesions, HIF-1α has been reported to have a direct effect on the cytokine profile (*24*). Jeong *et al*. also reported that activation of HIF-1α in mast cells could stimulate the expression of VEGF, leading to direct and indirect attraction of inflammatory cells into the intima in various stages of atherogenesis (*25,26*). Moreover, a number of studies have established the crucial role of HIF-1α in vascular smooth muscle cells proliferation exposed to hypoxic stimulus (*27*). However, the role of HIF-1α in endothelial cells has never been studied. In this study, we found that the ox-LDL was able to induce expression of HIF-1α. Then we manipulated the expression of HIF-1α level in HUVECs with HIF-1α-targeted siRNA and HIF-1α overexpressing plasmid, both did not show any marked effect on HUVECs apoptosis alone. However, HIF-1α-targeted siRNA significantly attenuated ox-LDL-induced apoptosis in HUVECs, suggesting that ox-LDL induced apoptosis, at least partly, was mediated *via* HIF-1α signaling. Interestingly, we found that OA was able to modulate the expression of HIF-1α, which led to its protective effect against ox-LDL induced apoptosis in HUVECs, as demonstrated by the effect of OA on ox-LDL induced apoptosis in HIF-1α knockdown or HIF-1α overexpressing HUVECs. Based on our results, we suggest that HIF-1α might serve as a novel target in the prevention and treatment of AS.

It is known that intracellular ROS positively correlated with HIF-1α level in a variety cells (*28*) and OA has been identified as a free radical scavenger (*29*). Consistent with previous studies, our results also confirmed the role of OA as a free radical scavenger in endothelial cells by demonstrating that OA treatment was able to reduce ox-LDL-induced ROS in a dose-dependent manner. Moreover, a combined use of OA with ROS inducer $CoCl_2$ or ROS inhibitor NAC showed that the modulating effect of OA on HIF-1α level in HUVECs was mediated by regulating the intracellular ROS levels. It was suggested that ROS may be involved in stabilizing HIF-1α, leading to elevated levels of HIF-1α (*30*). Therefore, interfering with the ROS level will affect the degradation of HIF-1α. Our experiments showed that OA decreased the level of HIF-1α in part by promoting HIF-1α degradation, supporting our argument that OA modulated HIF-1α level *via* eliminating intracellular ROS. However, it is to be noted that the regulatory effect of OA on HIF-1α level also involved its effect on translational level (our results showed that OA had no effect on HIF-1α mRNA but a dose-dependent effect on HIF-1α protein level in HUVECs), which indicated that OA might modulate HIF-1α level *via* more than a single pathway. Meanwhile, we also noticed that OA does not always work as a free radical scavenger. A recent study by Lin *et al* reported that activation of ROS/ASK1/p38 MAPK pathways is responsible for the apoptosis stimulated by OA in cancer cells (*31*), which suggests that the effect of OA on ROS might be cell specific.

First identified as an endothelial-specific scavenger receptor by Sawmura in 1997 (*18*), LOX-1 has been found to be expressed in endothelial cells, macrophages, vascular smooth muscle and platelets and can be upregulated by many factors such as ox-LDL, Ang II, shear stress, and advanced glycation (*32-34)*. A number of studies implicated that the apoptosis of endothelial cells induced by ox-LDL was predominantly mediated by LOX-1 whose expression is strongly induced by exposure to ox-LDL (*16,34*). More importantly, ox-LDL induces apoptosis in endothelial cells, which can be suppressed by agents that are able to downregulate LOX-1 expression (*16,35*). As shown in the present study, an approximately 3-fold expression of LOX-1 protein/mRNA was observed in ox-LDL treated HUVECs, compared to the control group while OA significantly decreased the induced expression of LOX-1 by ox-LDL in a dose-dependent manner. We also found that OA decreased the intracellular ROS level by downregulating LOX-1, which probably resulted from compromised ox-LDL intake by HUVECs due to less available LOX-1 (*18*). Moreover, by applying LOX-1-targeted siRNA and LOX-1 overexpressing plasmid in HUVECs, we were able to prove the contribution of downregulating LOX-1 in the anti-apoptotic effect of OA against ox-LDL induced apoptosis in HUVECs. Collectively, our results suggest that the LOX-1/ROS/HIF-1α signaling pathway might, at least partly, be involved in the anti-apoptotic effects of OA.

5. Conclusions

In summary, the present study on HUVECs provides evidence that OA protected ox-LDL induced cell apoptosis by suppressing sequential events cause by ox-LDL treatment including LOX-1 up-regulation, ROS overproduction, and HIF-1α overexpression. Our work provides new insights into therapeutic potential of OA in the treatment of AS.

Acknowledgements

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