Original Article

Activating Transcription Factor 6 Mediates Oxidized LDL-Induced Cholesterol Accumulation and Apoptosis in Macrophages by Up-Regulating CHOP Expression

Shutong Yao^{1,2}, Chuanlong Zong¹, Ying Zhang¹, Hui Sang^{1,2}, Mingfeng Yang¹, Peng Jiao¹, Yongqi Fang², Nana Yang¹, Guohua Song¹ and Shucun Qin^{1,2}

¹Institute of Atherosclerosis, Key Laboratory of Atherosclerosis in Universities of Shandong, Taishan Medical University, Taian, China

²College of Basic Medical Sciences, Taishan Medical University, Taian, China

Aim: This study was to explore whether activating transcription factor 6 (ATF6), an important sensor to endoplasmic reticulum (ER) stress, would mediate oxidized low-density lipoprotein (ox-LDL) - induced cholesterol accumulation and apoptosis in cultured macrophages and the underlying molecular mechanisms.

Methods: Intracellular lipid droplets and total cholesterol levels were assayed by oil red O staining and enzymatic colorimetry, respectively. Cell viability and apoptosis were determined using MTT assay and AnnexinV-FITC apoptosis detection kit, respectively. The nuclear translocation of ATF6 in cells was detected by immunofluorescence analysis. Protein and mRNA levels were examined by Western blot analysis and real time-PCR, respectively. ATF6 siRNA was transfected to RAW264.7 cells by lipofectamin.

Results: Exposure of cells to ox-LDL induced glucose-regulated protein 78 (GRP78). C/EBP homologous protein (CHOP), a key-signaling component of ER stress-induced apoptosis, was up-regulated in ox-LDL-treated cells. ATF6, a factor that positively regulates CHOP expression, was activated by ox-LDL in a concentration- and time- dependent manner. The role of the ATF6-mediated ER stress pathway was further confirmed through the siRNA-mediated knockdown of ATF6, which attenuated ox-LDL-induced upregulation of CHOP, cholesterol accumulation and apoptosis in macrophages. In addition, the phosphorylation of double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), another factor that positively regulates CHOP expression, was induced in the presence of ox-LDL, and PERK-specific siRNA also inhibited the ox-LDL-induced upregulation of CHOP and apoptosis in RAW264.7 cells.

Conclusion: These results demonstrate that ER stress-related proteins, particularly ATF6 and its downstream molecule CHOP, are involved in ox-LDL-induced cholesterol accumulation and apoptosis in macrophages.

J Atheroscler Thromb, 2013; 20:94-107.

Key words; Endoplasmic reticulum stress, Activating transcription factor 6, CHOP, Oxidized low-density lipoprotein, Macrophage, Apoptosis

Introduction

Apoptosis is a key event in the progression of

advanced atherosclerosis, a cardiovascular disorder that is the leading cause of death in developed as well as developing countries¹). Apoptosis of macrophage-derived foam cells, especially in advanced atherosclerotic lesions where phagocytic clearance of apoptotic macrophages is defective, leads to the development of a necrotic core of atherosclerotic plaque, and reduction of the excretion of cell matrix in the plaque region, which may contribute to plaque instability and the majority of cardiovascular complications²⁻⁴). Thus,

Address for correspondence: Shucun Qin, Institute of Atherosclerosis, Key Laboratory of Atherosclerosis in Universities of Shandong, Taishan Medical University, Taian 271000, China

E-mail: shucunqin@hotmail.com

Received: February 15, 2012

Accepted for publication: July 18, 2012

further elucidating the mechanisms of macrophage apoptosis in advanced lesions will be critical for the formation of novel therapeutic approaches to combat atherosclerotic lesion progression^{2, 5}.

Mitochondria play a crucial role in the control of cell death and apoptosis in most cells including macrophages^{6, 7}. Apart from mitochondria, other cell organelles take part in cell death and its regulation⁸. Recently, alteration in the function of the endoplasmic reticulum (ER), also known as ER stress, has been considered to be a cross-point to link cellular processes with multiple risk factors that exist in all stages of atherosclerosis⁹ and is believed to play a critical role in lipid metabolic disorder¹⁰, activation of inflammatory reactions¹¹, and cellular apoptosis¹². It is well known that the ER stress response is an adaptive mechanism by which cells react to perturbations in ER homeostasis through translational attenuation and the up-regulation of ER-resident chaperones such as glucose-regulated protein (GRP) 78 and GRP 94; however, when ER function is severely impaired, the organelle spreads apoptotic signals. One component of the ER stress-mediated apoptosis pathway is the activating transcription factor 6 (ATF6)-dependent activation of C/EBP homologous protein (CHOP)¹³⁻¹⁵. ATF6 is a type II transmembrane protein and its intra-ER domain is lined with GRP78, existing in an inactivated form. Under conditions of increased ER stress, ATF6 is dissociated from GRP 78, translocated to the Golgi apparatus, and processed by site 1 protease (S1P) or site 2 protease (S2P). Processed ATF6 translocates to the nucleus and promotes the expression of ER stress-responsive genes, including CHOP, a well-known transcription factor which mediates ER stress-induced apoptosis^{16, 17}.

Oxidized low-density lipoprotein (ox-LDL) has been suggested as a major inducer leading to cholesterol accumulation and apoptosis in macrophages^{7, 18, 19}. We have previously shown that ox-LDL can induce ER stress during the formation of macrophage-derived foam cells²⁰; therefore, we are interested in understanding whether ATF6 is involved in ox-LDL-induced cellular cholesterol accumulation and apoptosis in macrophages. In this study, we revealed that ox-LDL induces activation of the ER stress pathway, including the translocation of ATF6 from the cytoplasm to the nucleus and up-regulation of GRP78 and CHOP. An experiment using ATF6-specific small interfering RNA (siRNA) indicated that the activation of ATF6 is involved in ox-LDL-induced cholesterol accumulation and apoptosis in macrophages.

Materials and Methods

Reagents

Oil red O, tunicamycin (TM), siRNA against ATF6, double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK), and rabbit antibody against β -actin were obtained from Sigma (St Louis, MO, USA). Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco (BRL, Gaithersburg, MD, USA). RIPA lysis buffer, nuclear extraction kits and bicinchoninic acid (BCA) protein quantitative kits were purchased from Solarbio (Beijing, China). Rabbit anti-mouse polyclonal antibodies against GRP78, CHOP, PERK, phospho-PERK (p-PERK) and Histone H3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibody against ATF6 was purchased from Abcam (Cambridge, MA, USA). Horseradish peroxidase-labeled goat anti-rabbit IgG and SABC-Cy3 immunohistochemistry kits were obtained from Boshide (Wuhan, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Genview (Houston, TX, USA). The annexin V-FITC apoptosis detection kit was purchased from KeyGEN Biotech (Nanjing, China). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). Enhanced chemiluminescence (ECL) kits were purchased from Thermo Scientific Pierce (Rockford, IL, USA). Real-time PCR reagent kits were purchased from Tiangen Biological Chemistry (Beijing, China). Tissue/cell free cholesterol assay kits were obtained from Applygen (Beijing, China). All other reagents were analytical grade and obtained from commercial sources.

Isolation and Oxidation of Low Density Lipoprotein

Human LDL was isolated and oxidized as described recently²¹. In brief, LDL (density=1.019 to 1.063 g/mL) was isolated from the plasma of normolipidemic donors by sequential ultracentrifugation, and incubated with 10 μ mol/L CuSO₄ for 18 hours at 37°C. At the end of incubation, 0.1 mmol/L EDTA was added to prevent further oxidation and the oxidized LDL was concentrated to 1 mg/mL. The extent of LDL oxidation was assessed on the basis of increased mobility in an agarose gel (compared to that of native LDL) and also by the presence of increased levels of thiobarbituric acid-reactive substances (TBARS) in the sample. Typically, ox-LDL preparations had TBARS of >30 μ mol/g protein and a relative mobility index on agarose gels of 2.0-2.5 when compared to that of

native LDL. Lipoproteins were stored at 4°C in the dark and freshly prepared every two weeks.

Cells and Cell Culture

RAW264.7, a mouse macrophage cell line, was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 2 mM glutamine, antibiotics (100 U/mL penicillin A and 100 U/mL streptomycin) and 10% fetal bovine serum. The cultures were maintained at 37°C in a humidified incubator containing 5% CO₂ until sub-confluent, and then the medium was replaced with serum-free medium. The cells were stimulated with increasing concentrations (25, 50 and 100 mg/L) of ox-LDL for 12, 24 and 48 h, or in cubated with 5 mg/L TM (a classical ER stress inducer) for 12 h.

SiRNA Transfection

RAW264.7 cells (5×10^6) were seeded on six-well plates in growth medium without antibiotics and allowed to reach 30%-50% confluence. ATF6-specific or PERK-specific siRNA oligomer (200 pmol) and control siRNA oligomers (200 pmol) were diluted in 250 μ L Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA, USA) and combined with 5 μ L Lipofectamine 2000 (Invitrogen) pre-diluted in 250 μ L Opti-MEM. After 20 min incubation at room temperature, the complexes were added to the cells in a final volume of 2.5 mL medium. The ATF6 siRNAs were 5'-CUUUA AUGGUGCUAAGUGAdTdT-3' (sense) and 5'-UCACUUAGCACCAUU AAAGdTdT-3' (antisense), and the PERK siRNAs were 5'-GUAAUGGUGCACUUU CAAUdTdT-3' (sense) and 5'-AUUGAAAGUGCACCAUUACdTdT-3' (antisense). After transfection for 48 h, the cells were exposed to ox-LDL (100 mg/L) for 24 h. The silencing of ATF6 and PERK was validated by Western blotting and quantitative real-time PCR.

Immunofluorescence Assay for ATF6 Nuclear Relocation

The treated cells grown on glass coverslips were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton-X 100, and blocked in 3% BSA. Samples were incubated with ATF6 antibody (1:100 in PBS-1 % BSA) for 1 h at room temperature. Following incubation with the appropriate secondary antibody (1:200 in PBS-1% BSA), the samples were incubated with SABC-Cy3 complex and 4',6-diamidino-2-phenylindole (DAPI). Coverslips were then washed with PBS and mounted in antifade reagent. Stained cells were

observed using an Olympus BX51 microscope (Tokyo, Japan).

Isolation of Whole Cell and Nuclear Proteins and Western Blot Analysis

Total proteins and nuclear proteins from the treated cells were extracted using RIPA lysis buffer and nuclear extraction kits, respectively, following the manufacturer's instructions. Equal amounts of protein were subjected to 8% to 12% SDS-PAGE and transferred onto PVDF membranes by electroblotting. After blocking in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 10% nonfat dry milk for 2 h at room temperature, the membranes were incubated with primary antibodies for 3 h at room temperature or overnight at 4°C. After washing four times with TBS containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Immunoblots were revealed by ECL reaction and visualized using a high-performance chemiluminescence film. The integrated optical density (IOD) of immunoreactive bands was measured using Image-Pro Plus software (version 6.0; Media Cybernetics, MD, USA) and normalized by a house-keeping protein (β -actin or histone).

RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. After contaminated genomic DNA was digested with DNase I (Roche Diagnostics), first-strand cDNA was synthesized using MuLV Reverse Transcriptase (Applied Biosystems) from 2 μ g total RNA. The primers used for real-time PCR were synthesized by TaKaRa and listed in **Table 1**. Real-time PCR was performed with a Rotor-Gene Q real-time PCR cycler (Qiagen) using SYBR-green PCR master mix kits (TianGen Biotech). Initial denaturation was performed at 94°C for 10 minutes, followed by 40 cycles of denaturation at 94°C for 20 seconds, then annealing at 56°C for 30 seconds and extension at 68°C for 30 seconds. The data were analyzed using Rotor-Gene Q software (version 1.7; Qiagen), and relative mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method and normalized against β -actin. Each experiment was repeated three times.

Intracellular Lipid Droplet Observation by Oil Red O Staining

The treated cells grown on glass coverslips were washed with PBS, fixed with a 4% paraformaldehyde

Table 1. Primers used for PCR and qRT PCR analysis

Target mRNA	Forward primer (5' to 3')	Reverse primer (5' to 3')
ATF6	TGGGCAGGACTATGAAGTAATG	CAACGACTCAGGGATGGTGCTG
CHOP	CCACCACACCTGAAAGCAGAA	GGTGCCCCCAATTTTCATCT
GRP78	ACATGGACCTGTTCCGCTCTA	TGGCTCCTTGCCAT TGAAGA
β -Actin	CGGGGACCTGACTGACTACC	AGGAAGGCTGGAAGAGTGC

ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; GRP78, glucose- regulated proteins 78

solution for 20 min, stained with 0.5% Oil red O in isopropanol for 30 min, and counterstained with hematoxylin for another 5 min. The cells were observed using an Olympus BX51 microscope and analyzed with Image-Pro Plus software. The lipid droplet content was expressed as the average value of integrated optical density (IOD).

Intracellular Total Cholesterol (TC) Assay

The intracellular TC level was measured using a tissue/cell total cholesterol assay kit according to the manufacturer's instructions. In brief, the treated cells were harvested and divided into two equal portions to be processed for measurement of protein concentration and TC content, respectively. Lipids were extracted using the lysis solution provided in the kit. To determine the TC level, cholesterol ester hydrolase was used to convert cholesterol ester into free cholesterol. Then, cholesterol oxidase was used to generate H_2O_2 from the free cholesterol generated in the previous step, and peroxidase was used to catalyze the reaction of H_2O_2 with 4-amino-antipyrine and phenol to yield a stable rose-colored product. The absorbance of this colored product was measured at 550 nm with an Infinite F200 microplate reader (Tecan, Switzerland), and the concentration of TC was determined using a standard curve and normalized to the level of total cellular protein.

MTT Assay

Cell viability was determined using the MTT reduction assay according to the following method. Cells were seeded at a density of 5×10^3 cells/well in 96-well plates, transfected with ATF6 siRNA or PERK siRNA (80 nM), and subsequently treated with 100 mg/L ox-LDL for 24 h. Following incubation, MTT was added to a final concentration of 0.5 mg/ml and the samples were incubated at 37°C for 4 h. After removing the supernatant, 150 μ L dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals, and the optical density (OD) was detected at 490 nm using an Infinite F200 microplate reader (Tecan). Cell viability was expressed as the percentage of the control

group transfected with control siRNA (100%).

Detection of Apoptosis by Flow Cytometry Analysis and Laser Scanning Confocal Microscope

Annexin V-FITC/PI double-staining assay was used to quantify apoptosis, according to the manufacturer's protocol. Following treatment, cells were centrifuged, washed with PBS, resuspended in 500 μ L binding buffer, and incubated with 5 μ L Annexin V-FITC and 5 μ L propidium iodide (PI) solution for 10 min at room temperature in the dark. The samples were analyzed on a FAC Scan flow cytometer using Cell-Quest software (Becton-Dickinson, CA). Double staining of cells with Annexin V-FITC and PI permits the identification of different cell populations based on their staining patterns as follows: live cells (FITC⁻PI⁻), early apoptotic (FITC⁺PI⁻), late apoptotic (FITC⁺PI⁺) and necrotic cells (FITC⁻PI⁺).

Cells treated as described above were analyzed on glass coverslips using a laser scanning confocal microscope (Radiance 2100; Bio-Rad, USA) with 488-nm excitation and 525-nm emission wavelengths. Cells undergoing prophase apoptosis fluoresced bright green fluorescence due to Annexin V-FITC staining, whereas the nuclei of cells undergoing advanced stage apoptosis fluoresced cardinal red due to PI staining.

Statistical Analysis

Results are shown as the mean $\pm$ SEM for at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Student-Newmann-Keuls multiple comparison tests with SPSS13.0 software for Windows. $P < 0.05$ was considered significant.

Results

Induction of GRP78 and CHOP by Ox-LDL

The effect of ox-LDL on the expression of GRP78, an ER stress marker, was examined by immunoblotting analysis. GRP78 was present in untreated control cells and increased in response to treatment with TM (an inhibitor of N-linked glycosylation and

Fig. 1. Expression levels of GRP78 and CHOP in ox-LDL-incubated RAW264.7 cells. Cells were incubated with the indicated concentration of ox-LDL for 24 h, 5 mg/L tunicamycin (TM) for 12 h, or 100 mg/L ox-LDL for 12, 24, and 48 h. The protein expressions of GRP78 and CHOP were analyzed by Western blotting and normalized to the β -actin level (A and B). The mRNA levels of GRP78 and CHOP were measured by quantitative real-time PCR, which were measured relative to vehicle-treated controls and calculated after adjusting for β -actin using the $2^{-\Delta\Delta C_t}$ method (C and D). Data are presented as the mean $\pm$ SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus control.

a representative ER stress inducer). Similarly, ox-LDL, at concentrations of 25, 50, and 100 mg/L for 24 h, increased the level of GRP78 protein by 84.0%, 245.5%, and 299.8%, respectively (**Fig. 1A**). When RAW264.7 cells were incubated for 12, 24, and 48 h with 100 mg/L ox-LDL, the levels of GRP78 protein were increased by 256.6%, 374.4%, and 407.9%, respectively (**Fig. 1B**). Additionally, we examined the effect of ox-LDL on the expression of another ER

stress-related protein, CHOP, which induces apoptosis. CHOP was barely present in untreated cells, but was induced by TM. Ox-LDL also clearly induced CHOP expression in a dose- and time-dependent manner (**Fig. 1A** and **1B**).

The effect of ox-LDL on the transcript levels of GRP78 and CHOP was also determined by quantitative real-time PCR (**Fig. 1C** and **1D**). Consistent with the Western blot results, ox-LDL treatment increased

Fig. 2. Activation of ATF6 in ox-LDL-treated RAW264.7 macrophages. **A:** Immunofluorescence experiments show ATF6 visualized by Cy3 labeling (red) and nuclei stained with DAPI (blue). Cells were treated with ox-LDL (100 mg/L) for 24 h or TM (5 mg/L) for 12 h. Representative fluorescent images are shown. Scale bar = 20 μ m. **B and C:** The protein level of ATF6-p50 in nuclear extracts was analyzed by Western blotting and normalized to the histone level. Cells were treated as described in Fig. 1. Data are presented as the mean $\pm$ SEM of at least three independent experiments. ** $p < 0.01$ versus control.

the levels of GRP78 and CHOP mRNA in a dose- and time-dependent manner, suggesting that these ER stress-related proteins are induced at the level of transcription.

Activation of ATF6 by Ox-LDL

ER stress response element (ERSE) has been identified in the promoter of ER chaperones and CHOP genes²². ER transmembrane-localized ATF6-

Fig. 3. Effect of ATF6 siRNA on GRP78 and CHOP expression in ox-LDL-incubated RAW264.7 cells. Cells were transfected with siRNA against ATF6 or a negative control siRNA, followed by treatment with 100 mg/L ox-LDL for 24 h. The silencing of ATF6 was validated by Western blotting (A) and quantitative real-time PCR (B). Expressions of GRP78 and CHOP were evaluated by Western blotting (C) and quantitative real-time PCR (D) in transfected cells. Data are presented as the mean $\pm$ SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus control group transfected with con-siRNA; # $p < 0.05$, ## $p < 0.01$ versus ox-LDL group transfected with con-siRNA.

p90 (the inactive form of ATF6 for ERSE-dependent transcription) is cleaved into ATF6-p50, which translocates to the nucleus where it specifically binds to ERSE to activate the transcription from ERSE²³. The induction of GRP78 and CHOP by ox-LDL or TM (Fig. 1) suggests that ATF6-p90 is activated into ATF6-p50. Immunofluorescence analysis was used to confirm this hypothesis. As shown in Fig. 2A, ATF6

showed diffused cytoplasmic localization in untreated control cells, whereas clusters of ATF6 were detected in the nuclear region in ox-LDL- or TM-treated cells, indicative of activated ATF6.

For further confirmation of this point, the level of ATF6-p50 in the nucleus was measured by immunoblotting analysis (Fig. 2B and 2C). The ATF6-p50 level increased significantly in nuclear extracts pre-

Fig. 4. ATF6 silencing reduces ox-LDL-induced cholesterol accumulation in RAW264.7 cells. Cells were transfected with siRNA against ATF6 or a negative control siRNA, followed by treatment with 100 mg/L ox-LDL for 24 h. A: Intracellular lipid droplets were stained by Oil red O. Representative lipid droplet staining images are shown. Scale bar = 20 μ m. B: The average integrated optical density (IOD) of lipid droplets stained with Oil red O from differentiated macrophage foam cells. C: The intracellular total cholesterol (TC) content was measured using a tissue/cell total cholesterol assay kit. Data are presented as the mean $\pm$ SEM of at least three independent experiments. ** p < 0.01 versus control group transfected with con-siRNA; # p < 0.05 versus ox-LDL group transfected with con-siRNA.

pared from cells treated with ox-LDL in a dose- and time-dependent manner, as well as with TM. These results suggest that ATF6 is activated in the presence of ox-LDL.

Effect of ATF6 SiRNA on the Ox-LDL-Induced Expression of GRP78 and CHOP

To determine whether the activation of ATF6 could trigger ox-LDL-induced ER stress, the siRNA against ATF6 was transfected into RAW264.7 cells before incubating the cells with ox-LDL. To observe the efficiency of RNA interference, the protein and mRNA expression of ATF6 were investigated by Western blotting and quantitative real-time RT-PCR, respectively. As seen in **Fig. 3A** and **3B**, both protein and mRNA levels of ATF6 were downregulated by ATF6 siRNA in the absence or presence of ox-LDL. The effect of ATF6 on the upregulation of GRP78 and CHOP induced by ox-LDL was further determined. Results of **Fig. 3C** and **3D** indicated that pretreatment with ATF6 siRNA inhibited the ox-LDL-induced upregulation of CHOP at protein and

mRNA levels significantly, but had no significant effect on the expression of GRP78.

ATF6 SiRNA Inhibits Ox-LDL-Induced Cholesterol Accumulation in RAW264.7 Cells

Since intracellular cholesterol accumulation is a potent inducer of apoptosis in macrophage²⁴, we tested whether ATF6 could mediate the cholesterol accumulation induced by ox-LDL. Oil red O staining for analysis of lipid droplets indicated that many lipid droplets were present in RAW264.7 cells treated with ox-LDL (100 mg/L) for 24 h; however, the ox-LDL-induced accumulation of lipid droplets was attenuated by ATF6 siRNA (**Fig. 4A** and **4B**).

Consistently with Oil red O staining data, the intracellular TC quantitative assay also indicated that the increase in intracellular TC content in RAW264.7 cells induced by ox-LDL was markedly attenuated by transfection with ATF6 siRNA (**Fig. 4C**). These data indicated that ATF6 promoted ox-LDL-induced cholesterol accumulation in macrophages.

Fig. 5. ATF6 silencing inhibits ox-LDL-induced cell death. Cells were transfected with a siRNA against ATF6 or a negative control siRNA, followed by treatment with 100 mg/L ox-LDL for 24 h, and cell viability was measured by MTT assay and expressed as the percentage of the control group transfected with con-siRNA. Data are expressed as the mean $\pm$ SEM of at least three independent experiments. ** $p < 0.01$ versus control group transfected with con-siRNA; # $p < 0.05$ versus ox-LDL group transfected with con-siRNA.

ATF6 SiRNA Protects RAW264.7 Cells from Cell Death Induced by Ox-LDL

The specific role of ATF6 in ox-LDL-induced cell death was determined by ATF6 siRNA transfection. MTT assay indicated that addition of ox-LDL at 100 mg/L for 24 h decreased cell viability by approximately 53.2%; however, the ox-LDL-dependent decrease in cell viability was partially suppressed by transfection with ATF6 siRNA (**Fig. 5**).

ATF6 SiRNA Attenuates Ox-LDL-Induced Apoptosis of RAW264.7 Cells

To determine whether the activation of ATF6 is involved in ox-LDL-induced apoptosis, RAW264.7 cells were transfected with ATF6 siRNA prior to treatment with ox-LDL. Results of flow cytometry analysis showed that apoptosis was induced in RAW264.7 cells treated with 100 mg/L ox-LDL for 24 h, which was reduced significantly by transfection with ATF6 siRNA (**Fig. 6A**).

The morphologic changes of cells treated as described above were also observed using laser scanning confocal microscopy after Annexin V-FITC/PI staining of the cells. As illustrated in **Fig. 6B**, typical morphological changes, such as PI staining throughout the nuclei and a halo of bright green staining (Annexin V-FITC) on the plasma membrane, appeared when cells were treated with 100 mg/L ox-LDL for 24 h,

whereas the cells transfected with ATF6 siRNA prior to treatment with ox-LDL did not show these evident morphological changes associated with apoptosis.

PERK SiRNA Inhibits Ox-LDL-Induced Upregulation of P-PERK and CHOP

PERK is another upstream molecule that plays an important role in the induction of CHOP during ER stress²⁵. To determine whether the activation of PERK could also trigger ox-LDL-induced ER stress, the siRNA against PERK was transfected into RAW264.7 cells before incubating the cells with ox-LDL. To observe the efficiency of RNA interference, the protein expression of PERK was investigated by Western blotting. As seen in **Fig. 7A**, PERK was downregulated by PERK siRNA in the absence or presence of ox-LDL. **Fig. 7B** and **7C** indicate that treatment of cells with 100 mg/L ox-LDL for 24 h resulted in a significant increase in the phosphorylation of PERK and CHOP expression at both protein and mRNA levels; however, pretreatment with PERK siRNA inhibited significantly the ox-LDL-induced upregulation of p-PERK and CHOP.

PERK SiRNA Attenuates Ox-LDL-Induced Apoptosis of RAW264.7 Cells

The specific role of PERK in ox-LDL-induced cell death and apoptosis was determined by PERK siRNA transfection. MTT assay and flow cytometry analysis indicated that the ox-LDL-dependent decrease in cell viability and induction of apoptosis were suppressed significantly by transfection with PERK siRNA (**Fig. 8A** and **8B**).

Discussion

In this study, we have demonstrated that ox-LDL activates the ER transmembrane protein ATF6 via its cleavage, and subsequently stimulates the expression of CHOP. It is suggested that this upregulation of CHOP induced by ATF6 is involved in ox-LDL-induced apoptosis by showing that ATF6-specific siRNA transfection partially suppresses cellular cholesterol accumulation and apoptosis induced by ox-LDL.

CHOP, also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is a leucine zipper transcription factor that is present at a low level under normal conditions but is robustly expressed in response to ER stress²⁶. CHOP deficiency has been shown to attenuate myocardial reperfusion injury by inhibiting myocardial apoptosis and inflammation²⁷. Mice deficient in CHOP are also markedly protected from pancreatic β -cell apoptosis and diabetes²⁸.

Fig. 6. ATF6 silencing decreases apoptosis induced by ox-LDL in RAW264.7 cells. Cells were transfected with siRNA against ATF6 or a negative control siRNA, followed by treatment with 100 mg/L ox-LDL for 24 h. A: The ratio of apoptotic cells stained with Annexin V-FITC and PI was detected by flow cytometry analysis. Representative data of flow cytometry analysis are presented. The percentage of apoptotic cells under different treatments was measured and expressed as the mean $\pm$ SEM of at least three independent experiments. B: Morphological changes of apoptotic cells were observed with laser scanning confocal microscope by Annexin V-FITC/PI staining. Representative images are shown. Scale bar = 20 μ m.

Recently, CHOP-mediated apoptosis in macrophages has been demonstrated to contribute to the instability of atherosclerotic plaques, and CHOP deficiency has been found to protect macrophages from ER stress-induced apoptosis *in vitro* and in advanced atherosclerotic lesions of mice^{17, 24, 29, 30}. Thus, the induction of CHOP indicates an increase in ER-initiated apoptosis. In this study, we found that by mimicking TM, a classical ER stress inducer, ox-LDL upregulated the expression of CHOP in RAW264.7 cells in a concentration- and time-dependent manner. We also showed that not only the apoptotic ER stress response but also the adaptive response (induction of GRP78) is induced by ox-LDL. Previously, many papers have reported that the expression of GRP78 in cells suppresses apoptosis^{31, 32}; therefore, induction of GRP78 may be involved in the protection of macrophages from ox-LDL-induced apoptosis.

As described in the introduction, ATF6 is an important upstream molecule that plays an important role in the induction of CHOP in ER stress; however, the role of ATF6 in ox-LDL-induced cholesterol accumulation and apoptosis in macrophages remains unclear. This study showed that, similar to TM, ox-

LDL had an effect that resulted in the translocation of ATF6 from the cytoplasm into the nucleus. Interestingly, ATF6 silencing experiments showed that ATF6-specific siRNA inhibited the ox-LDL-induced upregulation of CHOP and apoptosis in RAW264.7 cells. This suggested that the ATF6-CHOP pathway is involved in the apoptosis of macrophage-derived foam cells. However, suppression of ox-LDL-induced upregulation of CHOP and apoptosis by ATF6-siRNA transfection was only partial (**Fig. 3** and **6**), suggesting that ATF6 pathway-independent mechanisms are also involved in ox-LDL-induced apoptosis in macrophages. PERK is one of the upstream kinase activators of the ER stress pathway. Activation of PERK in response to ER stress leads to eukaryotic initiation factor 2 α (eIF2 α) phosphorylation and transient protein synthesis inhibition. This block in translation leads to the synthesis of activating transcription factor 4 (ATF4), which binds to the C/EBP-ATF composite site and transactivates the CHOP promoter^{25, 33}. We here showed that the phosphorylation of PERK was induced in the presence of ox-LDL, and PERK-specific siRNA also inhibited the ox-LDL-induced upreg-

Fig. 7. PERK silencing inhibits ox-LDL-induced upregulation of p-PERK and CHOP in RAW264.7 cells. Cells were transfected with siRNA against PERK or a negative control siRNA, followed by treatment with 100 mg/L ox-LDL for 24 h. The silencing of PERK was validated by Western blotting (A). Protein levels of p-PERK and CHOP were evaluated by Western blot (B) and mRNA level of CHOP was determined by quantitative real-time PCR (C) in transfected cells. Data are presented as the mean $\pm$ SEM of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus control group transfected with con-siRNA; # $p < 0.05$, ## $p < 0.01$ versus ox-LDL group transfected with con-siRNA.

ulation of CHOP and apoptosis in RAW264.7 cells. This suggested that the PERK-CHOP pathway is also involved in ox-LDL-induced apoptosis in macrophages. In addition, ER stress activates apoptosis signal-regulating kinase 1 (ASK1) through inositol-requiring enzyme 1 (IRE1) and tumor necrosis factor receptor-associated factor 2 (TRAF2), and activated ASK1 induces apoptosis through c-Jun amino-terminal kinase (JNK)³⁴. On the other hand, caspase-12, an ER-associated caspase, may also be involved in ER stress-induced apoptosis. ER stress causes procaspase-12 to cluster at the ER membrane where it is activated via IRE1 and TRAF2, and subsequently activates caspase-9 and -3^{35, 36}.

The accumulation of intracellular cholesterol has been identified as an important inducer of macrophage death in advanced atherosclerotic lesions^{2, 4}. It has been reported that cholesterol accumulation can induce apoptosis in cultured macrophages^{21, 24}. Our data in this work demonstrated that ox-LDL could promote cholesterol accumulation in RAW264.7 macrophages and foam cell formation. Interestingly, we also found that ATF6-specific siRNA prevented ox-LDL-induced intercellular cholesterol accumulation, suggesting that ATF6 may mediate cholesterol accumulation in RAW264.7 macrophages induced by ox-LDL, but the associated mechanisms require further exploration.

In conclusion, the present study indicated that

Fig. 8. PERK silencing attenuates ox-LDL-induced apoptosis of RAW264.7 cells. Cells were transfected with siRNA against PERK or a negative control siRNA, followed by treatment with 100 mg/L ox-LDL for 24 h. A: Cell viability was measured by MTT assay and expressed as a percentage of the control group transfected with con-siRNA. Data are expressed as the mean $\pm$ SEM of at least three independent experiments. ** $p < 0.01$ versus control group transfected with con-siRNA; ## $p < 0.01$ versus ox-LDL group transfected with con-siRNA. B: The ratio of apoptotic cells stained with Annexin V - FITC and PI was detected by flow cytometry analysis. Representative data of flow cytometry analysis are presented. The percentage of apoptotic cells under different treatments was measured and expressed as the mean $\pm$ SEM of at least three independent experiments.

ox-LDL could induce cholesterol accumulation and apoptosis in RAW264.7 macrophages, which is accompanied by the activation of ATF6 and increased expression of CHOP. ATF6 siRNA could suppress cholesterol accumulation and apoptosis induced by

ox-LDL, and decrease ox-LDL-induced up-regulation of CHOP. These data suggest that ATF6 mediates ox-LDL-induced cholesterol accumulation and apoptosis in macrophages by up-regulating CHOP expression. Our findings provide an important demonstration that ATF6-mediated ER stress is associated with the formation and apoptosis of macrophage-derived foam cells, which may be considered a novel target in the prevention of atherosclerosis.

Acknowledgments

This research was supported by the Taishan Scholars Foundation of Shandong Province (zd056, zd057), Special Research Funding of Taishan Medical University (1065) and the National Natural Science Foundation of China (30971098). We also thank the colleagues in our laboratory for their discussion and support.

Conflict of Interest

None.

References

- 1) Laufer EM, Winkens HM, Corsten MF, Reutelingsperger CP, Narula J, Hofstra L: PET and SPECT imaging of apoptosis in vulnerable atherosclerotic plaques with radiolabeled Annexin A5. *Q J Nucl Med Mol Imaging*, 2009; 53: 26-34
- 2) 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: 2255-2264
- 3) Seimon T, Tabas I: Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J Lipid Res*, 2009; 50: S382-387
- 4) Tabas I: Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol. *Cell Death Differ*, 2004; 11: S12-16
- 5) Tiwari RL, Singh V, Barthwal MK: Macrophages: an elusive yet emerging therapeutic target of atherosclerosis. *Med Res Rev*, 2008; 28: 483-544
- 6) Aflaki E, Radovic B, Chandak PG, Kolb D, Eisenberg T, Ring J, Fertschai I, Uellen A, Wolinski H, Kohlwein SD, Zechner R, Levak-Frank S, Sattler W, Graier WF, Malli R, Madeo F, Kratky D: Triacylglycerol accumulation activates the mitochondrial apoptosis pathway in macrophages. *J Biol Chem*, 2011; 286: 7418-7428
- 7) Nowak M, Tardivel S, Sayegrih K, Robert V, Abreu S, Chaminade P, Vicca S, Grynberg A, Lacour B: Impact of polyunsaturated fatty acids on oxidized low density lipoprotein-induced U937 cell apoptosis. *J Atheroscler Thromb*, 2011; 18: 494-503
- 8) Ferri KF, Kroemer G: Organelle-specific initiation of cell death pathways. *Nat Cell Biol*, 2001; 3: 255-263
- 9) Hotamisligil GS: Endoplasmic reticulum stress and atherosclerosis. *Nat Med*, 2010; 16: 396-399

- 10) Pineau L, Colas J, Dupont S, Beney L, Fleurat-Lessard P, Berjeaud JM, Bergès T, Ferreira T: Lipid-induced ER stress: synergistic effects of sterols and saturated fatty acids. *Traffic*, 2009; 10: 673-690
- 11) Gora S, Maouche S, Atout R, Wanherdrick K, Lambeau G, Cambien F, Ninio E, Karabina SA: Phospholipolyzed LDL induces an inflammatory response in endothelial cells through endoplasmic reticulum stress signaling. *FASEB J*, 2010; 24: 3284-3297
- 12) Kedi X, Ming Y, Yongping W, Yi Y, Xiaoxiang Z: Free cholesterol overloading induced smooth muscle cells death and activated both ER- and mitochondrial-dependent death pathway. *Atherosclerosis*, 2009; 207: 123-130
- 13) Tsutsumi S, Gotoh T, Tomisato W, Mima S, Hoshino T, Hwang HJ, Takenaka H, Tsuchiya T, Mori M, Mizushima T: Endoplasmic reticulum stress response is involved in nonsteroidal anti-inflammatory drug-induced apoptosis. *Cell Death Differ*, 2004; 11: 1009-1016
- 14) Gotoh T, Oyadomari S, Mori K, Mori M: Nitric oxide-induced apoptosis in RAW 264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP. *J Biol Chem*, 2002; 277: 12343-12350
- 15) Senkal CE, Ponnusamy S, Bielawski J, Hannun YA, Ogretmen B: Antiapoptotic roles of ceramide-synthase-6-generated C16-ceramide via selective regulation of the ATF6/CHOP arm of ER-stress-response pathways. *FASEB J*, 2010; 24: 296-308
- 16) Adachi Y, Yamamoto K, Okada T, Yoshida H, Harada A, Mori K: ATF6 is a transcription factor specializing in the regulation of quality control proteins in the endoplasmic reticulum. *Cell Struct Funct*, 2008; 33: 75-89
- 17) Tsukano H, Gotoh T, Endo M, Miyata K, Tazume H, Kado-matsu T, Yano M, Iwakaki T, Kohno K, Araki K, Mizuta H, Oike Y: The endoplasmic reticulum stress-C/EBP homologous protein pathway-mediated apoptosis in macrophages contributes to the instability of atherosclerotic plaques. *Arterioscler Thromb Vasc Biol*, 2010; 30: 1925-1932
- 18) Wintergerst ES, Jelk J, Rahner C, Asmis R: Apoptosis induced by oxidized low density lipoprotein in human monocyte-derived macrophages involves CD36 and activation of caspase-3. *Eur J Biochem*, 2000; 267: 6050-6059
- 19) Martinet W, Kockx MM: Apoptosis in atherosclerosis: focus on oxidized lipids and inflammation. *Curr Opin Lipidol*, 2001; 12: 535-541
- 20) Yao ST, Sang H, Yang NN, Kang L, Tian H, Zhang Y, Song GH, Qin SC: Oxidative low density lipoprotein induces macrophage ER stress via CD 36. *Sheng Li Xue Bao*, 2010; 62: 433-440
- 21) Boullier A, Li Y, Quehenberger O, Palinski W, Tabas I, Witztum JL, Miller YI: Minimally oxidized LDL offsets the apoptotic effects of extensively oxidized LDL and free cholesterol in macrophages. *Arterioscler Thromb Vasc Biol*, 2006; 26: 1169-1176
- 22) Yoshida H, Haze K, Yanagi H, Yura T, Mori K: Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J Biol Chem*, 1998; 273: 33741-33749
- 23) Yoshida H, Okada T, Haze K, Yanagi H, Yura T, Negishi M, Mori K: ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Mol Cell Biol*, 2000; 20: 6755-6767
- 24) Devries-Seimon T, Li Y, Yao PM, Stone E, Wang Y, Davis RJ, Flavell R, Tabas I: Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor. *J Cell Biol*, 2005; 171: 61-73
- 25) Ma Y, Brewer JW, Diehl JA, Hendershot LM: Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J Mol Biol*, 2002; 318: 1351-1365
- 26) Tabas I, Ron D: Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol*, 2011; 13: 184-190
- 27) Miyazaki Y, Kaikita K, Endo M, Horio E, Miura M, Tsujita K, Hokimoto S, Yamamuro M, Iwakaki T, Gotoh T, Ogawa H, Oike Y: C/EBP homologous protein deficiency attenuates myocardial reperfusion injury by inhibiting myocardial apoptosis and inflammation. *Arterioscler Thromb Vasc Biol*, 2011; 31: 1124-1132
- 28) Song B, Scheuner D, Ron D, Pennathur S, Kaufman R: Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J Clin Invest*, 2008; 118: 3378-3389
- 29) Feng B, Yao PM, Li Y, Devlin CM, Zhang D, Harding HP, Sweeney M, Rong JX, Kuriakose G, Fisher EA, Marks AR, Ron D, Tabas I: The endoplasmic reticulum is the site of cholesterol-induced cytotoxicity in macrophages. *Nat Cell Biol*, 2003; 5: 781-792
- 30) Thorp E, Li G, Seimon TA, Kuriakose G, Ron D, Tabas I: Reduced apoptosis and plaque necrosis in advanced atherosclerotic lesions of Apoe^{-/-} and Ldlr^{-/-} mice lacking CHOP. *Cell Metabolism*, 2009; 9: 474-481
- 31) Bilecová-Rabajdová M, Urban P, Mašlanková J, Veselá J, Mareková M: Analysis of changes in pro (Gadd153) and anti apoptotic (Grp78) gene expression after ischemic-reperfusion injury of the small intestine. *Prague Med Rep*, 2010; 111: 249-256
- 32) Zhou H, Zhang Y, Fu Y, Chan L, Lee AS: Novel mechanism of anti-apoptotic function of 78-kDa glucose-regulated protein (GRP78): endocrine resistance factor in breast cancer, through release of B-cell lymphoma 2 (BCL-2) from BCL-2-interacting killer (BIK). *J Biol Chem*, 2011; 286: 25687-25696
- 33) Bromati CR, Lellis-Santos C, Yamanaoka TS, Nogueira TC, Leonelli M, Caperuto LC, Gorjão R, Leite AR, Anhê GF, Bordin S: UPR induces transient burst of apoptosis in islets of early lactating rats through reduced AKT phosphorylation via ATF4/CHOP stimulation of TRB3 expression. *Am J Physiol Regul Integr Comp Physiol*, 2011; 300: R92-100
- 34) Kim YH, Joo HS, Kim DS: Nitric oxide induction of IRE1-alpha-dependent CREB phosphorylation in human glioma cells. *Nitric Oxide*, 2010; 23: 112-120
- 35) Morishima N, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y: An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem*, 2002; 277: 34287-34294
- 36) Reijonen S, Putkonen N, Nørremølle A, Lindholm D,

Korhonen L: Inhibition of endoplasmic reticulum stress counteracts neuronal cell death and protein aggregation

caused by N-terminal mutant huntingtin proteins. *Exp Cell Res*, 2008; 314: 950-960