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Hypoxia triggers endothelial endoplasmic reticulum stress and apoptosis via induction of VLDL receptor

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ABSTRACT

Endothelial cells express very low density lipoprotein receptor (VLDLr). Beyond the function as peripheral lipoprotein receptor, other roles of VLDLr in endothelial cells have not been completely unraveled. In the present study, human umbilical vein endothelial cells were subjected to hypoxia, and VLDLr expression, endoplasmic reticulum (ER) stress, and apoptosis were assessed. Hypoxia triggered endothelial ER stress and apoptosis, and induced VLDLr expression. Silencing or stabilization of HIF-1α reduced and enhanced VLDLr expression, respectively. HIF-1α affected *vldlr* promoter activity by interacting with a hypoxia-responsive element (HRE). Knockdown or overexpression of VLDLr alleviated and exacerbated hypoxia-induced ER stress and apoptosis, respectively. Thus, hypoxia induces VLDLr expression through the interaction of HIF-1α with HRE at the *vldlr* promoter. VLDLr then mediates ER stress and apoptosis.

1. Introduction

The endoplasmic reticulum (ER) is a highly dynamic organelle in eukaryotic cells, dysfunction of which results in ER stress and disturbed protein folding processes, also termed as unfolded protein response (UPR). Commonly, a persistent UPR is correlated with cellular malfunction. A large number of regulatory molecules involved in UPR procedure and ER stress, such as glucose-regulated protein 78 (GRP78, also termed Bip), a calcium dependent molecular chaperone riched in the ER lumen, protein kinase activated by double-stranded RNA (PKR)-like ER kinase (PERK), a transmembrane protein which is associated with GRP78 in unstressed cells, and CHOP, a proapoptotic protein, expression of which is strongly promoted by PERK signaling when prosurvival exertion does not overcome the sustained ER stress. As an important organelle, ER controls cell survival and death in its own particular way. Both intro- and extra-cellular factors influence ER homeostasis. There is compelling evidence that disturbed ER functions play crucial roles in a number of endothelial pathological processes and cardiovascular disease, such as ischemic vascular disorder, neovascularization, and atherosclerosis [1–3].

The very low density lipoprotein (VLDL) receptor (VLDLr) is a member of the low density lipoprotein (LDL) receptor family which composed more than ten growing receptors. VLDLr is highly expressed in fatty acid tissues. Tissues from the adipose layer, skeletal muscle, and heart express VLDLr. Additionally, VLDLr is present in endothelial cells as well. Beyond the function of VLDLr as a peripheral lipoprotein receptor, the possibilities of its biological roles have been extended to the process of signal transduction, angiogenesis, and tumor growth [4]. Recently, some study suggests a regulatory role of VLDLr in the endothelial pathology. The activation of retinal vascular endothelial cells and promotion of angiogenesis were mediated through VLDLr [5,6]. VLDLr modulated fibrin-dependent transendothelial migration of leukocytes [7]. VLDLr was used in microvascular endothelial cells to inhibit cell division [8]. However, no further study was presented to clarify the effects of VLDLr in the other endothelial pathological events, such as cellular ER stress and apoptosis.

The aim of the current investigation was to detect the effect of VLDLr on regulation of endothelial ER stress and apoptosis in the condition of hypoxia. We provided evidence that hypoxia induced VLDLr expression through a HIF-1α-dependent style, and the

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induced VLDLr expression triggered endothelial ER stress and apoptosis.

2. Materials and methods

2.1. Antibodies and reagents

Anti-VLDL receptor (VLDLr) antibody was obtained form R&R Systems, and anti-cleaved caspase-3, anti-cleaved caspase-8, anti-cleaved caspase-9, anti-Bax, anti-Bcl-2, anti-β-actin and HRP-conjugated secondary antibodies were from Cell Signaling Technology. Anti-phospho-PERK, anti-PERK, anti-GRP78, anti-CHOP, and anti-cleaved caspase-4 were purchased from Santa Cruz Biotechnology. Dimethyloxalylglycine (DMOG) was obtained from Cayman Chemical. All other chemicals were obtained from Sigma.

2.2. Cell culture and hypoxia incubation

Human umbilical vein endothelial cells (HUVECs) (Sciencell) were cultured in endothelial cell medium (ECM) supplemented with endothelial cell growth supplement (ECGS), 5% fetal bovine serum (FBS), and penicillin/streptomycin (P/S) solution (Sciencell) in an incubator with 37 °C, 5% CO_2. Cells of passage No. 4–6 were grown until confluence for experimental use. For experimental hypoxia, cells were incubated with the serum-free medium and placed in an airtight humidified chamber with 37 °C, 5% CO_2, and 95% N_2. The corresponding normoxia control cells were cultured in a humidified incubator with 37 °C, 5% CO_2, and 21% O_2.

2.3. Transfection of siRNA and adenovirus

The VLDLr siRNA, HIF-1α siRNA, HIF-2α siRNA, and non-targeting siRNA used in our study were designed by Dharmacon. HUVECs were transfected with siRNA (50 nmol/L) using DharmaFECT1 as described previously [9]. The non-targeting siRNA was used as transfection control for non-sequence-specific effects of the transfected siRNAs. Recombinant adenoviruses expressing VLDLr or control viruses were constructed using the Ad-Easy system. The cultures were infected routinely at a multiplicity of infection of five with an infection efficiency up to 80%.

2.4. Promoter constructs and luciferase reporter gene assay

The promoter fragments of *vldlr* were PCR-amplified from genomic DNA and cloned into a luciferase vector at the Kpn1 and Bgl II site (pGL3; Promega, Madison, WI). The primers used for PCR amplification were as follows: 5′-GAGGGTACCAGTCGAGCCCCT-3′ (sense); 5′-CTCAGATCTCATGCTGCCCGC-3′ (antisense). The mutant promoter pGL3vldlrHREmut was constructed by altering the putative hypoxia-responsive element (HRE) sequence CGTG (−161 to −158 bp) to **GATC** (bold indicates changes). HUVECs were seeded in 96-well plates and transfected with luciferase vector (100 ng, pGL3 constructs), and Renilla vector (25 ng, pRLTK) using Lipofectamine 2000 (Invitrogen). Twenty-four hours later, cells were subjected to hypoxia with or without SalA pretreatment and then lysed. The ratios between firefly luciferase and Renilla luciferase activity were determined with a dual-luciferase assay (Promega).

2.5. Quantitative realtime RT-PCR analysis

Total RNA was extracted from HUVECs with Trizol (Invitrogen). Realtime PCR was performed with Bio-RAD iQ5 Multicolor Real-Time PCR Detection System using SYBR Green as fluorescent and ROX (Takara) as reference dyes as described previously [9]. The specific primers used were as follows: human VLDLr: 5′-GTGGAAAATGTGATGGGGATG-3′ (forward), 5′-CCATTGTTGCACACGAAGTC-3′ (reverse); human GRP78: 5′-GCCGCAGGCGCTGGAAAGAT-3′ (forward), 5′-GCCGATGAGTCGCTTGGCGT-3′ (reverse); human CHOP: 5′-GGTGGCAGCGACAGAGCCAA-3′ (forward), 5′-CATGCGTTGCTTGCCAGCCC-3′ (reverse); human GAPDH: 5′-GACCCCTTCATTGACCTC-3′ (forward), 5′-GCTAAGCAGTTGGTGGTG-3′ (reverse).

2.6. Immunoblot analysis

Immunoblotting was performed according to the previous protocols [10]. Briefly, total protein was extracted from HUVECs using lysis buffer. Protein (50–60 μg) from each sample was separated with SDS–PAGE, then transferred to nitrocellulose membranes, and probed using primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. The chemiluminescence signal was measured with a densitometry program (Gel-pro 4.5 Analyzer, Media Cybernetics). To calculate the protein signal, we subtracted background, normalized the value to β-actin. To quantify the phospho-specific protein, we normalized the value to the

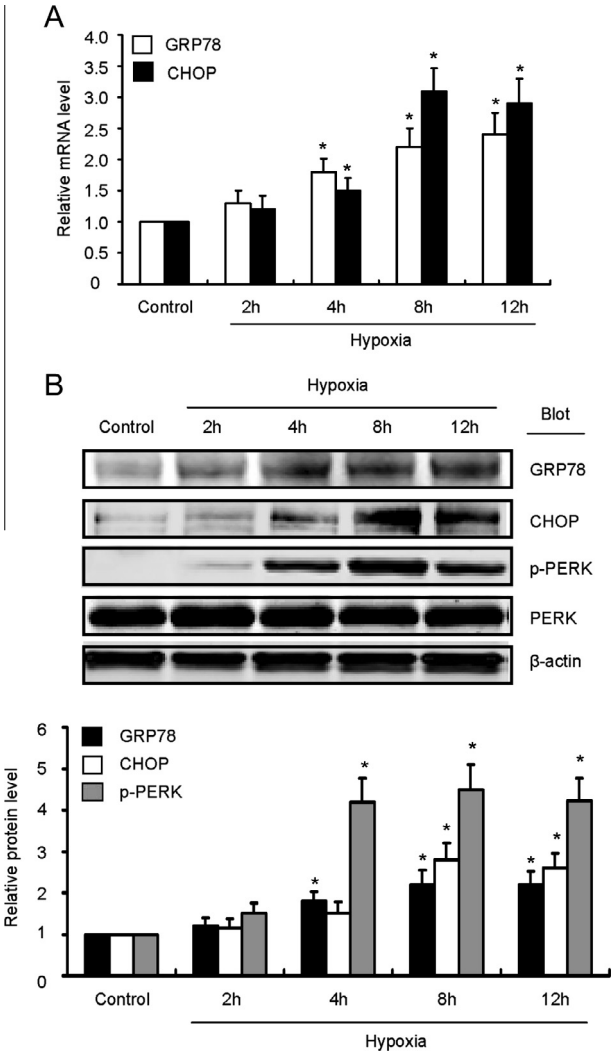

Fig. 1. Hypoxia triggers endothelial ER stress. HUVECs were treated with hypoxia for the indicated time points. (A) The relative expression levels of GRP78 and CHOP were detected by q-PT-PCR ($n = 6$, means ± SE). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control cells. (B) The protein levels of GRP78, CHOP, p-PERK, PERK, and β-actin were detected by immunoblot analysis using indicated antibodies. A representative blot (top) was shown ($n = 4$). Quantitative analysis of GRP78, CHOP, and p-PERK (bottom) was expressed as fold increase above control group ($n = 4$, means ± SE). $^{*}P < 0.05$ vs. control cells.

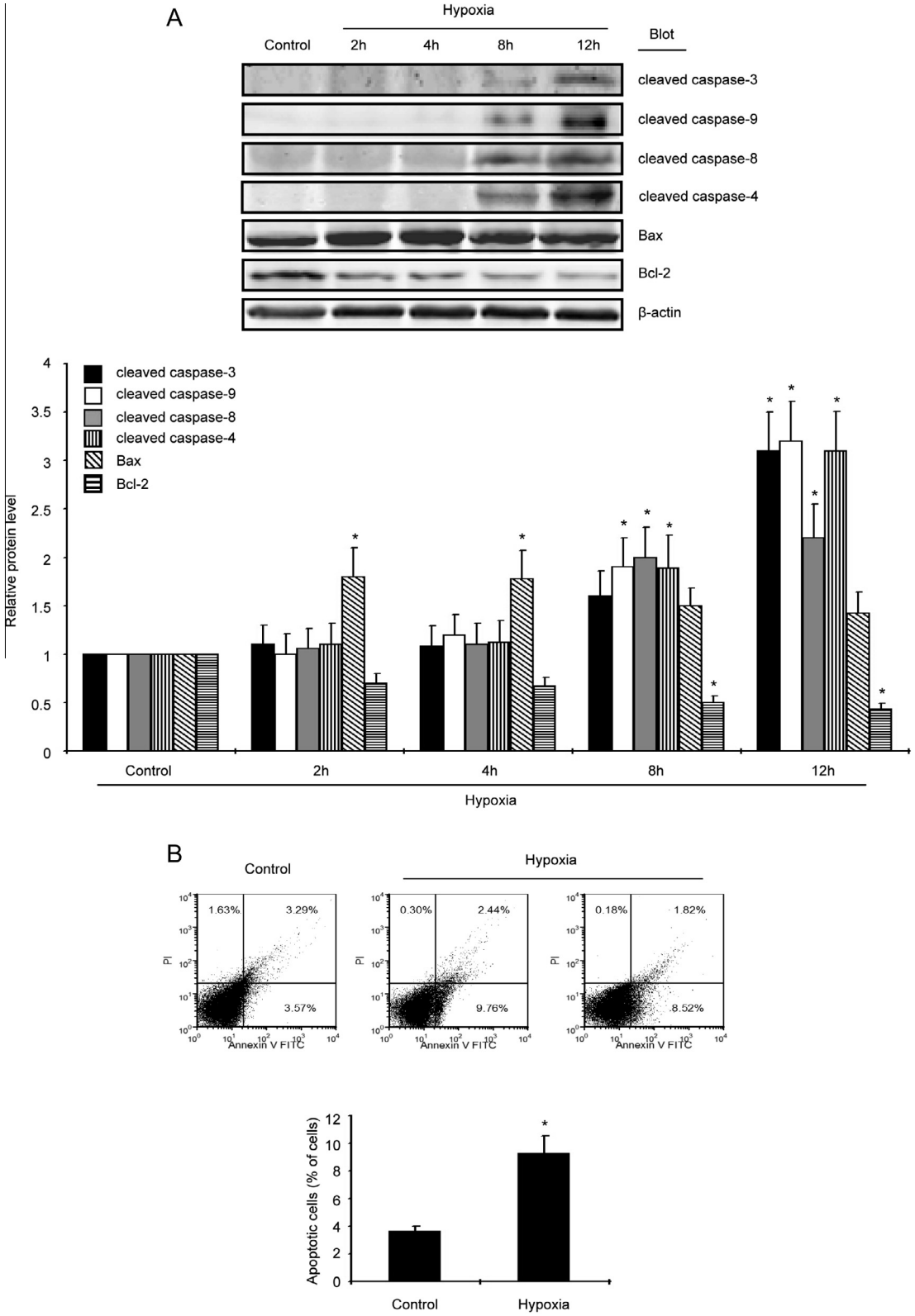

Fig. 2. Hypoxia induces endothelial apoptosis. HUVECs were treated with hypoxia for the indicated time points. (A) The protein levels of cleaved caspase-3, cleaved caspase-9, cleaved caspase-8, cleaved caspase-4, Bcl-2, Bax, and β-actin were detected by immunoblot analysis using indicated antibodies. A representative blot (top) was shown ($n = 4$). Quantitative analysis of cleaved caspase-3, cleaved caspase-9, cleaved caspase-8, cleaved caspase-4, Bcl-2, and Bax (bottom) was expressed as fold increase above control group ($n = 4$, means ± SE). $^{*}P < 0.05$ vs. control cells. (B) The cells were treated with hypoxia for 12 h and the apoptosis was measured by flow cytometry analysis. Representative photos of different groups were shown (top). The apoptotic cells were quantified ($n = 4$, means ± SE) (bottom). The values represent the mean percentages of apoptotic cells in different groups. $^{*}P < 0.05$ vs. control cells.

signal of total target protein and β-actin. The data of each group were presented as a percentage of the control.

2.7. Flow cytometry assay

Cell apoptosis was analyzed by flow cytometry using annexin V-FITC/propidium iodide (PI) staining according to the manufacturer's instructions (Invitrogen, USA). Briefly, cells were rinsed twice with ice-cold PBS and resuspended in 250 μl binding buffer with a concentration of 2–5 × 10^5/ml. Then cells were added with 5 μl of annexin V-FITC stock solution and rinsed for 3 min at 4 °C. After that, cells were added with 10 μl PI (20 μg/ml) and incubated 10 min in the dark at room temperature. Cell apoptosis was analyzed using flow cytometry (FACSCalibur, BD Biosciences, USA) with Cell Quest software. Approximately 1 × 10^4 cells were analyzed with each sample.

2.8. Statistical analysis

Each experiment was performed at least in triplicate. All the data were shown as means ± SE. Differences between groups were assessed using Student's *t*-test to determine statistical significance. In each case, $P < 0.05$ was considered significant.

3. Results

3.1. Hypoxia triggers endothelial ER stress

Recent evidence indicated that hypoxia induced ER stress, which was involved in the pathogenesis of cardiovascular disease [3,11]. We thus examined the ER stress in endothelial cells. Experimentally induced hypoxia in endothelial cells triggered elevated mRNA level of GRP78 and CHOP (Fig. 1A). In agreement with the mRNA levels, the GRP78 and CHOP protein levels were also promoted by hypoxia (Fig. 1B). In addition, the hypoxia markedly promoted phosphorylation of PERK (Fig. 1B). These results suggested a significant promotion of ER stress was elicited by hypoxia in endothelial cells.

3.2. Hypoxia induces endothelial apoptosis

In concert with previous studies, our result indicated that treatment of HUVECs with hypoxia significantly induced the expression of proapoptotic protein Bax, whereas the antiapoptotic protein Bcl-2 was inhibited by hypoxia (Fig. 2A). Furthermore, hypoxia remarkably increase the levels of cleaved caspase-3, caspase-9, caspase-8, and caspase-4 (Fig. 2A). In addition, cellular apoptosis was detected by flow cytometry analysis. In the bottom left quadrant located viable cells (annexin V-FITC-/PI-). Annexin V-FITC +/PI-cells located in the bottom right quadrant represent cellular status with early apoptosis. The result indicated that hypoxia for 12 h induced a remarkable apoptosis of endothelial cells (Fig. 2B).

3.3. Hypoxia induces VLDLr expression in endothelial cells

In physiological conditions, the endothelial cells express low amounts of VLDLr. Previous evidences suggested that VLDLr mediated endothelial proliferation and angiogenesis. In our results, quantitative RT-PCR and immunoblot analyses demonstrated that VLDLr mRNA and protein levels increased significantly in the hypoxic endothelial cells (Fig. 3A and B). In addition, we also detected high expression level of HIF-1α, which was markedly elicited by hypoxia (Fig. 3B).

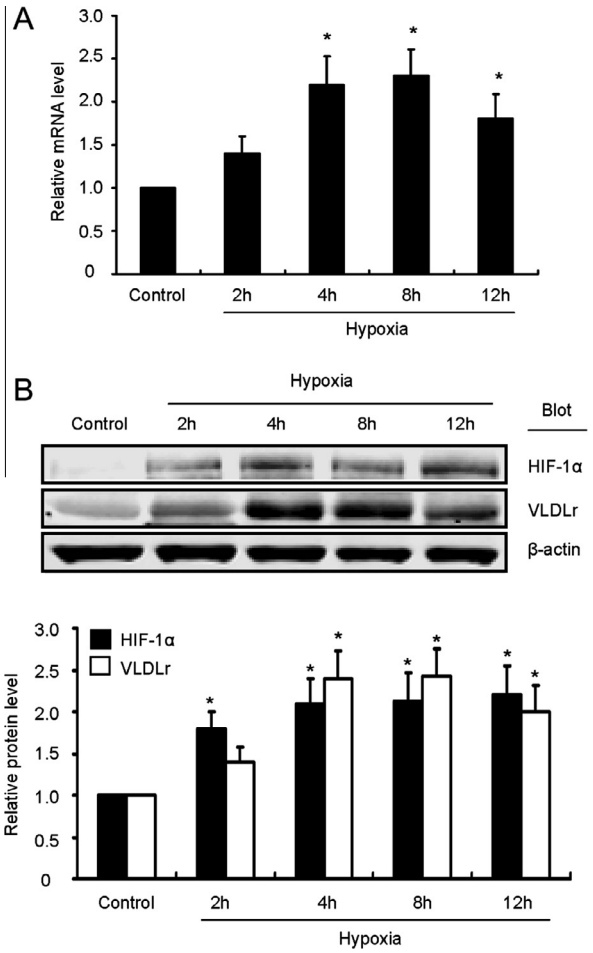

Fig. 3. Hypoxia induces expression of VLDLr and HIF-1α. HUVECs were treated with hypoxia for the indicated time points. (A) The relative mRNA level of VLDLr was detected by q-PT-PCR ($n = 6$, means ± SE). *$P < 0.05$ vs. control cells. (B) The protein levels of VLDLr, HIF-1α, and β-actin were detected by immunoblot analysis using indicated antibodies. A representative blot (top) was shown ($n = 4$). Quantitative analysis of VLDLr and HIF-1α (bottom) was expressed as fold increase above control group ($n = 4$, means ± SE). *$P < 0.05$ vs. control cells.

3.4. Enhanced expression of VLDLr is dependent on HIF-1α

It seemed that induction of VLDLr expression was a common responsiveness to hypoxic stress [12]. We therefore detected whether HIF-1α was involved in the induction of VLDLr expression under hypoxia condition. As expected, transfection of HUVECs with HIF-1α siRNA hampered the hypoxia-induced upregulation of VLDLr expression (Fig. 4A). Additionally, stabilization of HIF-1α by dimethyloxalylglycine (DMOG) led to increased VLDLr expression (Fig. 4B). Further, Cobalt (II) Chloride ($CoCl_2$), a chemical inducer of HIF-1α, increased HIF-1α expression, as well as expression of VLDLr (Fig. 4C). Moreover, overexpression of VLDLr in the condition of HIF-1α inhibition also induced ER stress and apoptosis under hypoxia (Supplementary Fig. 1). In addition, hypoxia also induces HIF-2α expression. However HIF-2α siRNA showed no effect on VLDLr expression (Supplementary Fig. 2A and B). Taken these findings together, hypoxia-induced enhancement in VLDLr expression might depend on HIF-1α.

To further study the essential role of HIF-1α in the regulation of VLDLr expression, reporter gene assays was carried out by coupling the *vldlr* promoter to the luciferase gene. The data showed that

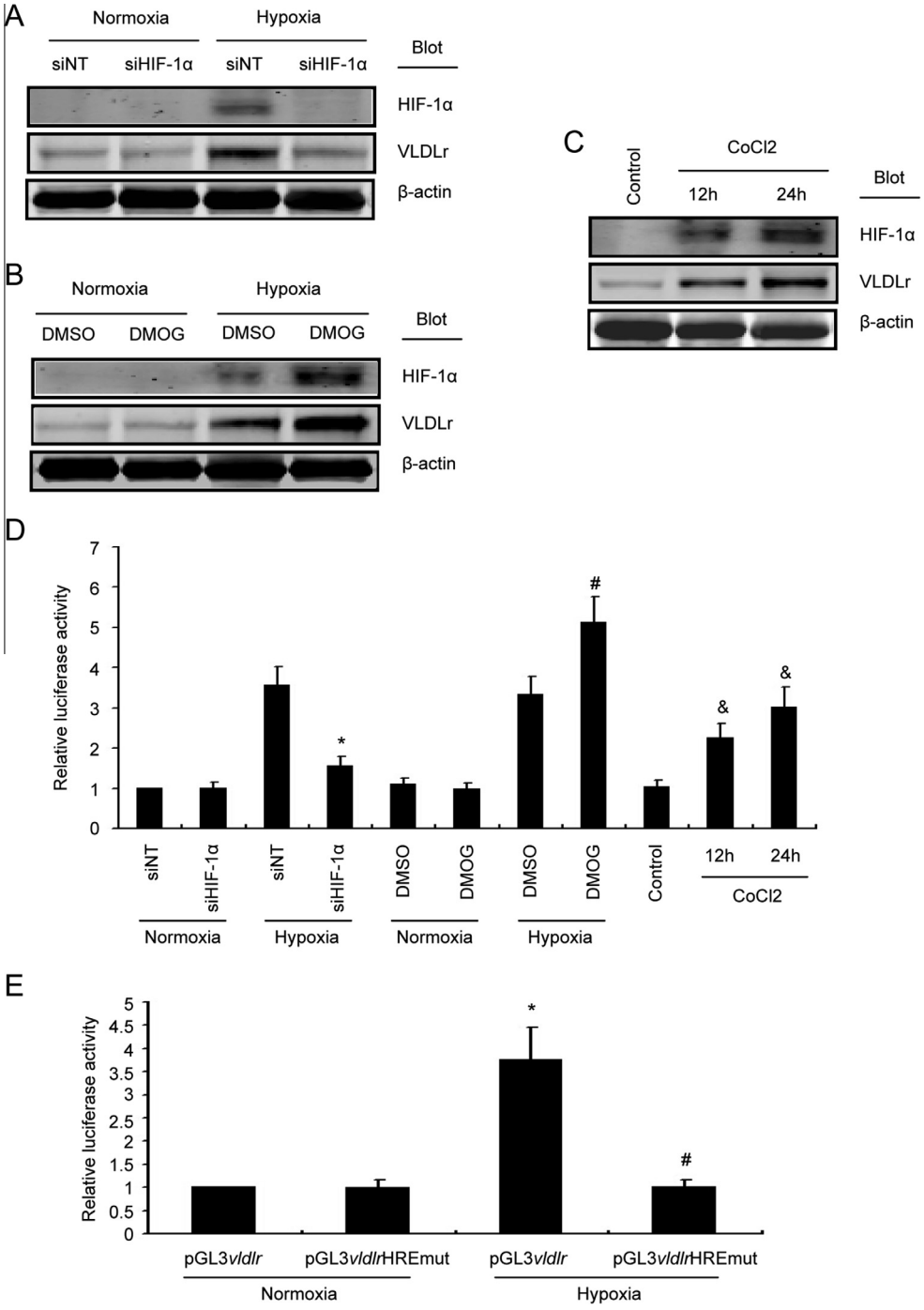

Fig. 4. Hypoxia induces expression of VLDLr through HIF-1α-dependent manner. (A) HUVECs were transfected with non-targeting control siRNA (siNT) or HIF-1α siRNA (siHIF-1α), and after 48 h, cells were treated with normoxia or hypoxia for 8 h. (B) HUVECs were pretreated with DMSO or the HIF-1α stabilizer DMOG (1 mmol/L) for 30 min prior to normoxia or hypoxia incubation for 8 h. (C) HUVECs were treated with $CoCl_2$ (150 μM) for 12 or 24 h. The protein levels of VLDLr, HIF-1α, and β-actin were detected by immunoblot analysis using indicated antibodies. A representative blot (top) was shown ($n = 4$). (D) HUVECs were transfected with luciferase constructs containing fragments of *vldlr* promoter, and cells were cotransfected with siRNA against HIF-1α and then cultured in normoxia or hypoxia for 8 h, or cells were pretreated with DMSO or DMOG (1 mmol/L) for 30 min prior to 8 h normoxia or hypoxia incubation, or cells were treated with $CoCl_2$ (150 μM) for 12 or 24 h. Luciferase activity was measured using Dual-luciferase Reporter Assay System ($n = 6$, means ± SE). $^{*}P < 0.05$ vs. hypoxia siNT cells; $^{\#}P < 0.05$ vs. hypoxia DMSO cells; $^{\&}P < 0.05$ vs. parallel normoxia cells. (E) Luciferase activity of cells transfected with luciferase constructs containing fragments of *vldlr* promoter (pGL3*vldlr*) or the mutant promoter (pGL3*vldlr*HREmut) and incubated in normoxia or hypoxia for 8 h ($n = 6$, means ± SE). $^{*}P < 0.05$ vs. normoxia pGL3*vldlr* cells; $^{\#}P < 0.05$ vs. hypoxia pGL3*vldlr* cells.

silencing or stabilization of HIF-1α significantly abolished or increased the influence of hypoxia on the promotion of luciferase activity, respectively (Fig. 4D). Furthermore, HIF-2α siRNA displayed no effect on the luciferase activity (Supplementary Fig. 1C). These results suggested the importance of HIF-1α in the promoter activity of *vldlr*. In silico analysis indicated the presence of a potential HRE between −161 and −158 bp. Mutation of this site completely inhibited the luciferase activity induced by hypoxia (Fig. 4E). Collectively, we determined that HIF-1α is crucial for the HRE activity in the *vldlr* promoter.

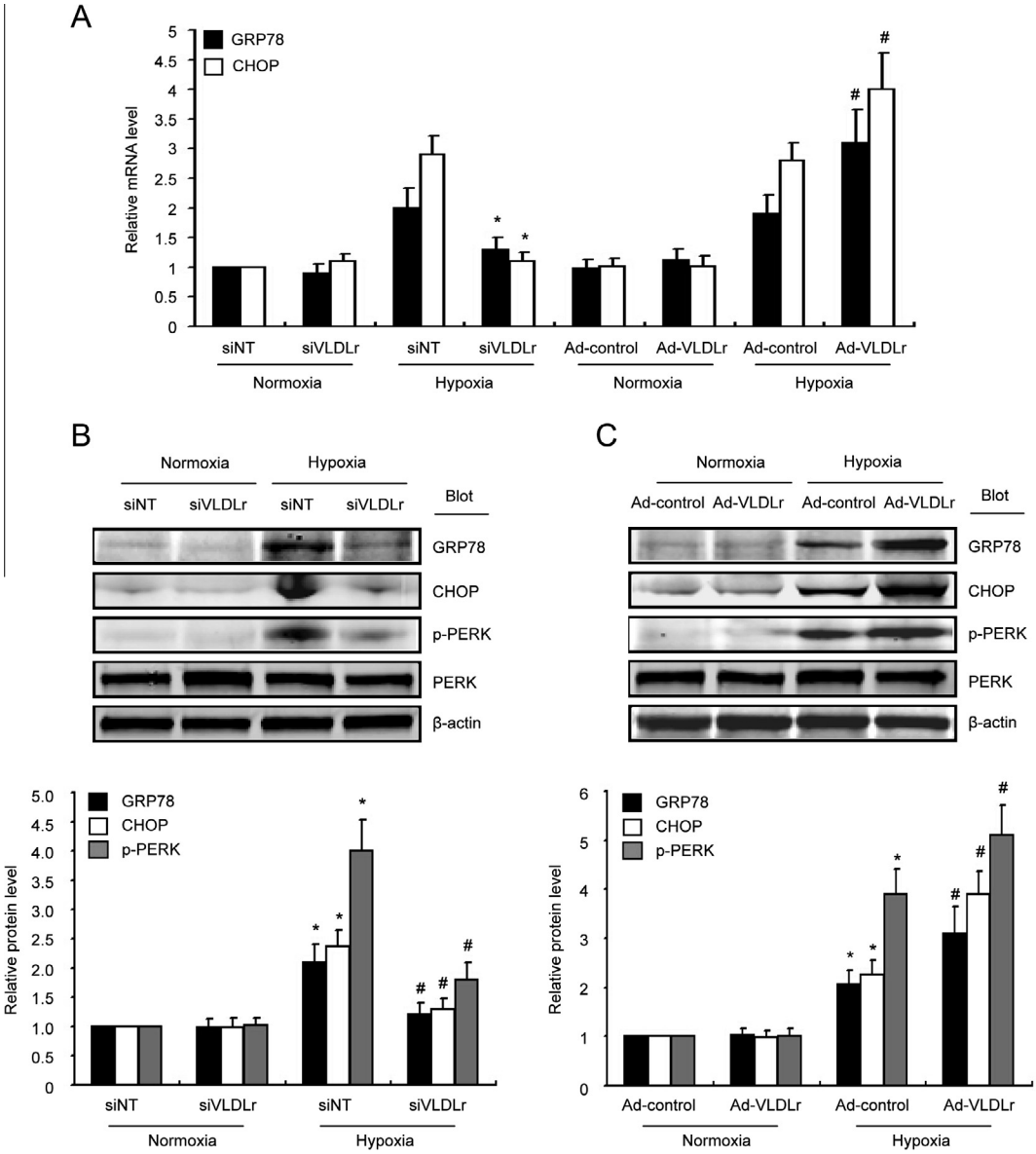

Fig. 5. VLDLr is essential for Hypoxia-triggered ER stress. HUVECs were transfected with non-targeting control siRNA or VLDLr siRNA, or HUVECs were transfected with adenovirus vector (Ad-control) or VLDLr (Ad-VLDLr), and after 48 h, cells were incubated in normoxia or hypoxia for 8 h. (A) The relative mRNA levels of GRP78 and CHOP were detected by q-PT-PCR ($n = 6$, means ± SE). $^{*}P < 0.05$ vs. hypoxia siNT cells; $^{\#}P < 0.05$ vs. hypoxia Ad-control cells. (B and C) The protein levels of GRP78, CHOP, p-PERK, PERK, and β-actin were detected by immunoblot analysis using indicated antibodies. A representative blot (top) was shown ($n = 4$). Quantitative analysis of GRP78, CHOP, and p-PERK (bottom) was expressed as fold increase above control group ($n = 4$, means ± SE). $^{*}P < 0.05$ vs. parallel normoxia control cells; $^{\#}P < 0.05$ vs. parallel hypoxia control cells.

3.5. VLDLr mediates hypoxia-induced endothelial ER stress

We examined whether hypoxia-induced VLDLr expression might involved in promoting endothelial ER stress. Compared with cells transfected with non-target in siRNA, knockdown of VLDLr by siRNA abolished increased expression levels of GRP78 and CHOP (Fig. 5A and B). Instead, overexpression of VLDLr exacerbated GRP78 and CHOP expression under hypoxia condition (Fig. 5A and C). In addition, we showed that hypoxia-induced increases in phosphorylation of PERK were markedly inhibited or promoted in VLDLr-silenced or VLDLr-overexpressed cells, respectively (Fig. 5). These findings thus indicated the essentiality of VLDLr for hypoxia-triggered endothelial ER stress.

3.6. VLDLr mediates hypoxia-induced endothelial apoptosis

To determine whether VLDLr affect hypoxia-induced endothelial apoptosis, the expression levels of antiapoptotic Bcl-2, proapoptotic Bax, and activation of caspases was examined by immunoblot in VLDLr-differently-expressed or VLDLr-overexpressed cells. Knockdown or overexpression of VLDLr significantly abolished or exacerbated cleaved levels of caspase-3, caspase-9, and caspase-4, respectively (Fig. 6A and B). However no obvious difference was detected in cleaved caspase-8 (Fig. 6A and B). In addition, the antiapoptotic Bcl-2 was inhibited by hypoxia, and the inhibited effect was largely attenuated or promoted by transfection of cells with VLDLr siRNA or VLDLr expression adenovirus

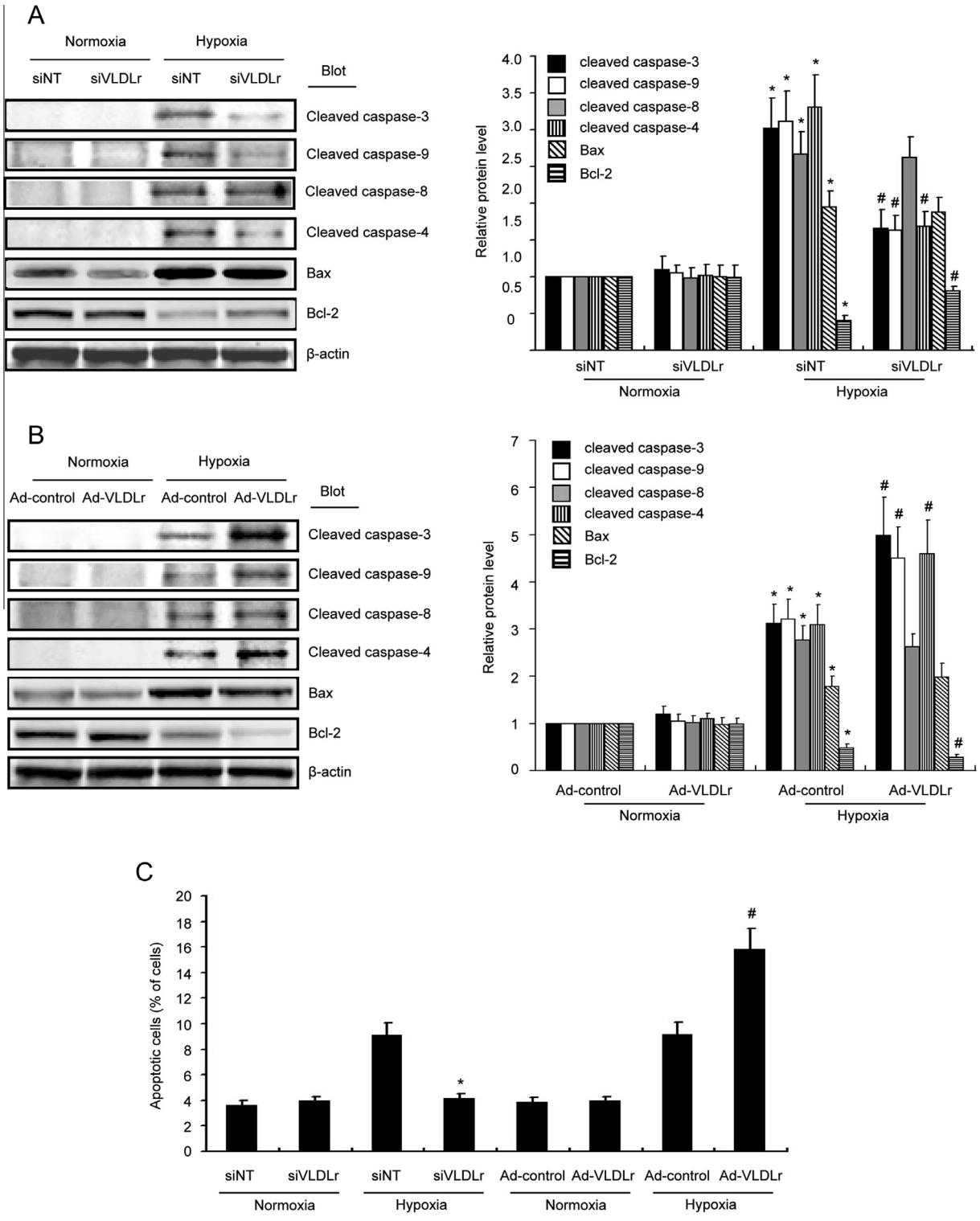

Fig. 6. VLDLr is essential for Hypoxia-induced apoptosis. HUVECs were transfected with non-targeting control siRNA or VLDLr siRNA, or HUVECs were transfected with Ad-control or Ad-VLDLr, and after 48 h, cells were incubated in normoxia or hypoxia for 12 h. (A and B) The protein levels of cleaved caspase-3, cleaved caspase-9, cleaved caspase-8, cleaved caspase-4, Bcl-2, Bax, and β-actin were detected by immunoblot analysis using indicated antibodies. A representative blot (left) was shown ($n = 4$). Quantitative analysis of cleaved caspase-3, cleaved caspase-9, cleaved caspase-8, cleaved caspase-4, Bcl-2, and Bax (right) was expressed as fold increase above control group ($n = 4$, means ± SE). $^{*}P < 0.05$ vs. parallel normoxia control cells; $^{\#}P < 0.05$ vs. parallel hypoxia control cells. (C) The cell apoptosis was measured by flow cytometry analysis and quantitative analysis of apoptotic cells was shown ($n = 4$, means ± SE). The values represent the mean percentages of apoptotic cells in different groups. $^{*}P < 0.05$ vs. hypoxia siNT cells; $^{\#}P < 0.05$ vs. hypoxia Ad-control cells.

vector, respectively (Fig. 6A and B). Interestingly, we did not detect significant changes in the expression of proapoptotic Bax between different expression levels of VLDLr (Fig. 6A and B). Furthermore, flow cytometry analysis showed that cells knockout or overexpression of VLDLr presented a lower or elevated apoptotic level compared with parallel VLDLr normally expressed cells (Fig. 6C). Taken together, these results suggested a causal correlation between VLDLr and endothelial apoptosis in hypoxia condition.

4. Discussion

In the current study, we demonstrated for the first time that hypoxia induced VLDLr expression which mediated the generation of endothelial ER stress and apoptosis. The induction of VLDLr expression by hypoxia was mediated by the interaction between HIF-1α and HRE in the *vldlr* promoter. Thus, our findings present novel molecular mechanisms that could contribute to the elucidation of endothelial ER stress and apoptosis under hypoxia condition.

The vascular endothelium maintains vascular homeostasis through its important functions such as regulation of blood pressure and coagulation, involvement of inflammatory processes, and mediation of atherosclerotic lesions and stroke. The endoplasmic reticulum (ER) is a highly dynamic organelle existed in all eukaryotic cells [13]. It plays a crucial roles in the folding of membrane and secretory proteins, lipid biosynthesis, and calcium homeostasis. The disturbed ER homeostasis results in accumulation of misfolded or unfolded proteins in the ER, thus directing to the activation of a special cellular process called UPR, which oftentimes represents the event of ER stress [14]. Compelling evidences indicated the importance of UPR and ER stress in the vascular complications [15]. Damage of endothelial cells can be due to not only oxidative stress, but also condition of ER stress [16]. Endothelial cells are always predisposed to ER stress, since they were exposed to multi-factor and multi-event status in blood, and possesses a high volume of protein synthesis [17]. In our results, hypoxia triggered endothelial ER stress manifested by elevated mRNA and protein levels of GRP78 and CHOP and increase of phosphorylated PERK (Fig. 1), a finding that is in concert with some previous studies [18,19]. Primarily, the goal of UPR is to alleviate ER stress and maintain cell survival. If ER stress lasted much longer or too severe, signaling switches from pro-survival to pro-death, thus triggering ER stress-induced apoptosis [20,21]. As a counterpart, endothelial apoptosis after hypoxia was also detected, and death receptor-initiated (cleaved caspase-8), mitochondrial-dependent (cleaved caspase-9, Bax, and Bcl-2), and ER stress-induced (cleaved caspase-4) apoptosis pathways were triggered (Fig. 2).

VLDLr is found in most tissues of the body, with particularly high expression in fatty acid tissues due to their high level of VLDLr's primary ligand, triglycerides. Endothelial cells express low amount of VLDLr physiologically [22,23]. Previous studies indicated that hypoxia promoted VLDLr mRNA expression in human monocytes and Müller cells [24,25]. In concert with these findings, we provided evidence that hypoxic stress induced VLDLr expression in endothelial cells (Fig. 3). We also showed that HIF-1α was important for the hypoxia-induced VLDLr expression in endothelial cells. Silencing of HIF-1α expression or stabilization of HIF-1α significantly inhibited or enhanced VLDLr expression, respectively (Fig. 4A and B). $CoCl_2$, a chemical inducer of HIF-1α, enhanced expression of VLDLr (Fig. 4C). Moreover, using reporter gene assay, we found a crucial role of HIF-1α in triggering *vldlr* promoter activity by its interaction with HRE in the promoter (Fig. 4D and E). Thus we concluded that hypoxia-induced VLDLr expression was mediated by binding of HIF-1α to HRE in the *vldlr* promoter.

As a peripheral remnant lipoprotein receiver, VLDLr is generally linked to the lipid metabolism. More recently, the possibility of its biological function extended to include signal transduction, cellular proliferation, and tumor pathogenesis [4]. With respect to endothelial cells, there were some interesting findings. VLDLr, as a novel endothelial receptor for fibrin, promoted fibrin-dependent transmigration of leukocyte and thereby the subsequent inflammation [7]. The suppression of endothelial cell proliferation by tissue factor pathway inhibitor was mediated by VLDLr [26]. Loss of VLDLr activated retinal vascular endothelial cells and promoted angiogenesis [6]. Further, it was reported that VLDLr was essential for the inhibition of microvascular endothelial cell division [8]. Therefore, all these evidences proposed an important action of VLDLr on regulation of endothelial biology. Our results provided a causal relationship between VLDLr and endothelial ER stress, a finding manifested by the responsiveness that the expression of GRP78, CHOP, and phosphorylated PERK was affected by differentially expressed VLDLr (Fig. 5). Furthermore, VLDLr was also an active mediator within endothelial apoptosis. Knockdown or overexpression of VLDLr inhibited or aggravated apoptosis induced by hypoxia for 12 h (Fig. 6). Interestingly, although VLDLr influenced hypoxia-mediated activation of caspase-3, caspase-9, and caspase-4 and downregulation of Bcl-2, there were no significant alterations with respect to Bax and cleaved caspase-8 (Fig. 6A and B). Thus, it seemed that VLDLr mediated endothelial apoptosis special somehow. However, in our results, VLDLr did not affect apoptosis significantly under hypoxia for 24 h (data not shown). It was shown that VLDLr expression was elevated at early stage of hypoxia, and after 12 h of hypoxia it declined (Fig. 3). Thus, we speculated that VLDLr was responsible for the apoptosis in the early stage of hypoxia. Due to the decline of VLDLr expression, the apoptosis was not mediated by VLDLr in the late stage of hypoxia. Of course, there may be negative mediator involved in the modulation of VLDLr expression beyond the positive regulator of HIF-1α.

In conclusion, our study provided evidence, for the first time, that hypoxia triggers endothelial ER stress and apoptosis through induction of VLDLr expression which involves the enhanced *vldlr* promoter activity by HIF-1α interaction with HRE in the promoter. Elucidation of the molecular mechanisms by which hypoxia induces endothelial ER stress and apoptosis may have significance for therapy to reduce vascular ischemic injury.

Disclosures

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.09.046.

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