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Original Article

Pollen Typhae Total Flavone Inhibits Endoplasmic Reticulum Stress-Induced Apoptosis in Human Aortic-Vascular Smooth Muscle Cells through Down-Regulating PERK-eIF2α-ATF4-CHOP Pathway*

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ABSTRACT **Objective:** To test the hypothesis that the inhibition of endoplasmic reticulum (ER) stress-induced apoptosis in oxidized low-density lipoproteins (ox-LDL)-induced human aortic-vascular smooth muscle cells (HA-VSMCs) was associated with suppression of the protein kinase RNA-like ER kinase (PERK)-eukaryotic translation initiation factor 2α (eIF2α)-activating transcription factor 4 (ATF4)-CCAAT/enhancer binding protein homologous protein (CHOP) signaling pathway by *Pollen Typhae* total flavone (PTF). **Methods:** Primary HA-VSMCs were cultured and identified. The cultured HA-VSMCs were randomized into 5 groups, including a normal control group, an ox-LDL group (70 μg/mL high ox-LDL), an HPTF group (70 μg/mL high ox-LDL+500 μg/mL PTF), an MPTF group (70 μg/mL high ox-LDL+250 μg/mL PTF), and a LPTF group (70 μg/mL high ox-LDL+100 μg/mL PTF) in the first part; and a normal control group, an ox-LDL group (70 μg/mL high ox-LDL), an MPTF group (70 μg/mL high ox-LDL+250 μg/mL PTF), a shRNA group (transducted with PERK shRNA lentiviral particles), a scramble shRNA group (transducted with control shRNA lentiviral particles), an MPTF+ox-LDL+shRNA group (250 μg/mL PTF+70 μg/mL high ox-LDL+PERK shRNA lentiviral particles) and an ox-LDL+shRNA group (70 μg/mL high ox-LDL+PERK shRNA lentiviral particles) in the second part. The protein expression levels of ER-associated apoptosis proteins were detected by Western blot, and their mRNA expression levels were detected by quantitative real-time reverse transcription-polymerase chain reaction. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was applied to test cell viability, and the level of apoptosis was monitored by flow cytometry. **Results:** The MTT assay and flow cytometry showed that the ox-LDL group had a significant increase in apoptosis, which was attenuated in PTF treatment groups and shRNA groups. Moreover, the ox-LDL group had increased protein and mRNA levels of binding immunoglobulin protein and ER-associated apoptosis proteins, such as PERK, eIF2α, ATF4 and CHOP, which were attenuated in PTF treatment groups and shRNA groups. **Conclusions:** The apoptosis induced by ox-LDL had a strong relation to ER stress. The protective effect of PTF on ER stress-induced apoptosis was associated with inhibition of the PERK-eIF2α-ATF4-CHOP pathway, which might be a potential therapeutic strategy for enhancing the stability of atherosclerotic plaques.

KEYWORDS *Pollen Typhae* total flavone, endoplasmic reticulum stress, protein kinase RNA-like endoplasmic reticulum kinase-eukaryotic translation initiation factor 2α-activating transcription factor 4-CCAAT/enhancer binding protein homologous protein pathway, apoptosis, vulnerable atherosclerotic plaque

The rupture of vulnerable plaques contributed to thrombotic clogging of blood vessels, leading to angina, acute myocardial infarction, and atherothrombotic stroke.(1) By summarizing previous studies, the name "vulnerable plaque" was proposed by Muller, et al in 1989, for unstable plaques that had a susceptibility to rupture.(2) Vulnerable plaques were characterized by an enlarged necrotic lipid core with an overlying thin fibrous cap that tended to rupture, which was infiltrated by macrophages and lymphocytes.(3) In addition, there were few vascular

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smooth muscle cells (VSMCs) within the fibrous cap.[4] VSMCs, endothelial cells (ECs), and fibroblasts mainly composed the blood vessel wall. ECs dysfunction stimulated the development and progression of atherosclerosis in the early stage,[5,6] while apoptosis of VSMCs, causing plaques to become vulnerable, played an important role in the late stage of atherosclerosis.[7,8] The fibrous cap of normal plaques was mainly composed of VSMCs and the extracellular matrix that maintained the stability of the cap. In contrast, increased apoptosis of VSMCs and reduced collagen within the thin fibrous cap contributed to plaque vulnerability, leading to rupture.[9]

Previous studies on VSMCs induced by oxidized low-density lipoproteins (ox-LDL) and animal models, such as mice, with atherosclerosis have shown that VSMC apoptosis caused the formation and development of vulnerable plaques.[10,11] Therefore, increased apoptosis of VSMCs in the late stage of atherosclerosis gave rise to cellular loss in the plaque, weakening plaque stability and causing rupture events.[12] Ox-LDL, which was one type of modified lipoprotein, could contribute to atherogenesis through a variety of ways, including formation of foam cells, inflammatory reaction, and apoptosis of VSMCs induced by endoplasmic reticulum (ER) stress.[12,13] A growing amount of evidence indicated that ER stress was strongly associated with the initiation and development of atherosclerosis.[14] ER function was impaired in the pathological progression of atherosclerosis.[15] Therefore, the unfolded protein response (UPR) acted as a physiological protective reaction to maintain ER homeostasis. The UPR was stimulated by the activation of 3 major ER sensors: protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1α (IRE1α), and activating transcription factor 6 (ATF6). ER stress and the activation of UPR have been observed at all stages of formation of atherosclerotic plaques.

When ER homeostasis was disturbed, PERK dissociated from binding immunoglobulin protein (BiP) and became self-phosphorylated. After self-phosphorylation, PERK suppressed eukaryotic translation initiation factor 2α (eIF2α) function, leading to the reduction of mRNA and proteins. However, transcriptional factor ATF4 translocated to the nucleus and activated genes related to preventing ER stress. The extended duration of ER stress in lesions leaded to enhanced UPR signaling accompanying the process of atherosclerosis; meanwhile, the increase in ATF4 promoted the expression of CCAAT/enhancer binding protein homologous protein (CHOP), which induced cell apoptosis by down-regulating the anti-apoptotic B-cell lymphoma (BCL) family and up-regulating endoplasmic reticulum oxidoreductin 1α (ERO1α), which mediated the Ca^{2+}-dependent apoptotic pathway.[16,17] As evidence showed, CHOP expression was remarkably increased in VSMCs in vulnerable plaques.[18] Consequently, the PERK/CHOP pathway induced expression of death effectors, activating apoptosis signaling pathways to cause apoptosis of VSMCs in the advanced stage of atherosclerosis, when the vulnerable plaque was likely to rapture.[19]

Pollen Typhae (pollen of *Typha angustifolia* L.) was a Chinese herb medicine and was reported to alleviate clinical symptoms of coronary heart disease. Previous investigations of *Pollen Typhae* total flavone (PTF), which was one of the active ingredients of *Pollen Typhae*, showed that PTF was able to suppress the progression of atherosclerosis.[20,21] PTF mainly contained flavone glycosides, isorhamnetin and quercetin. The proposed underlying mechanisms included modulation of lipid metabolism, activities of antioxidant enzymes such as superoxide dismutase, and inhibition of apoptosis-related signals such as the CAAT/enhancer binding protein.[22] Moreover, there was some evidence indicating that PTF might also regulate the production of nitric oxide, which has been shown to have a double-edged effect on ECs in atherogenesis.[23] Among all of these mechanisms, what drew our attention most was the inhibitory effect of PTF on apoptosis-related signaling pathways in anti-atherosclerosis. However, there was no direct evidence to demonstrate that PTF could affect the stability of atherosclerotic plaques or that PTF regulated the stability of atherosclerotic plaques through the PERK/CHOP signaling pathway. In the present study, we used human aortic (HA)-VSMCs to explore the potential mechanisms for the effects of PTF on angiogenesis.

METHODS

Materials

Purified PTF (>98%) was purchased from Shanghai Tauto Biotech Co., Ltd. (China).

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HA-VSMCs and smooth muscle cell medium (SMCM) were purchased from ScienCell Research Laboratories (USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-GAPDH antibody was purchased from Abcam (Hong Kong, China). Horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against PERK, phosphorylated (p)-PERK, p-eIF2α, p-IRE1α, CHOP, BiP, cleaved ATF6 and ATF4 were purchased from Cell Signaling Technology, Inc. (Shanghai, China). PTF (100, 250, or 500 μg/mL) was dissolved in 0.1% DMSO and stored at 4 ℃. PERK shRNA lentiviral particles (sc-36213), control shRNA lentiviral particles (sc-108080), siRNA transfection reagent (sc-29528) and siRNA transfection medium (sc-36868) were purchased from Santa Cruz Biotechnology, Inc. (USA). Solution A: siRNA duplex solution, 2–8 μL siRNA duplex (sc-36213 or sc-108080) diluted with 100 μL sc-36868. Solution B: 2–8 μL sc-29528 diluted with 100 μL sc-36868.

Culture, Identification and Grouping of HA-VSMCs

HA-VSMCs were maintained in SMCM, consisting of 500 mL basal medium, 10 mL fetal bovine serum, 5 mL smooth muscle cell growth supplement, and 5 mL penicillin/streptomycin, and incubated under air with 5% CO_2 at 37 ℃. The cells for experimentation were used from the 3rd to 8th passages, ensuring genetic stability. The growth medium was changed every 2–3 days until cells reached a confluence of 80%, after which they were detached using 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) and subcultured again.

Primary cultured HA-VSMCs showed the typical "hills and valleys" growth pattern. The identification of HA-VSMCs was carried out according to the α-smooth muscle actin assay kit experimental procedure by immunofluorescence identification (Appendix 1).

To establish the ox-LDL-induced HA-VSMCs, which were serum-starved for 24 h, high ox-LDL (Luwen Biotechnologies, China) was used. HA-VSMCs were randomly divided into 5 groups, including a normal control group, an ox-LDL group (70 μg/mL high ox-LDL), an HPTF group (70 μg/mL high ox-LDL+500 μg/mL PTF), an MPTF group (70 μg/mL high ox-LDL+250 μg/mL PTF), and a LPTF group (70 μg/mL high ox-LDL+100 μg/mL PTF).

Stable Lentiviral Transduction

The PERK shRNA (sc-36213) was used as PERK/eIF2α signaling inhibitor to specifically knockdown PERK gene expression. For stable lentiviral transduction, HA-VSMCs were seeded in 6-well culture plates, 2×10^5 cells per well in 2 mL antibiotic-free normal growth medium, and incubated at 37 ℃ in a 5% CO_2 incubator until the cells were 80% confluent. Then the solution A was added directly to the dilute solution B. After the mixture was incubated 30 min at room temperature, the cells were washed once with 2 mL of siRNA transfection medium. For each transfection, 0.8 mL siRNA transfection medium was added to each tube containing the siRNA transfection reagent mixture (solution A+solution B). The HA-VSMCs overlayed with the mixture were incubated for 6 h at 37 ℃ in a 5% CO_2 incubator. Afterwards, the SMCM was added into cells without removing the transfection mixture and the cells were incubated for an additional 24 h. After transfection for 24 h, HA-VSMCs were divided into 4 groups including a shRNA group (transducted with sc-36213), a scramble shRNA group (transducted with sc-108080), an MPTF+ox-LDL+shRNA group (250 μg/mL PTF+70 μg/mL high ox-LDL+sc-36213), and an ox-LDL+shRNA group (70 μg/mL high ox-LDL+sc-36213).

Measurement of Cell Proliferation Inhibiting Rate Using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effects of PTF on cell viability in HA-VSMCs. The digested cell suspension (10^4 cells/well) was seeded into 96-well culture plates and cultured for 24 h. After cultured cells adhered to the wells, the culture medium was removed, and an appropriate medium was added according to the different experimental groups as previously described. Each experimental group was carried out in 5 wells. The plates were transferred to a 37 ℃ incubator with air containing 5% CO_2 for 48 h, after which 20 μL of MTT was added to each well. Four hours later, the culture was terminated, and 150 mL of DMSO was added to each well, and the plate was placed on the micro-oscillator at 37 ℃ for 10 min, and the absorbance value (A) was determined at 490 nm. The formula of proliferation inhibition rate was as follow: The proliferation inhibition rate (%)=(1–$A_{experimental\ group}$/$A_{normal\ control\ group}$)×100%.

Measurement of Cell Cycle and Apoptosis Rate Using Flow Cytometry

The HA-VSMCs were incubated for 48 h as previously described. Subsequently, the cells were washed twice with pre-cooled phosphate-buffered saline (PBS), fixed in pre-cooled 75% ethanol, and centrifuged at 2,000×*g* for 5 min at 4 ℃. The cells were washed with PBS twice and harvested, after which the cells were incubated in 1×Annexin V buffer containing Annexin V and propidium iodide (PI) for 15 min. Stained cells were quantified with a FacsCalibur flow cytometer (BD Biosciences, USA), using 10,000 cells per measurement. All experiments were repeated at least 3 times.

Western Blot

HA-VSMCs in the logarithmic growth phase were used in the experiments, in which each experimental group was treated as previously described. Cell lysates were prepared by incubation on ice with lysis buffer [20 mmol/L Tris-HCl, pH 7.5, 16 mmol/L 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS), 1 mmol/L Na_2-EDTA, and 1 mmol/L DL-dithiothreitol (DTT)] with protease inhibitors [1 mmol/L benzamidine, 1 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, and 0.5 mmol/L phenylmethyl sulfonylfluoride (PMSF)] and centrifuged at 12,000×*g* for 20 min at 4 ℃. The samples were boiled for 15 min, and the protein concentration was measured by a bicinchoninic acid protein assay kit. After resolution of equal protein for each sample by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the protein was electrophoretically transferred onto polyvinylidene fluoride membranes (BIO RAD, Shanghai, China). Membranes were blocked with 5% non-fat dried milk in TBST [20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, and 0.1% Tween 20] for 2 h at room temperature on a rocker; membranes were incubated with primary antibodies (1:2000, anti-BiP, anti-PERK, anti-p-PERK, anti-p-eIF2α, anti-ATF4, anti-CHOP, anti-p-IRE1α, and anti-cleaved ATF6) overnight at 4 ℃, then secondary antibody (1:3000 HRP-conjugated anti-goat or anti-rabbit IgG) for 1 h at 37 ℃. After washing with TBST, the membrane reaction was visualized using an enhanced chemiluminescence detection kit. The band intensities of corresponding protein expression were observed and analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, USA) and normalized to band intensities of GAPDH. All experiments were repeated 3 times.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA from HA-VSMCs was extracted using the standard Trizol RNA isolation protocol. Two microgram of total RNA was reverse transcribed using the SuperScript RT kit from Invitrogen (Carlsbad, CA). Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) with SYBR Green Master Mix. The primers used in this experiment are listed in Table 1. Expression was normalized with respect to that of the housekeeping gene GAPDH.

Table 1. Primer Sequence for Quantitative Real-Time RT-PCR Analysis

Gene name	Sense primer (5'–3')	Antisense primer (5'–3')
GAPDH	ACAGTCAGCCGCATCTTC	CTCCGACCTTCACCTTCC
BiP	AGACGGGCAAAGATGTCAGG	GCCCGTTTGGCCTTTTCTAC
PERK	ATCCCCCATGGAACGACCTG	ACCCGCCAGGGACAAAAATG
ATF4	GGGTTTTGGATTGGTGGGGT	CGCTCGTTAAATCGCTTCCC
CHOP	CACCTTTCCCAGAAGTGGCT	TGCGTATGTGGGATTGAGGG
IRE1α	ATTGTGTACCGGGGCATGTT	CTCACGGTCTGCGAAGCTAA
ATF6	ACGGAGTATTTTGTCCGCCT	GGCTCCCCCATTTCACAAGT

Statistical Analyses

Data from at least 4 independent experiments were used for statistical analysis carried out using SPSS 20.0 software. All values were expressed as the mean ± standard deviation ($\bar{x} \pm s$). The significance of differences among experimental groups was assessed by one-way analysis of variance and inter-group pairwise comparison known as the Student-Newman-Keuls method to detect the difference of each index. Results were considered statistically significant when the *P*-value was<0.05.

RESULTS

Effects of PTF on Inhibiting Apoptosis in HA-VSMCs

The proliferation inhibition rates of HA-VSMCs in the HPTF, MPTF and LPTF groups were significantly decreased compared with the ox-LDL group ($P<0.05$). Furthermore, compared with the ox-LDL group,

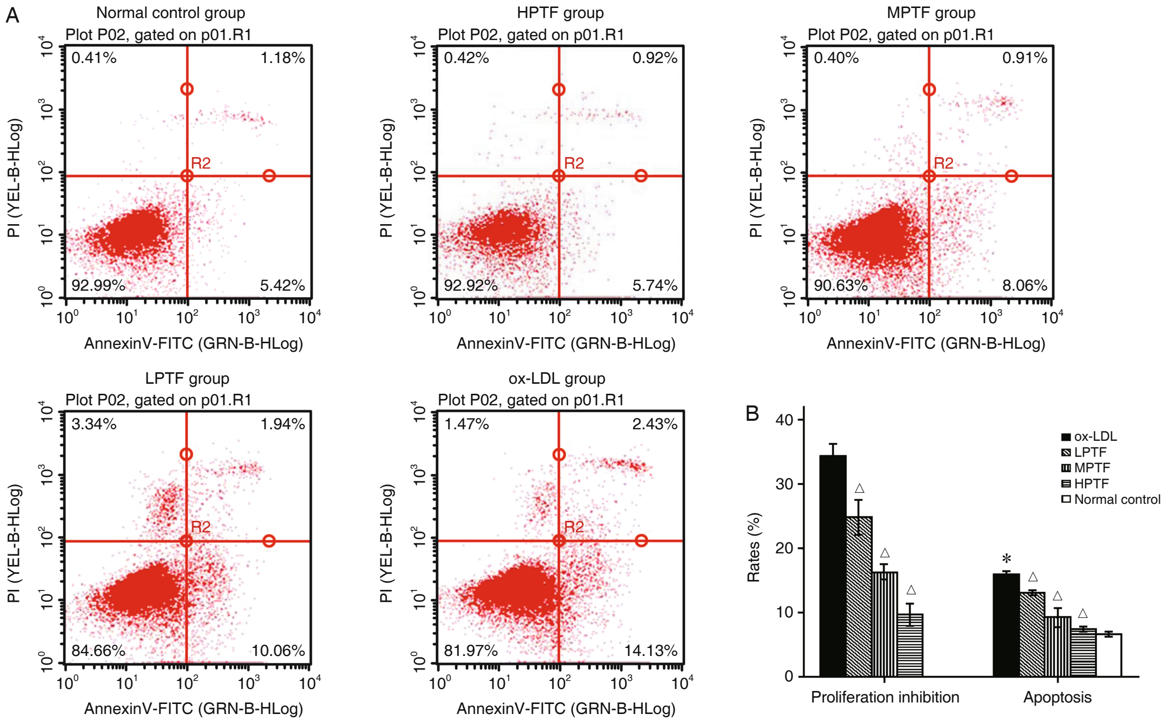

Figure 1. Effects of PTF on Proliferation Inhibition Rates and Apoptosis Rates of HA-VSMCs

Notes: A: Apoptosis rates of HA-VSMCs by flow cytometry. The Annexin V^+PI^- cells (lower right quadrant) represented early apoptotic cells, and the Annexin V^+PI^+cells (upper right quadrant) represented advanced apoptotic cells and/or necrotic cells. B: Proliferation inhibition rates and apoptosis rates of HA-VSMCs. $^*P<0.05$ *vs*. normal control group; $^{\triangle}P<0.05$ *vs*. ox-LDL group

the HPTF, MPTF and LPTF groups showed lower apoptosis rates and proportions of Annexin V labeling ($P<0.05$, Figure 1).

Compared with the ox-LDL+shRNA group, the ox-LDL and ox-LDL+MPTF groups showed higher apoptosis rates and proportions of Annexin V labeling ($P<0.05$, Figure 2). However, the apoptosis rate had no significant difference among the MPTF+ox-LDL+shRNA group, ox-LDL+shRNA group, scramble shRNA group, and shRNA group ($P>0.05$).

PTF Relieves ER Stress in HA-VSMCs

Western blot analysis showed that BiP protein was highly expressed in the ox-LDL group compared with the normal control group ($P<0.05$). Nevertheless, the protein expression levels of BiP in the MPTF and HPTF groups were relatively lower compared with the ox-LDL group ($P<0.05$). Moreover, the mRNA expression level of BiP was highly expressed in the ox-LDL group compared with the normal control group ($P<0.05$), and the mRNA expression levels of BiP in the LPTF, MPTF and HPTF groups were relatively lower compared with the ox-LDL group in quantitative real-time RT-PCR analysis ($P<0.05$, Figure 3).

PTF Suppresses ER-Associated Apoptosis Pathways in HA-VSMCs

Western blot analysis showed significantly increased protein levels of p-PERK, p-eIF2α, ATF4 and CHOP in the ox-LDL group when compared with the normal control group ($P<0.05$). Moreover, protein expressions of p-PERK, p-eIF2α, ATF4 and CHOP in the MPTF and HPTF treatment groups were significantly lower than those in the ox-LDL group ($P<0.05$). In addition, the protein expressions of p-PERK, p-eIF2α, ATF4 and CHOP in the HPTF treatment group were significantly lower than those in the LPTF group ($P<0.05$, Figure 4A).

The mRNA levels of ATF4 and CHOP were significantly higher in the ox-LDL group compared with the normal control group ($P<0.05$) and the mRNA levels of ATF4 and CHOP were significantly lower in the LPTF, MPTF and HPTF treatment groups compared with the ox-LDL group ($P<0.05$). Furthermore, the mRNA expression levels of ATF4 and CHOP in the HPTF group were significantly lower than those in the LPTF group ($P<0.05$, Figure 4B). Besides, there was no significant difference between the expression levels of ATF6 and IRE1α in the PTF treatment groups and those in the ox-LDL group ($P>0.05$, Figure 5).

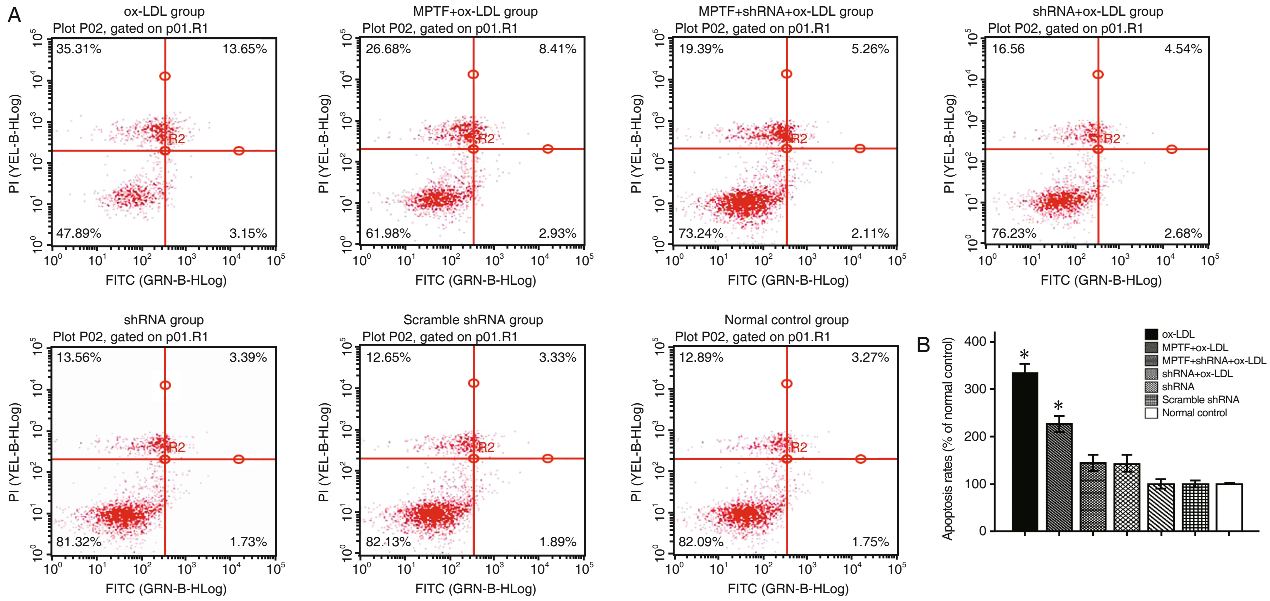

Figure 2. Effect of PERK shRNA on Apoptosis of HA-VSMCs

Notes: A: Apoptosis rates of HA-VSMCs by flow cytometry. The Annexin V^+PI^- cells (lower right quadrant) represented early Apoptotic cells, and the Annexin V^+PI^+cells (upper right quadrant) represented advanced apoptotic cells and/or necrotic cells. B: Apoptosis rates of HA-VSMCs. **P*<0.05 *vs*. shRNA+ox-LDL group

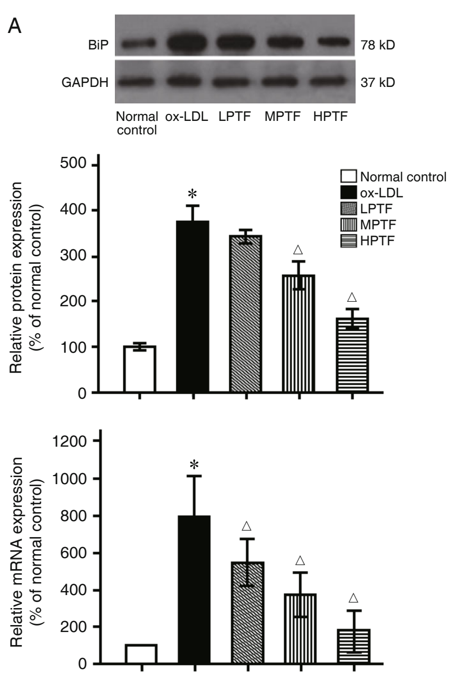

Figure 3. Effect of PTF on BiP Protein and mRNA Expressions in HA-VSMCs

Notes: A: Protein expression of BiP in HA-VSMCs by Western blot; B: mRNA expression of BiP in HA-VSMCs by quantitative real-time RT-PCR. **P*<0.05 *vs*. normal control group; △*P*<0.05 *vs*. ox-LDL group

As shown in Figure 6, compared with the normal control group and ox-LDL+shRNA group, the PERK protein and mRNA expressions showed lower levels in the shRNA group, while the protein expression of PERK, p-eIF2α, ATF4 and CHOP showed higher levels in the

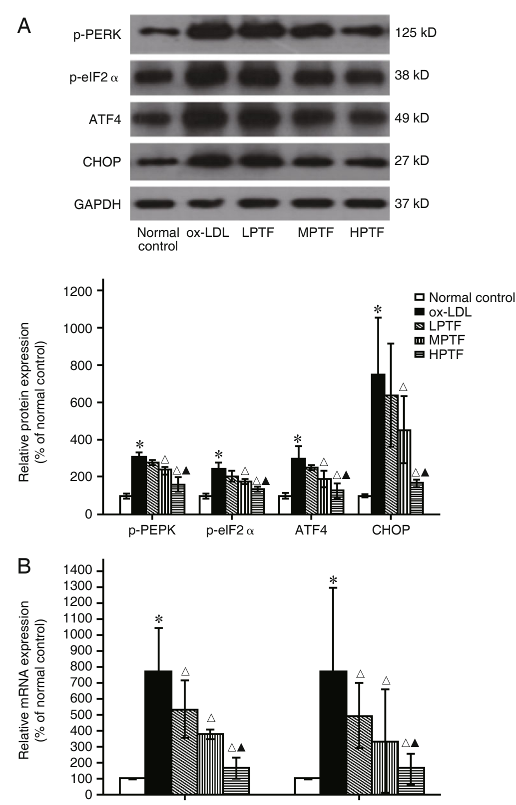

Figure 4. Effects of PTF on Protein Expressions of p-PERK, p-eIF2α, ATF4 and CHOP and mRNA Expressions of ATF4 and CHOP in HA-VSMCs

Notes: A: Protein Expressions of p-PERK, p-eIF2α, ATF4 and CHOP in HA-VSMCs by Western blot; B: mRNA expressions of ATF4 and CHOP in HA-VSMCs by quantitative real-time RT-PCR. **P*<0.05 *vs*. normal control group; △*P*<0.05 *vs*. ox-LDL group; ▲*P*<0.05 *vs*. LPTF group

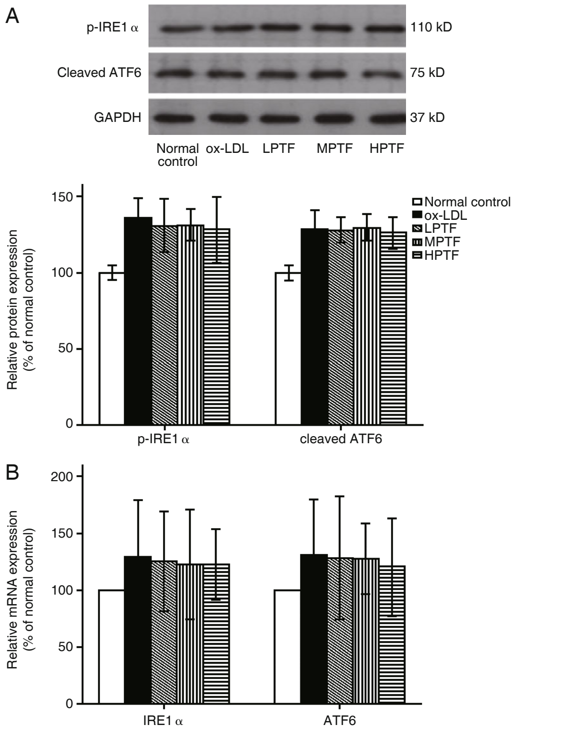

Figure 5. Effects of PTF on Protein Expressions of p-IRE1α and Cleaved ATF6 and mRNA Expressions of IRE1α and ATF6 in HA-VSMCs

Notes: A: Protein expressions of p-IRE1α and cleaved ATF6 in HA-VSMCs by Western blot; B: mRNA expressions of IRE1α and ATF6 in HA-VSMCs by quantitative real-time RT-PCR

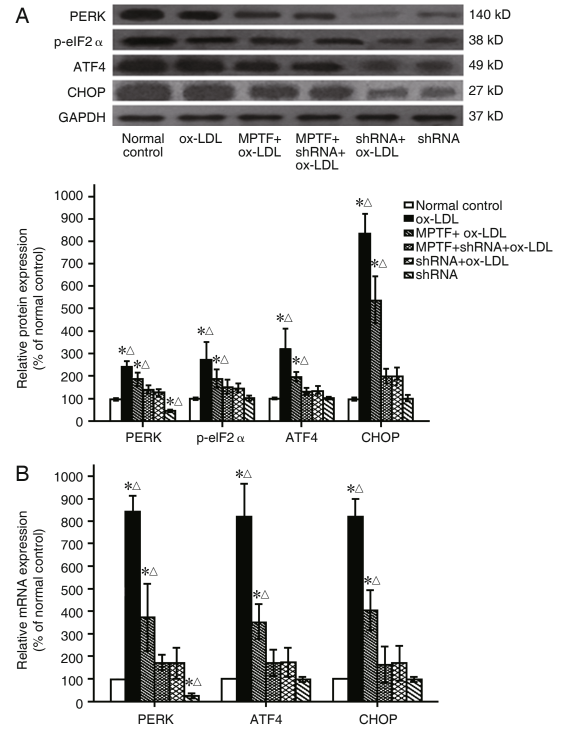

Figure 6. Effects of PTF on PERK, p-eIF2α, ATF4 and CHOP Protein Expressions and PERK, ATF4, CHOP mRNA Expressions in HA-VSMCs

Notes: A: Protein expressions of PERK, p-eIF2α, ATF4 and CHOP in HA-VSMCs by Western blot; B: mRNA expressions of PERK, ATF4 and CHOP in HA-VSMCs by quantitative real-time RT-PCR. *P<0.05 *vs*. normal control group; △P<0.05 *vs*. shRNA+ox-LDL group

ox-LDL group and the MPTF+ox-LDL group (P<0.05). The mRNA levels of PERK, ATF4 and CHOP in the ox-LDL+shRNA group, the normal control group, the ox-LDL group and the MPTF+ox-LDL group paralleled with their protein expression (P<0.05). Nevertheless, the protein and mRNA levels of PERK, ATF4 and CHOP had no significant differences between the MPTF+shRNA+ox-LDL group and the ox-LDL+shRNA group (P>0.05).

DISCUSSION

The involvement of ER stress in the rupture of vulnerable atherosclerotic plaques was a relatively new area of research. Targeting inhibition of ER stress might provide new therapeutic approaches for the rupture of vulnerable atherosclerotic plaques. Accumulating evidence demonstrated that ER stress played an important role in the formation and development of atherosclerosis.[14,18,24] What interested us most was the molecular mechanism of ER stress in vulnerable atherosclerotic plaque rupture, which was a common feature in several varieties of cardiovascular and cerebrovascular diseases. The tendency of vulnerable plaques to rupture was characterized by an enlarged necrotic lipid core with an overlying thin fibrous cap, which contained less VSMCs.[3] Previous studies reported that high doses of ox-LDL could induce apoptosis in VSMCs and that overloading free cholesterol could induce smooth muscle cell death via activating ER stress pathways.[25,26] Former investigations of PTF have shown a suppressing effect on the progression of atherosclerosis.[21] However, it was still unknown whether PTF could inhibit apoptosis of high lipid-induced VSMCs, and the exact mechanism of apoptosis of high lipid-induced VSMCs was also unknown. Therefore, we investigated the effects of PTF on ER stress and apoptosis of ox-LDL-induced HA-VSMCs.

The ER chaperone protein BiP was known as an ER stress marker protein.[27] When ER stress happened, PERK, ATF6, IRE1α, and ER stress sensors dissociated from BiP and were autophosphorylated and activated.[28] Subsequently, the expressions of these proteins were transcriptionally regulated during ER stress responses. In this study, BiP protein expression was highly expressed in ox-LDL HA-VSMCs compared with the normal control HA-VSMCs. The protein expression levels of BiP in the PTF groups were significantly lower than the ox-LDL group. In addition, the mRNA expression level of BiP showed a similar trend. These results indicated that ox-LDL could induce ER stress and PTF could relieve ER stress in ox-LDL-induced HA-VSMCs.

As mentioned above, in response to ER stress, 3 major proteins, PERK, IRE1α and ATF6, activated UPR.(29) PERK was a transmembrane protein with an ER stress-sensing domain that binded BiP.(30) A previous study reported that CHOP was considered to be a transcription factor that induced apoptosis in ER stress-induced apoptosis.(31) Transcription of the CHOP gene could be mediated by PERK. When ER stress was prolonged, PERK activation leaded to phosphorylation of eIF2α, which inhibited general protein translation. ATF4 drove the transcription of the specific UPR target gene CHOP.(32) In this study, ox-LDL-induced HA-VSMCs were found to have increased protein expressions of BiP, p-PERK, p-eIF2α, ATF4 and CHOP and increased mRNA levels of BiP, ATF4 and CHOP. On the contrary, the results in PTF treatment groups were significantly attenuated. In addition, to determine whether the activation of PERK could trigger ox-LDL-induced ER stress, the PERK shRNA was transfected into HA-VSMCs to knockdown the PERK gene. Compared with the ox-LDL+shRNA group and the normal control group, protein and mRNA expressions of PERK, p-eIF2α, ATF4 and CHOP showed higher levels in the ox-LDL and MPTF+ox-LDL groups. The protein and mRNA levels of PERK, ATF4 and CHOP had no significant differences between the MPTF+ox-LDL+shRNA group and the ox-LDL+shRNA group. These results prompted that PERK could trigger ox-LDL-induced ER stress strongly related to PERK-eIF2α-ATF4-CHOP signaling. Moreover, what mentioned above indicated that PTF was able to relieve ER stress strongly associated with down-regulating PERK-eIF2α-ATF4-CHOP signaling. Besides, the effects of PTF on ATF6 and IRE1α activation have also been examined. The levels of ATF6 and IRE1α were not affected by PTF treatment, indicating the effect of PTF was focused on PERK/CHOP signaling.

In this study, flow cytometry was used to detect apoptosis and increased apoptosis was detected in ox-LDL-induced HA-VSMCs while less apoptosis was detected in the PTF treatment groups. Compared with the ox-LDL+shRNA group, the ox-LDL group and the MPTF+ox-LDL group showed a higher apoptosis rate and proportion of Annexin V labeling. Moreover, apoptosis was significantly increased in the ox-LDL-induced HA-VSMCs, and p-PERK and p-eIF2α were decreased in the PTF treatment and shRNA groups. Furthermore, there was no significant difference between the apoptosis rates of MPTF+ox-LDL+shRNA group and that of ox-LDL+shRNA group. These results indicated that the apoptosis of ox-LDL-induced HA-VSMCs was associated with ER stress and that PTF was likely to suppress the apoptosis of ox-LDL-induced HA-VSMCs via inhibiting ER stress.

In this research, the results above indicated that PTF had a protective effect on preventing apoptosis induced by ER stress through inhibiting expression of PERK, eIF2α, ATF4 and CHOP. Furthermore, protein expression levels of p-PERK, p-eIF2α and ATF4, the CHOP upstream kinases, were significantly increased in ox-LDL-induced HA-VSMCs and were suppressed by PTF treatment. Consequently, it could be concluded that there was a strong relationship between the protective effect of PTF and suppression of apoptosis induced by ER stress through inhibiting the PERK-eIF2α-ATF4-CHOP pathway.

In conclusion, this research demonstrated that apoptosis induced by ER stress in ox-LDL-induced HA-VSMCs was associated with activation of the PERK-eIF2α-ATF4-CHOP signaling pathway and that PTF inhibited ER stress-induced apoptosis possibly through suppression of this signaling pathway. The findings above might provide an alternative therapeutic treatment for preventing the rupture of atherosclerotic plaques through inhibiting HA-VSMCs apoptosis to improve structure and stability of plaques.

Conflict of Interest

All authors declare that they have no conflict of interest.

Author Contributions

Zhang Z and Wang L conceived the study and drafted the protocol. Chen MT, Huang RL and Ou LJ contributed to cell culture and identification. Chen YN and Men L contributed to conducting Western blot. Chang X and Yang YZ contributed to conducting RT-PCR. All authors have approved the final manuscript.

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