Original Article

Role of PERK/eIF2α/CHOP Endoplasmic Reticulum Stress Pathway in Oxidized Low-density Lipoprotein Mediated Induction of Endothelial Apoptosis*

TAO Yong Kang[1,&], YU Pu Lin[2,&], BAI Yong Ping[3], YAN Sheng Tao[1], ZHAO Shui Ping[4,#], and ZHANG Guo Qiang[1,#]

1. Departments of Emergency, China-Japan Friendship Hospital, Beijing 100029, China; 2. Beijing Institution of Geriatrics, Beijing Hospital, Beijing 100730, China; 3. Department of Geriatric Medicine, Xiangya Hospital, Central South University, Changsha 410008, Hunan, China; 4. Department of Cardiology, The Second Xiangya Hospital, Central South University, Changsha 410011, Hunan, China

Abstract

Objective PERK/eIF2α/CHOP is a major signaling pathway mediating endoplasmic reticulum (ER) stress related with atherosclerosis. Oxidized LDL (ox-LDL) also induces endothelial apoptosis and plays a vital role in the initiation and progression of atherosclerosis. The present study was conducted to explore the regulatory effect of ox-LDL on PERK/eIF2α/CHOP signaling pathway in vascular endothelial cells.

Methods The effects of ox-LDL on PERK and p-eIF2α protein expression of primary human umbilical vein endothelial cells (HUVECs) were investigated by Western blot analysis. PERK gene silencing and selective eIF2α phosphatase inhibitor, salubrinal were used to inhibit the process of ox-LDL induced endothelial cell apoptosis, caspase-3 activity, and CHOP mRNA level.

Results Ox-LDL treatment significantly increased the expression of PERK, PERK-mediated inactivation of eIF2α phosphorylation, and the expression of CHOP, as well as the caspase-3 activity and apoptosis. The effects of ox-LDL were markedly decreased by knocking down PERK with stable transduction of lentiviral shRNA or by selective eIF2α phosphatase inhibitor, salubrinal.

Conclusion This study provides the first evidence that ox-LDL induces apoptosis in vascular endothelial cells mediated largely via the PERK/eIF2α/CHOP ER-stress pathway. It adds new insights into the molecular mechanisms underlying the pathogenesis and progression of atherosclerosis.

Key words: PERK; eIF2α; CHOP; Endoplasmic reticulum stress; Oxidized low-density lipoprotein; Endothelial cell; Apoptosis; Atherosclerosis; Caspase-3

Biomed Environ Sci, 2016; 29(12): 868-876 *doi: 10.3967/bes2016.116* *ISSN: 0895-3988*

www.besjournal.com (full text) *CN: 11-2816/Q*

INTRODUCTION

Cardiovascular disease is currently the leading cause of death and illness in developed countries[1]. Notably, atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries, is the single most important contributor to cardiovascular diseases[1]. Previous studies have revealed several mechanisms that

*This work was supported by State Key Clinical Specialty Construction Project, China.

&The authors contributed equally to this work as co-first author.

#Correspondence should be addressed to: ZHANG Guo Qiang, MD, Tel: 86-10-84205174, E-mail: zhangchong2003@vip.sina.com; ZHAO Shui Ping, MD, Tel: 86-731-84805463, E-mail: zhaosp@medmail.com.cn

Biographical notes of the first authors: TAO Yong Kang, male, born in 1982, MD, majoring in emergency medicine and cardiology; YU Pu Lin, male, born in 1963, PHD, majoring in Geriatric medicine.

contribute to the development of atherosclerosis, including 1) uptake and modification of the apoB lipoprotein; 2) migration and activation of inflammatory cells, particularly macrophages; 3) migration and proliferation of vascular smooth muscle cells; 4) metabolism of collagen; and 5) activation of coagulation factors and platelets[2-5]. Recent studies have indicated that endoplasmic reticulum (ER) stress is a new mechanism underlying the pathogenesis of atherosclerosis and its complications[6].

The ER is a key subcellular compartment responsible for the synthesis and folding of proteins[7]. Different physiological and pathological perturbations interfere with protein folding processes in the ER lumen, leading to accumulation of unfolded or misfolded proteins, a cellular condition termed as ER stress[7]. The ER responds to ER stress by activating intracellular signal transduction pathways, collectively termed as the unfolded protein response (UPR)[7]. If this response is unable to recover ER homeostasis, it can result in cell apoptosis[7]. ER stress activates three stress sensors, e.g., protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring kinase 1 (IRE1), representing three branches of UPR[8]. PERK is an ER transmembrane protein kinase that inhibits protein translation through inactivation of eukaryotic translation initiation factor 2 α-subunit (eIF2α) by serine phosphorylation, which results in increased translation of ATF4, a transcription factor that upregulates a subset of UPR genes including CCAAT/Enhancer-Binding Protein Homologous Protein (CHOP)[8]. Also, known as growth arrest and DNA damage inducible gene (GADD153), CHOP has a pro-apoptotic activity and is critical for triggering apoptosis in response to ER stress[8-9].

Development of atherosclerosis disease is closely linked to both oxidative stress and endothelial cell dysfunction[10]. The production of reactive oxygen species (ROS) in vascular endothelial cells induces oxidation of low-density lipoproteins (LDL) and results in increased levels of oxidized LDL (ox-LDL), a key mediator of atherosclerosis[11]. Accumulating evidence has demonstrated that ox-LDL plays a vital role in the mediation of endothelial dysfunction and induces endothelial apoptosis as well as inflammation, which contributes to the initiation and progression of atherosclerosis[12-13].

In the present study, we explored the regulatory effect of ox-LDL on PERK/eIF2α/CHOP signaling pathway in vascular endothelial cells, and its impact on endothelial apoptosis.

MATERIALS AND METHODS

Human Umbilical Vein Endothelial Cell (HUVEC) Culture

Primary HUVECs (ATCC PCS-100-010) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were grown in vascular cell basal medium (ATCC PCS-100-030) supplemented with endothelial cell growth kit-BBE (ATCC PCS-1-040) (ATCC) and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, Beijing, China) in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Preparation of Ox-LDL

Plasma was obtained from healthy volunteers after obtaining their informed consent, and lipoproteins were isolated by sequential flotation ultracentrifugation[14]. Isolated LDL was desalted on an Econo-Pac 10 DG chromatography column (Bio-Rad, Hercules, CA, USA) and sterile-filtered (0.22 μm pore size; Millipore, Bedford, MA, USA). To oxidize LDL, the lipoprotein (0.5 mg/mL in sterile PBS) was incubated with 5 μm $CuSO_4$ at 37 °C for 20 h. Ox-LDL was concentrated by centrifuging in Amicon Centriplus YM-100 tubes (Millipore, Bedford, MA, USA) for 2 h at 3000 ×*g* and 8 °C, and subsequently sterile-filtered. The oxidation was confirmed by measuring thiobarbituric acid-reactive substances (TBARS) using tetra-ethoxy-propane (TEP) as a standard[15]. We used the ox-LDL within 24 h of preparation, and endothelial cells were incubated with ox-LDL dissolved in culture medium containing 1% FBS with or without selective eIF2α phosphatase inhibitor, salubrinal (Sigma-Aldrich)[16].

Stable Lentiviral Transduction

The PERK (sc-36213-V) shRNA lentiviral particles purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) contain expression constructs encoding target-specific shRNA designed to specifically knockdown PERK gene expression. The control shRNA lentiviral particles (sc-108080; Santa Cruz Biotechnology) contain a non-targeting shRNA sequence that will not lead to specific degradation of any cellular mRNA. Lentiviral transduction was performed and pools of stable transductants were generated via selection with puromycin (4.5 μg/mL,

Sigma-Aldrich) by the manufacturer's protocol (Santa Cruz Biotechnology).

Western Blot Analysis

HUVECs were lysed with a hypotonic buffer containing 2% Nonidet-P and a protease inhibitor cocktail (Sigma-Aldrich) by sonication three times for 3 seconds on ice. The supernatant obtained after centrifugation at 2000 ×*g* for 15 min at 4 °C was used for protein concentration determination by the Coomassie blue method and for subsequent steps. Equal amount of proteins for each sample were separated by 8%-15% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride microporous membrane (Millipore). Membranes were incubated for 1 h with rabbit anti-human PERK polyclonal antibody (H-300) (sc-13973) (1:1000 dilution), goat anti-human eIF2α polyclonal antibody (K-17) (sc-30882) (1:100 dilution), rabbit anti-human phosphorylated eIF2α polyclonal antibody (sc-101670) (1:1000 dilution), or mouse anti-human β-actin monoclonal antibody (ACTBD11B7) (sc-81178) (1:1000 dilution), and then washed and incubated for 1 h with 1:5000 dilution of secondary antibodies including bovine anti-rabbit IgG-horseradish peroxidase (HRP) (sc-2370), bovine anti-goat IgG-HRP (sc-230), and bovine anti-mouse IgG-HRP (sc-2371), respectively. Peroxidase was determined with a GE Healthcare ECL kit (Shanghai, China). Three independent experiments were performed.

Real-time Quantitative RT-PCR

RNA was prepared from cells using TRIzol reagent, and cDNAs were synthesized using SuperScript II reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Real-time quantitative PCR was performed on an Abi-Prism 7700 Sequence Detection System, with use of the fluorescent dye SYBR Green Master Mix (Applied Biosystems, Beijing, China) as described by the manufacturer. The primers used are as follows: for PERK, 5′-ATCCCCCATGGAACGACCTG-3′ (forward) and 5′-ACCCGCCAGGGACAAAAATG-3′ (reverse); for CHOP, 5′-GCCTTTCTCCTTTGGGACACTGTCCAGC-3′ (forward) and 5′-CTCGGCGAGTCGCCTCTACTTCCC-3′ (reverse); for GAPDH, 5′-CCAGCAAGAGCACAAGAGGAA-3′ (forward) and 5′-ATGGTACATGACAAGGTGCGG-3′ (reverse). Relative quantification of the mRNA level of Bmi1 was determined using the $2^{-\Delta\Delta Ct}$ method and normalized against that of GAPDH in the same sample[17]. Each experiment was repeated for three times in duplicates.

Cell Apoptosis Assay

HUVECs were cultured at 9×10^4 cells per well in 96-well tissue culture plates and incubated at 37 °C for 24 h under ox-LDL (100 μg/mL) treatment with or without salubrinal (40 μmol/L). Cell apoptosis was measured at 24 h with a microplate reader-based Titer TACS in situ apoptosis detection kit (4822-96-K; R&D systems, Minneapolis, MN, USA) as the manufacturer's instructions[18]. Each experiment was repeated for three times in duplicates.

Caspase-3 Activity Assay

The caspase-3 activity was determined with the colorimetric CaspACE Assay System (G7351) purchased from Promega (Madison, WI, USA)[19]. Briefly, HUVECs were grown in 60-mm petri dishes and incubated at 37 °C for 24 h under ox-LDL (100 μg/mL) treatment with or without salubrinal (40 μm). Cells were processed for 24 h according to the manufacturer's protocol (Promega). Supernatants of cell extracts were inoculated into microtiter wells containing caspase assay buffer, dimethyl sulfoxide, 100 mm dithiothreitol, and colorimetric caspase-3 substrate labeled with the chromophore, *p*-nitroaniline (Ac-DEVD-pNA). The plates were incubated at 37 °C for 3 h and absorbance was measured at 405 nm with a microtiter plate spectrophotometer.

Statistical Analysis

Statistical analyses were performed with SPSS for Windows 19.0 (IBM, Chicago, IL, USA). All continuous variable values were expressed as Mean ± SD. Comparison of means between two groups was performed with student *t*-test. Comparison of means among multiple groups was performed with one-way ANOVA followed by *post hoc* pairwise comparisons using Tukey's tests. A two-tailed $P < 0.05$ was considered statistically significant in this study.

RESULTS

Ox-LDL Induces Expression of PERK and Phosphorylation of eIF2α in HUVECs

To explore the potential regulatory effects of ox-LDL on PERK/eIF2α/CHOP signaling pathway in vascular endothelial cells, we treated HUVECs with ox-LDL (100 μg/mL) for 0, 12, 24, and 48 h. As shown in Figure 1, ox-LDL induced the expression of PERK and phosphorylation of eIF2α at serine 51, in a time-dependent manner, which reportedly is critical

for PERK-induced inactivation of eIF2α[7]. As PERK/eIF2α signaling is a major pathway for ER stress[7], the results suggest that ox-LDL may induce ER stress in vascular endothelial cells. To determine the role of PERK/eIF2α signaling in mediating the effects of ox-LDL on vascular endothelial cells, we knocked down PERK in HUVECs with shRNA. As shown in Figure 2, stable transduction of HUVECs with lentiviral PERK shRNA decreased the endogenous PERK protein level by about 80%, compared with the scramble shRNA control.

Effects of Inhibiting PERK/eIF2α on Apoptosis and Caspase-3 Activity Induced by Ox-LDL in HUVECs

The role of PERK/eIF2α signaling in ox-LDL-induced apoptosis in HUVECs was also analyzed. As shown in Figure 3A, ox-LDL treatment (100 μg/mL for 24 h) increased apoptosis in HUVECs by approximately 5-folds compared with the controls. The apoptotic effect of ox-LDL was decreased by approximately 75% by knocking down PERK with shRNA. Expectedly, PERK shRNA showed no

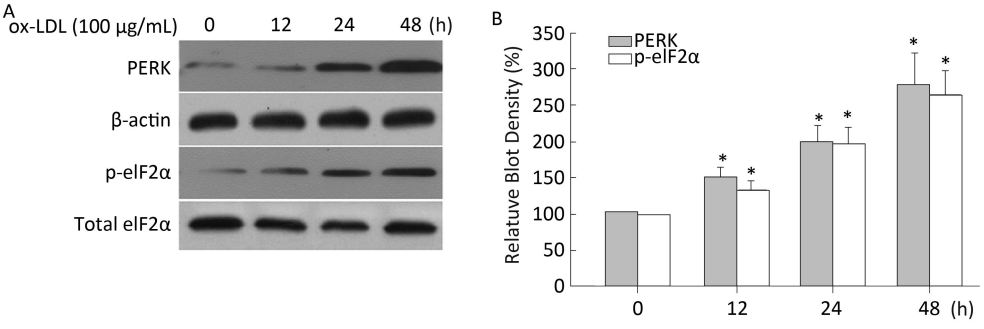

Figure 1. Effect of oxidized low-density lipoprotein (ox-LDL) on the expression of PERK and phosphorylation of eIF2α in HUVECs. A, HUVECs were treated with ox-LDL (100 μg/mL) for 0, 12, 24, and 48 h. Protein levels of PERK, phosphorylated eIF2α (at serine 51) (p-eIF2α), and total eIF2α were determined by Western blot analyses. β-actin blotting was used as a loading control. B, This figure shows the relative blot density, expressed as percentage to the relative PERK or p-eIF2α blot density in untreated control cells (0 h, designated as 100%). Density of the Western blots was measured by densitometry. Density of the PERK blot was normalized against that of β-actin to obtain a relative PERK blot density, and density of the p-eIF2α blot was normalized against that of total eIF2α to obtain a relative p-eIF2α blot density. $^{*}P < 0.05$ *vs.* control (0 h).

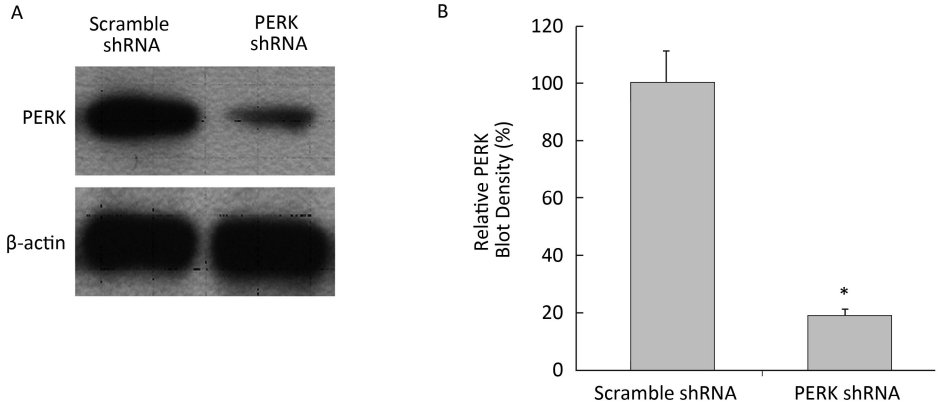

Figure 2. Stable knockdown of PERK in HUVECs. A, Lentivirial shRNA specifically against PERK was stably transduced into HUVECs. Non-targeting scramble lentiviral shRNA was used as control. Protein levels of PERK in the stable transductants were determined by Western blot analysis. β-actin blotting was used as a loading control. B, This figure shows the relative PERK blot density, expressed as percentage to the relative PERK blot density in HUVECs stably transduced with scramble shRNA (designated as 100%). Density of the Western blots was measured by densitometry. Density of the PERK blot was normalized against that of β-actin to obtain a relative PERK blot density. $^{*}P < 0.05$ *vs.* scramble shRNA.

significant effects on untreated control cells. As shown in Figure 3B, while selective eIF2α phosphatase inhibitor, salubrinal, showed no significant effects on untreated control cells, it decreased ox-LDL-induced apoptosis in HUVECs by more than 50%.

Caspase-3 is one of the key activated caspases present in apoptotic cells[20]. Apoptosis mediated by caspase-3 may be modulated by cellular oxidative stress. An increase in caspase-3-like protease activity in human endothelial cells is associated with the induction of apoptosis by ox-LDL[21]. Thus, we examined the caspase-3 activity in the experimental groups. As shown in Figure 4A, ox-LDL treatment (100 μg/mL for 24 h) increased the caspase-3 activity in HUVECs by approximately 3-folds compared with the controls. The inducing effect of ox-LDL was decreased by about 50% by PERK shRNA, which had no significant effect on untreated control cells. As shown in Figure 4B, while salubrinal showed no significant effects on untreated control cells, it decreased ox-LDL-induced caspase-3 activity in HUVECs by about 65%.

Therefore, these results suggest that ox-LDL induces apoptosis in vascular endothelial cells largely mediated via the PERK/eIF2α signaling pathway, specifically through caspase-3.

Ox-LDL Regulates PERK/eIF2α/CHOP Signaling in HUVECs

The above results suggest that PERK/eIF2α mediates the effects of ox-LDL on HUVECs. Compared with the controls, ox-LDL treatment (100 μg/mL for 24 h) increased phosphorylation of eIF2α (at serine 51) in HUVECs by over 2-folds, which was reduced by about 50% by knocking down PERK (Figure 5). CHOP, a stress-inducible transcription factor that regulates the genes encoding components involved in apoptosis, is a known downstream effector of PERK/eIF2α signaling pathway[8-9]. To confirm the crosstalk between ox-LDL and PERK/eIF2α signaling pathway, we next examined the effects of ox-LDL on the mRNA level of CHOP in the presence or absence of PERK shRNA or salubrinal. Compared with the controls, ox-LDL treatment (100 μg/mL for 24 h) increased the mRNA level of CHOP in HUVECs by 5-folds; the inducing effect of ox-LDL was decreased by about 75% by PERK shRNA, which showed no significant effects on untreated control cells (Figure 6A). As shown in Figure 6B, while salubrinal showed no significant effects on untreated control cells, it decreased the

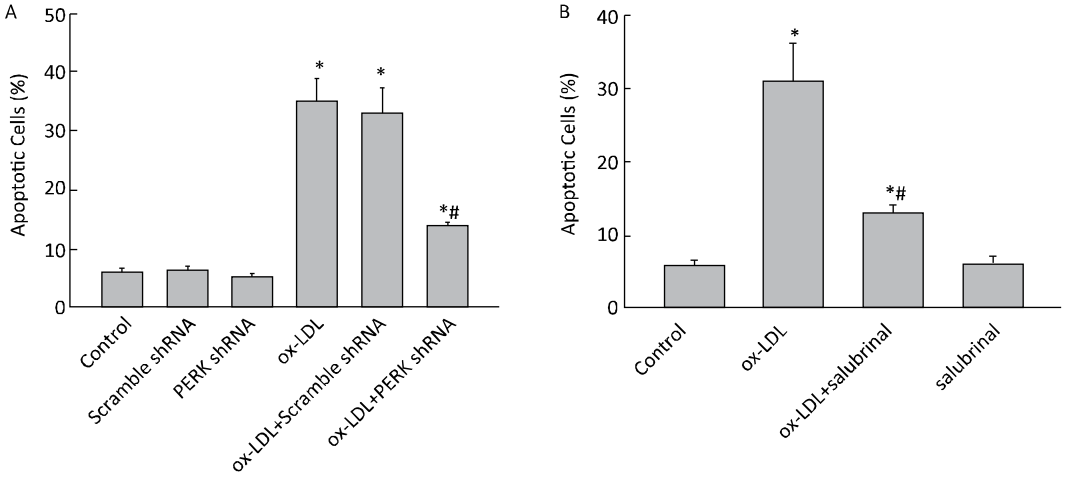

Figure 3. Effects of inhibiting PERK/eIF2α on oxidized low-density lipoprotein (ox-LDL) induced apoptosis in HUVECs. Apoptosis was measured with a microplate reader-based TiterTACS in situ apoptosis detection kit (R&D systems) in HUVECs. A, This figure depicts the apoptosis rates, expressed as percentages, in control cells, cells stably transduced with scramble shRNA, cells stably transduced with PERK shRNA, cells treated with ox-LDL (100 μg/mL for 24 h), cells stably transduced with scramble shRNA and treated with ox-LDL (100 μg/mL for 24 h), and cells stably transduced with PERK shRNA and treated with ox-LDL (100 μg/mL for 24 h). $^{*}P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* ox-LDL and ox-LDL + Scramble shRNA. B, This figure depicts the apoptosis rates, expressed as percentages, in control cells, cells treated with ox-LDL (100 μg/mL for 24 h), cells pre-treated with selective eIF2α phosphatase inhibitor salubrinal (40 μmol/L) for 1 h and then treated with ox-LDL (100 μg/mL) for 24 h, and cells pre-treated with salubrinal (40 μmol/L) for 1 h. $^{*}P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* ox-LDL.

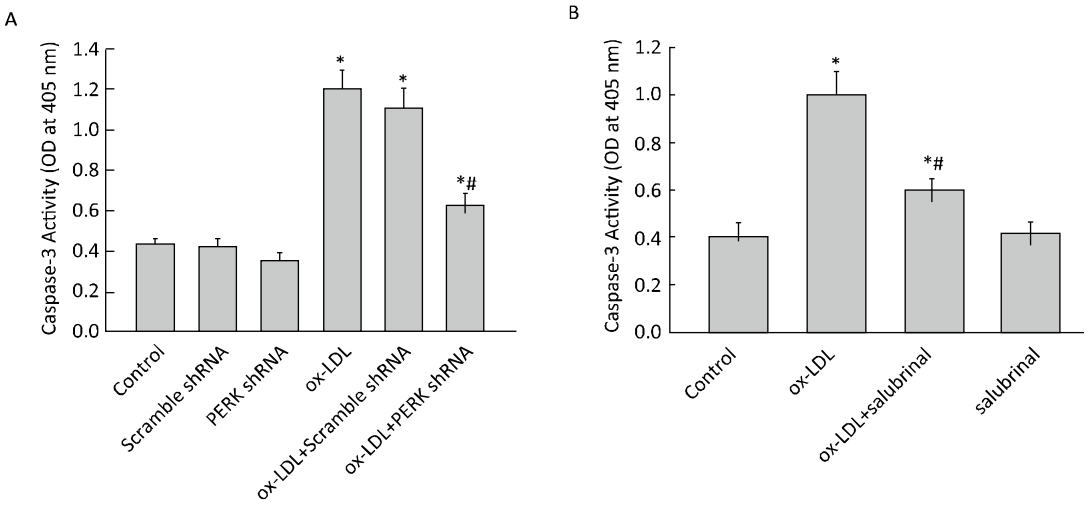

Figure 4. Effects of inhibiting PERK/eIF2α on caspase-3 activity induced by oxidized low-density lipoprotein (ox-LDL) in HUVECs. The caspase-3 activity was measured with the colorimetric CaspACE Assay System (Promega) in HUVECs and is expressed as optical density (OD) at 405 nm. A, This figure shows the caspase-3 activity was in control cells, cells stably transduced with scramble shRNA, cells stably transduced with PERK shRNA, cells treated with ox-LDL (100 μg/mL for 24 h), cells stably transduced with scramble shRNA and treated with ox-LDL (100 μg/mL for 24 h), and cells stably transduced with PERK shRNA and treated with ox-LDL (100 μg/mL for 24 h). $^{*}P < 0.05$ *vs*. control; $^{\#}P < 0.05$ *vs.* ox-LDL and ox-LDL + Scramble shRNA. B, This figure shows the caspase-3 activity in control cells, cells treated with ox-LDL (100 μg/mL for 24 h), cells pre-treated with selective eIF2α phosphatase inhibitor salubrinal (40 μmol/L) for 1 h and then treated with ox-LDL (100 μg/mL) for 24 h, and cells pre-treated with salubrinal (40 μmol/L) for 1 h. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ *vs.* ox-LDL.

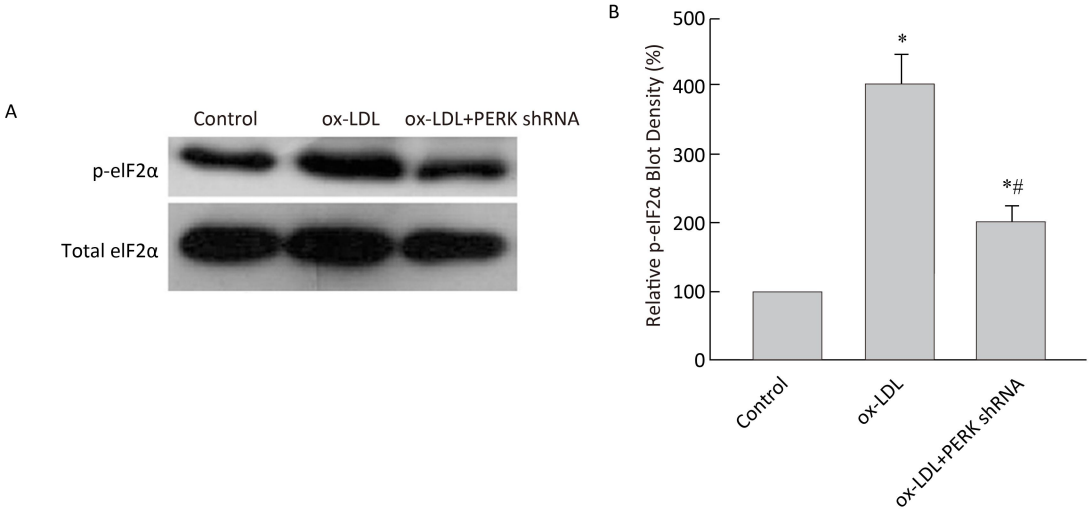

Figure 5. Effects of inhibiting PERK on phosphorylation of eIF2α induced by oxidized low-density lipoprotein (ox-LDL) in HUVECs. A, In HUVECs, protein levels of phosphorylated eIF2α (at serine 51) (p-eIF2α) and total eIF2α were determined by Western blot analyses in control cells, cells treated with ox-LDL (100 μg/mL for 24 h), and cells stably transduced with PERK shRNA and treated with ox-LDL (100 μg/mL for 24 h). B, This figure shows the relative p-eIF2α blot density, expressed as percentage to that in untreated control cells (designated as 100%). Density of the Western blots was measured by densitometry. Density of the p-eIF2α blot was normalized against that of the control group to obtain a relative p-eIF2α blot density. $^{*}P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* ox-LDL.

ox-LDL-induced mRNA level of CHOP in HUVECs by about 45%. Therefore, these results confirm that ox-LDL regulates PERK/eIF2α/CHOP signaling in HUVECs.

DISCUSSION

Atherosclerosis is the single most important contributor to the cardiovascular diseases. Ox-LDL is one of the most important risk factors of atherosclerosis. However, the exact role of ox-LDL in the pathophysiology of atherosclerosis is not clear. Recent studies have indicated that ER stress is a new mechanism underlying the pathogenesis of atherosclerosis and its complications and PERK/eIF2α/CHOP is a major signaling pathway which mediates ER stress[7-8]. Accumulating evidence has demonstrated that ox-LDL induces endothelial apoptosis and plays a vital role in the initiation and progression of atherosclerosis[12-13]. Our study's results suggest that ox-LDL induces apoptosis in vascular endothelial cells largely via PERK/eIF2α/CHOP signaling pathway.

Primary HUVECs were used as a human vascular endothelial cell model in our study. Previous studies have shown that ox-LDL treatment at 100 μg/mL for 24 h was effective on different effectors in HUVECs[22-23]. Indeed, we found that ox-LDL at 100 μg/mL increased the expression of PERK in HUVECs in a time-dependent manner; and ox-LDL at 100 μg/mL for 24 h effectively activated the PERK/eIF2α/CHOP signaling pathway in HUVECs by inducing the expression of PERK, PERK-mediated inactivation of eIF2α phosphorylation (at serine 51), and the expression of CHOP[6-8]. As PERK/eIF2α/CHOP is a major signaling pathway mediating ER stress[6-8], our results suggest that ox-LDL induces ER stress in vascular endothelial cells.

ER responds to stress by activating UPR and if UPR is insufficient to restore ER homeostasis, cells undergo apoptosis[7]. The PERK branch of UPR is strongly protective at the modest levels of signaling, but can contribute to cell death pathways, mediated by its downstream effector, CHOP, which has a pro-apoptotic activity and is critical for triggering apoptosis in response to ER stress[7,9]. In our study, ox-LDL treatment resulted in activation of PERK/

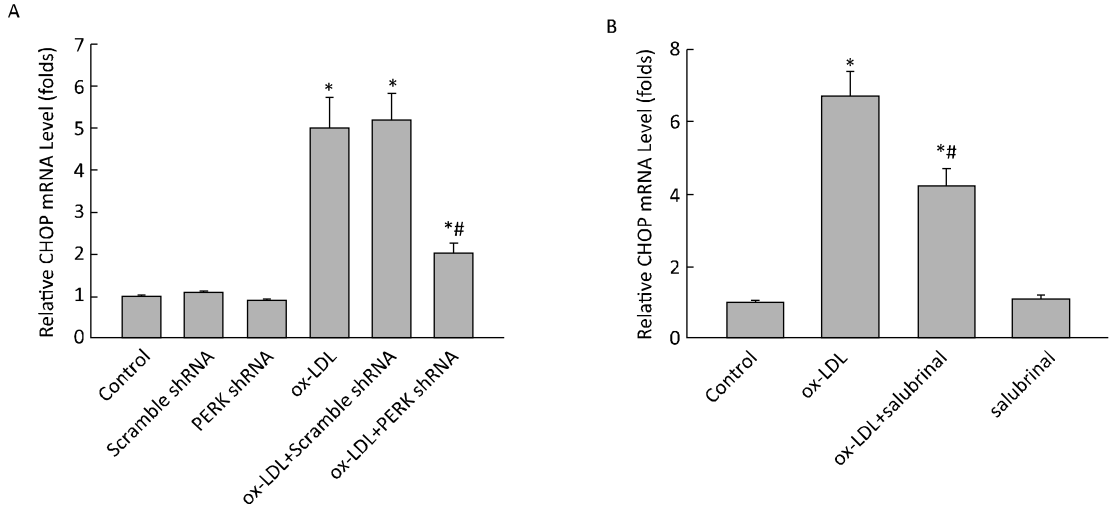

Figure 6. Effects of inhibiting PERK/eIF2α on expression of CHOP mRNA induced by oxidized low-density lipoprotein (ox-LDL) in HUVECs. The mRNA level of CHOP was measured by real-time RT-PCR in HUVECs. A. The mRNA level of CHOP was expressed as folds compared to that of the controls cells (designated as 1) in cells stably transduced with scramble shRNA, cells stably transduced with PERK shRNA, cells treated with ox-LDL (100 μg/mL for 24 h), cells stably transduced with scramble shRNA and treated with ox-LDL (100 μg/mL for 24 h), and cells stably transduced with PERK shRNA and treated with ox-LDL (100 μg/mL for 24 h). $^{*}P < 0.05$ *vs*. control; $^{\#}P < 0.05$ *vs.* ox-LDL and ox-LDL + Scramble shRNA. B, This figure depicts the relative CHOP mRNA level, expressed as folds compared to that of the controls cells (designated as 1) in cells treated with ox-LDL (100 μg/mL for 24 h), cells pre-treated with selective eIF2α phosphatase inhibitor salubrinal (40 μmol/L) for 1 h and then treated with ox-LDL (100 μg/mL) for 24 h, and cells pre-treated with salubrinal (40 μmol/L) for 1 h. $^{*}P < 0.05$ *vs*. control; $^{\#}P < 0.05$ *vs.* ox-LDL.

eIF2α/CHOP signaling pathway as well as significant apoptosis in HUVECs, suggesting that ox-LDL is an effective inducer of irreversible ER stress in endothelial cells. This is in agreement with previous studies showing that ox-LDL plays a vital role in the mediation of endothelial dysfunction and induction of endothelial apoptosis[12-13]. The apoptosis-inducing effect of ox-LDL was markedly decreased by knocking down PERK as well as by selective eIF2α phosphatase inhibitor, salubrinal[16], confirming our findings that ox-LDL induces apoptosis in vascular endothelial cells largely via PERK/eIF2α/CHOP signaling pathway. These results are in agreement with the concept of the bi-functional role of PERK in the regulation of ER stress, and apoptosis is likely played out at the level of phosphorylated eIF2a and is exemplified by the effects of inhibition of its specific phosphatases[7]. The results of the present study are also in agreement with the previous reports indicating that salubrinal protects cell from ER stress-induced apoptosis[16,24].

The UPR is mediated by three principal classes of stress sensors including PERK, ATF6, and IRE1[25]. The three UPR branches operate in parallel and use unique mechanisms of signal transduction to control the expression of specific transcription factors and signaling events that modulate a variety of UPR downstream responses, orchestrating adaptation to ER stress[7,25]. In this study, we only explored the regulatory effect of ox-LDL on the PERK branch of UPR and assessed its impact on endothelial apoptosis. The ATF6 and the IRE1 branches of UPR were not examined. We noted that the inducing effect of ox-LDL on apoptosis and caspase-3 activity in HUVECs was only abolished by 50%-75% by knocking down PERK or by salubrinal, suggesting the presence of other pathways mediating ox-LDL-induced apoptosis in vascular endothelial cells besides the PERK/eIF2α/CHOP signaling pathway. Further studies are warranted to investigate the role of ATF6 and/or the IRE1 ER-stress pathways in ox-LDL-induced endothelial apoptosis.

In conclusion, this is an advanced study to provide the evidence that ox-LDL induces apoptosis in vascular endothelial cells largely via the PERK/eIF2α/CHOP ER-stress signaling pathway. It adds new insights into the molecular mechanisms underlying the pathogenesis and progression of atherosclerosis.

Received: July 16, 2016;
Accepted: November 25, 2016

REFERENCES

1. Libby P. Inflammation in atherosclerosis. Nature, 2002; 420, 868-74.
2. Libby P. Changing concepts of atherogenesis. J Intern Med, 2000; 247, 349-58.
3. Virmani R, Burke AP, Kolodgie FD, et al. Vulnerable plaque: the pathology of unstable coronary lesions. J Interv Cardiol, 2002; 15, 439-46.
4. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. Arterioscler Thromb Vasc Biol, 2005; 25, 2255-64.
5. Kaneto H. Oxidative stress and ER stress in diabetes. Nihon Rinsho, 2011; 69(suppl), 171-5.
6. Liu ZW, Wang HY, Guan L, et al. Regulatory effects of hydrogen sulfide on alveolar epithelial cell endoplasmic reticulum stress in rats with acute lung injury. World J Emerg Med, 2015; 6, 67-73.
7. Walter P, Ron D. The unfolded protein response: from stress pathway to homeostatic regulation. Science, 2011; 334, 1081-6.
8. Hetz C, Martinon F, Rodriguez D, et al. The unfolded protein response: integrating stress signals through the stress sensor IRE1α. Physiol Rev, 2011; 91, 1219-43.
9. Zinszner H, Kuroda M, Wang X, et al. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev, 1998; 12, 982-95.
10. Morawietz H. Acromegaly and oxidative stress: impact on endothelial dysfunction and atherosclerosis. Horm Metab Res, 2013; 45, 255-6.
11. Bonomini F, Tengattini S, Fabiano A, et al. Atherosclerosis and oxidative stress. Histol Histopathol, 2008; 23, 381-90.
12. Ou HC, Lee WJ, Lee IT, et al. Ginkgo biloba extract attenuates oxLDL-induced oxidative functional damages in endothelial cells. J Appl Physiol, 2009; 106, 1674-85.
13. Tsai KL, Huang YH, Kao CL, et al. A novel mechanism of coenzyme Q10 protects against human endothelial cells from oxidative stress-induced injury by modulating NO-related pathways. J nutr biochem, 2012; 23, 458-68.
14. Mackness MI, Durrington PN. Lipoprotein separation and analysis for clinical studies. In Lipoprotein Analysis: a Practical Approach. Converse CA and Skinner ER (eds.) Oxford University Press, Oxford, 1992; pp. 1-42.
15. Sparrow CP, Parthasarathy S, Steinberg D. Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A2 mimics cell-mediated oxidative modification. J Lipid Res, 1988; 29, 745-53.
16. Wu L, Luo N, Zhao HR, et al. Salubrinal protects against rotenone-induced SH-SY5Y cell death via ATF4-parkin pathway. Brain Res, 2014; 1549, 52-62.
17. Wang H, Zhou M, Brand J, et al. Inflammation activates the interferon signaling pathways in taste bud cells. J Neurosci,

2007; 27, 10703-13.
18.Byun K, Bayarsaikhan E, Kim CY, et al. Induction of neuronal death by microglial AGE-albumin: implications for Alzheimer's disease. PLoS One, 2012; 7, e37917.
19.Babich H, Krupka ME, Nissim HA, et al. Differential in vitro cytotoxicity of (-)-epicatechin gallate (ECG) to cancer and normal cells from the human oral cavity. Toxicol In Vitro, 2005; 19, 231-42.
20.Woo M, Hakem R, Soengas MS, et al. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev, 1998; 12, 806-19.
21.Dimmeler S, Haendeler J, Galle J, et al. Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases. A mechanistic clue to the 'response to injury' hypothesis. Circulation, 1997; 95, 1760-3.
22.Dong Q, Xiang R, Zhang DY, et al. Ox-LDL increases OX40L in endothelial cells through a LOX-1-dependent mechanism. Braz J Med Biol Res, 2013; 46, 765-70.
23.Huang Y, Mironova M, Lopes-Virella MF. Oxidized LDL stimulates matrix metalloproteinase-1 expression in human vascular endothelial cells. Arterioscler Thromb Vasc Biol, 1999; 19, 2640-7.
24.Boyce M, Bryant KF, Jousse C, et al. A selective inhibitor of eIF2alpha dephosphorylation protects cells from ER stress. Science, 2005; 307, 935-9.
25.Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nat Rev Mol Cell Biol, 2007; 8, 519-29.