# Cholesterol overloading leads to hepatic L02 cell damage through activation of the unfolded protein response

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**Abstract.** Reported data indicate that cholesterol loading in the liver can cause hepatic injury. To explore the possible mechanisms of cell damage resulting from cholesterol overloading in hepatocytes, cell apoptosis, the unfolded protein response (UPR) and the correlation between them were assessed in the cholesterol-overloaded normal human hepatic cell line L02. L02 cells were incubated with 200 $\mu$g/ml of low density lipoprotein (LDL) for 24 h with or without 20 $\mu$g/ml 58035, an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT). In the LDL+58035 group, the intracellular cholesterol level was dramatically increased, which was measured by an enzymatic combined high performance liquid chromatography assay. Expression of immunoglobulin-binding protein, X-box binding protein 1, activating transcription factor 6, activating transcription factor 4, CCAAT/enhancer-binding protein homologous protein-10, markers of endoplasmic reticulum stress (ERS)/UPR, were up-regulated as determined using reverse transcription-polymerase chain reaction (RT-PCR) or Western blot analysis. The rate of cell apoptic death increased 21.3±2.4%. Meanwhile, the active caspase-3 protein expression was increased 8.4-fold compared to the active caspase-3 protein expression in the controls. Furthermore, 4-phenylbutyric acid, an inhibitor of UPR, partly reduced cell apoptosis and activation of caspase-3. This study suggests that cholesterol overloading in hepatic L02 cells induces ERS and activates the UPR which, in part, leads to the apoptotic damage of cells.

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## Introduction

In eukaryotic cells, the endoplasmic reticulum (ER) is the main organelle which performs the folding and maturation of secretory and membrane proteins. ER stress (ERS) is an adaptive response caused by accumulation of unfolded/misfolded proteins in the ER under conditions of stress. In order to maintain ER function, the unfolded protein response (UPR) is activated to up-regulate the ER chaperone and folding enzyme expression (1,2). ERS/UPR regulates intracellular gene expression to alleviate stress through three main signaling pathways. However, sustained UPR can lead to various diseases such as diabetes, Alzheimer's and Parkinson's disease (1,3-5). Research has shown that free cholesterol loading can induce the ERS/UPR in macrophages, resulting in the disorder of lipid metabolism and apoptosis, which subsequently contributes to the development and progression of atherosclerosis (6-8).

The liver is the principal organ involved in cholesterol metabolism (9,10), and many factors can cause disorder of lipid metabolism in the liver, such as alcohol abuse (11), high fat diet (12-14), hyperhomocysteinemia (15) or hepatic virus infections (16), which can induce ERS/UPR (1). These lead to the accumulation of lipids including cholesterol and the development of alcoholic and nonalcoholic hepatic steatosis (1,17). It has been demonstrated that cholesterol loading in liver fat causes hepatocytes to be susceptible to inflammatory factors, which then promotes steatohepatitis (18). In Niemann-Pick type C (NPC) disease which is caused by NPC1 and NPC2 protein mutations, cholesterol loading is closely correlated with liver cell death (19). Yet, it is still unknown whether cholesterol loading can induce the UPR of hepatocytes, and the underlined mechanism of hepatic injury resulting from cholesterol loading remains to be elucidated.

In this study, normal human hepatic L02 cells were treated with low density lipoprotein (LDL) with or without acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor 58035 (20), and the intracellular cholesterol, the markers of activated ERS/UPR and apoptosis were assessed. Based on the effect of cholesterol on macrophages, we postulated that cholesterol loading in hepatocytes activates UPR to induce cell apoptotic

damage. Our results showed that cholesterol overloading in L02 cells induced ER stress and activated the UPR which led to cell apoptosis.

## Materials and methods

*Materials*. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Trizol reagent were obtained from Invitrogen (Carlsbad, CA, USA). LDL (density, 1.006-1.063 g/ml) was isolated from the plasma of healthy human volunteers by sequential ultracentrifugation. 4-Phenylbutyric acid (PBA) was from Calbiochem (San Diego, CA, USA). The Annexin V-FITC Apoptosis Detection Kit Plus was purchased from Alexis (Switzerland). Antibodies against ATF6, BiP, active caspase-3 and ß-actin were purchased from Santa Cruz Biotechnology (CA, USA). All other chemicals and reagents, including compound 58035 (an inhibitor of ACAT), cholesterol oxidase and cholesterol esterase, were from Sigma (St. Louis, MO). The human hepatic cell line L02 was obtained from the China Center for Type Culture Collection (Wuhan, China).

*Cell culture*. L02 cells were cultured in DMEM containing 10% FBS and maintained at 37˚C in a 5% $CO_2$ humidified incubator. Cells were applied for experiments until 80% confluent. Cells were randomly divided into three groups and incubated for 24 h respectively: normal control group, LDL (200 $\mu$g/ml) group, and LDL (200 $\mu$g/ml)+58035 (20 $\mu$g/ml) group.

*Measurement of intracellular cholesterol*. After treatment for 24 h, the medium was discarded, and cells were washed twice in PBS. One milliliter of Triton X-100 was added for cell disruption. The total cholesterol (TC), free cholesterol (FC) and cholesterol ester (CE) contents were measured using a combination of an enzymatic method and high performance liquid chromatography (HPLC) assay as previously described (21). The protein concentration was measured using the Lowry method (Pierce, Rockford, IL, USA).

*Reverse transcription-polymerase chain reaction (RT-PCR) analysis*. After incubation for 24 h, total cell RNA was isolated using Trizol reagent following the manufacturer's instructions. The Qiagen OneStep RT-PCR Kit was used for RT-PCR. The primers used for immunoglobulin-binding protein (BiP), X-box binding protein 1 (XBP1), activating transcription factor 6 (ATF6), activating transcription factor 4 (ATF4), CCAAT/enhancer-binding protein homologous protein-10 (CHOP) and ß-actin are provided in Table I. The XBP1 primers were designed according to XBP1 cDNA encompassing the region of the restriction site of *Pst*1 (13). PCR products identified by 2% agarose gel electrophoresis were analyzed using Image Quant software.

*Western blot analysis*. The cell pellet was lysed in buffer containing 10 mmol/l Tris-HCl (pH 8.0), 1 mmol/l $MgCl_2$, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/l phenylmethysulfonyl fluoride, 1 mg/l aprotinin and 0.02 mg/l leupeptin. The proteins separated on the PAGE gel were transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in TBST solution with 50 mmol/l Tris-HCl (pH 7.6), 150 mmol/l NaCl, and 0.1% Tween-20. After incubation with the primary antibody, the membrane was incubation with the corresponding secondary antibodies conjugated to horseradish peroxidase. Proteins were visualized using an enhanced chemiluminescence system (Pierce, Rockford, IL, USA).

Table I. Primer sequences for PCR.

| Gene | Primer sequence (5'→3') | |
|---|---|---|
| XBP1 | Forward | TCGAAAGAAGGCTCGAATGAGTAG |
| | Reverse | AGGGCATTTGAAGAACATGACTGC |
| BiP | Forward | GGGGAGGGAGTATTTGGTATGTTG |
| | Reverse | CCCTTGCCTGAGTAAAGATGTGG |
| ATF6 | Forward | GAACCATTGCTTTACATTCCTCCAC |
| | Reverse | CTGCTTGACTTGGTCCTTTCTACTTC |
| ATF4 | Forward | TTCCAGCAAAGCACCGCAAC |
| | Reverse | AGGGCATCCAAGTCGAACTCCT |
| CHOP | Forward | TGAACGGCTCAAGCAGGAAATCG |
| | Reverse | GGATTGAGGGTCACATCATTGGCACT |
| ß-actin | Forward | TGAGACCTTCAACACCCCAG |
| | Reverse | GCCATCTCTTGCTCGAAGTC |

*Fluorescent-stained cell apoptosis assays*. After incubing the L02 cells with LDL, LDL+58035 or LDL+58035+3 mmol/l PBA, apoptosis was assayed by staining with fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI) according to the manufacturer's manual. Three to six representative fields were viewed immediately with an Olympus IX-70 inverted fluorescence microscope. Each field contained ~1000 cells. The number of positive fluorescent-stained cells and the total number of cells were determined. The rate of cell apoptosis was equal to the total number of cells divided by the total number of positive cells.

*Statistical analysis*. Quantitative data were expressed as the mean ± SD. Statistical analysis was performed using the Student's t-test or ANOVA with SPSS software package (version 12.0). A P-value $<0.05$ was considered statistically significant.

## Results

*Intracellular cholesterol levels are increased in the LDL-loaded L02 cells with or without 58035*. In the LDL group, the TC and FC contents in L02 cells were increased by 7.05±0.28 and 5.27±0.71 $\mu$g/mg, respectively. However, the TC and FC contents were increased dramatically in the LDL+58035 group, particularly the FC content which was increased by 9.3±1.02 $\mu$g/mg (Table II). FC was significantly overloaded.

Table II. The cholesterol content[a] in L02 cells of each group.

| Groups | TC | FC | CE |
|---|---|---|---|
| Control | 6.97±0.25 | 5.90±0.36 | 1.07±0.15 |
| 200 $\mu$g/ml LDL | 14.03±0.47[b] | 11.17±0.35[b] | 2.97±0.25[b] |
| 200 $\mu$g/ml LDL+ 20 $\mu$g/ml 58035 | 14.91±0.64[b] | 13.20±0.66[b,c] | 1.70±0.10[b,c] |

[a]$\mu$g/mg cell protein. Data are the mean ± SD, n=3. [b]p<0.05 vs control group, [c]p<0.05 vs 200 $\mu$g/ml LDL group. TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester.

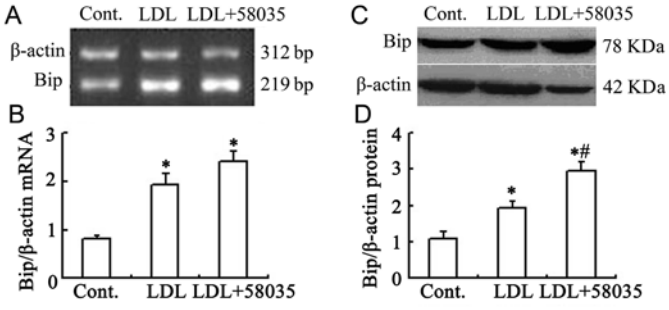


Figure 1. Expression of BiP in L02 cells with cholesterol loading. (A) BiP mRNA of each group with RT-PCR; (B) relative values of BiP mRNA of each group; (C) Western blot analysis of L02 cells of each group; and (D) relative values of BiP protein expression of each group. Data are the mean ± SD; *p<0.05 vs control group, #p<0.05 vs LDL group.

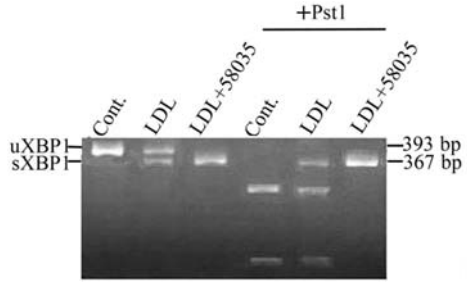


Figure 2. XBP1 mRNA analysis of each group by RT-PCR. PCR products were digested by the *Pst*1 restriction enzyme for 16 h. Intact and *Pst*1-digested PCR fragments were revealed on 2% agarose gel. A 393-bp fragment of XBP1 mRNA was amplifed by RT-PCR and digested to two bands by *Pst*1. The 367-bp fragment of sXBP1 mRNA lacking the restriction site could not be digested by *Pst*1.

*Expression of BiP is increased in the cholesterol-overloaded L02 cells*. Chaperone BiP expression reflects the activation of UPR (22). Compared to the control, the BiP mRNA level in cells treated with LDL was increased by 112±33% compared to that in the control cells, while the BiP mRNA level was increased by 200±34% in the LDL+58035 group (Fig. 1B). The protein expression in the LDL and LDL+58035 groups was increased 193±21% and 297±24% compared with that in the control cells, respectively (Fig. 1C and D).

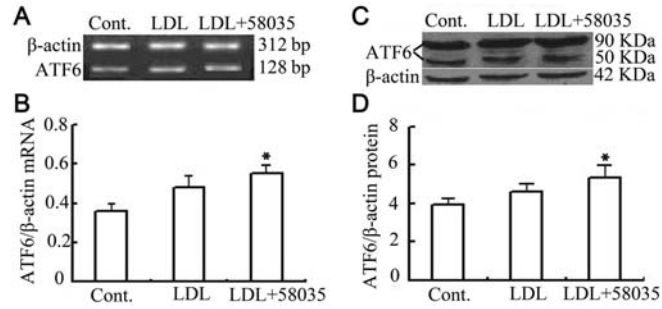


Figure 3. Expression of ATF6 in the L02 cells with cholesterol loading of each group. (A) ATF6 mRNA of each group by RT-PCR; (B) relative values of ATF6 mRNA of each group; (C) Western blot analysis of the L02 cells of each group and (D) relative values of ATF6 protein expression of each group. Data are the mean ± SD; *p<0.05 vs control groups.

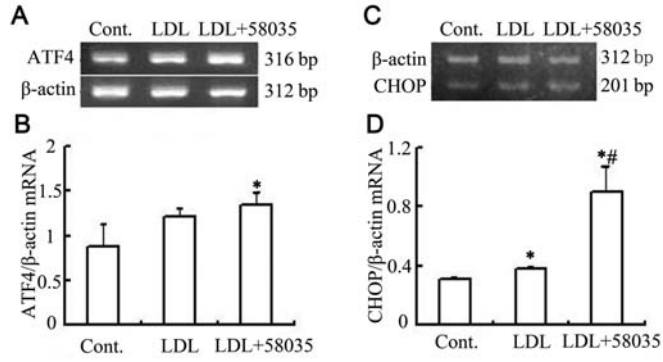


Figure 4. mRNA level of ATF4 and CHOP in the L02 cells with FC loading for each group. (A) ATF4 mRNA of each group by RT-PCR; (B) relative values of ATF4 mRNA of each group; (C) CHOP mRNA of each group by RT-PCR; and (D) relative values of CHOP mRNA of each group. Data are the mean ± SD; *p<0.05 vs control group, #p<0.05 vs LDL group.

*Spliced XBP1 mRNA is induced in cholesterol-overloaded L02 cells*. In our experiments, spliced XBP1 (sXBP1) mRNA was observed in the cholesterol-loaded L02 cells, and the more free cholesterol loading, the higher the sXBP1 mRNA level. In the control group, only unspliced XBP1 (uXBP1) mRNA was detected. Two bands of 256 and 137 bp were obtained by digestion of the PCR product of uXBP1 with *Pst*1, while sXBP1 lacking the restriction site could not be digested by *Pst*1 (Fig. 2) (13,22).

*Expression of ATF6 is increased in cholesterol-overloaded L02 cells*. In the LDL group, there were no significant changes in the mRNA and protein expression of ATF6 compared to that in the control group. However, in the LDL+58035 group, the ATF6 mRNA level was increased by 57±9% compared to the ATF6 mRNA level in the control cells (Fig. 3A and B), and the protein expression was increased 32±5% compared to that in the controls (Fig. 3C and D).

*ATF4 and CHOP mRNA expression is induced in LDL-loaded L02 cells with or without 58035*. The mRNA level of ATF4 was not apparently changed in the LDL group, while in the LDL+58035 group, it was increased 1.5±0.11-fold compared

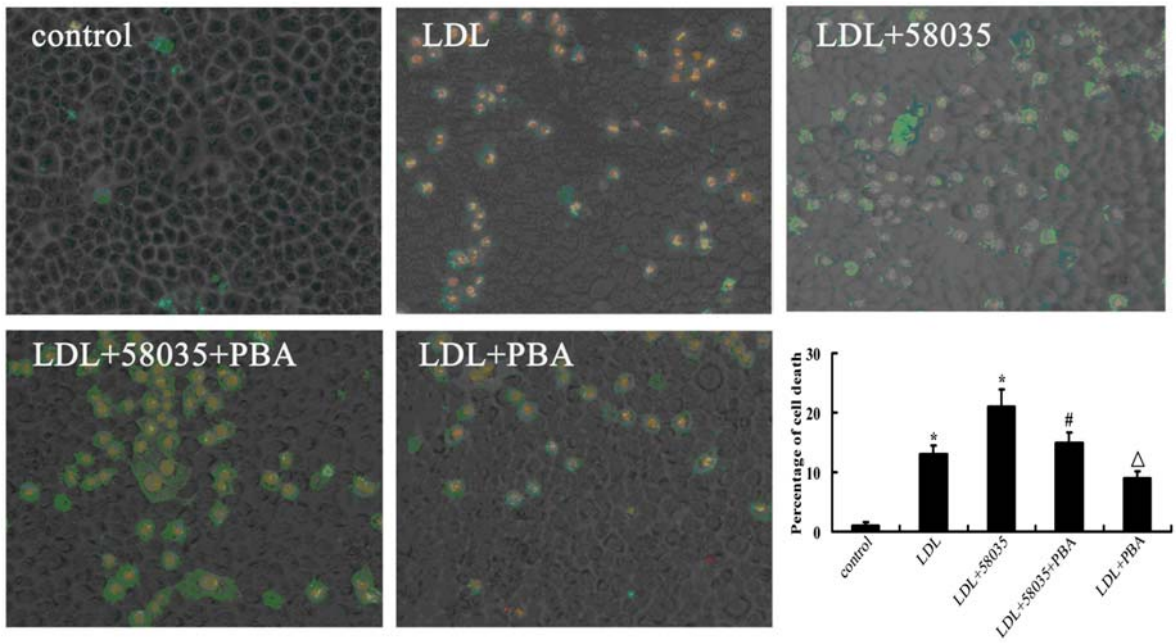


Figure 5. Assay of cholesterol-loading-induced apoptosis in L02 cells with FITC-labeled Annexin V and PI staining. Three to six representative fields were viewed with an Olympus IX-70 inverted fluorescence microscope. Each field had ~1000 cells. The number of fluorescent-stained positive cells and the total number of cells were determined. The rate of cell apoptosis was equal to the total number of cells divided by the total number of positive cells. Data are the mean ± SD; *p<0.05 vs control group, △p<0.05 vs LDL group, #p<0.05 vs LDL+58035 group.

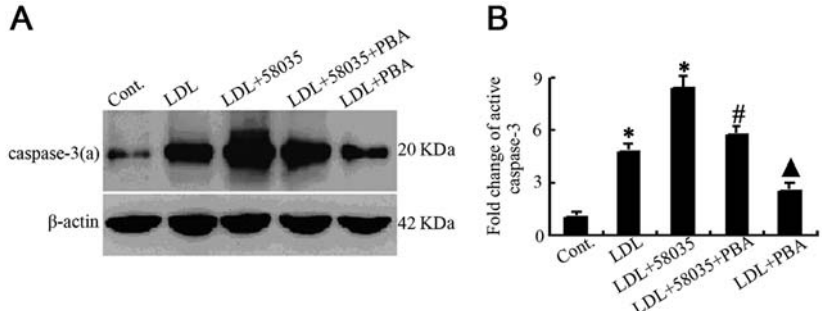


Figure 6. Assay of active caspase 3 in L02 cells with cholesterol loading. (A) Western blot analysis of active caspase 3 and ß-actin in L02 cells. (B) The relative values of active caspase 3 of each group. Data are the mean ± SD; *p<0.05 vs control group, ▲p<0.05 vs LDL group, #p<0.05 vs LDL+58035 group.

to that in the controls (Fig. 4A and B). The CHOP mRNA level in the LDL and LDL+58035 groups was enhanced 1.2±0.07- and 2.9±0.3-fold, respectively (Fig. 4C and D).

*Cholesterol overloading in L02 cells results in apoptosis, which is be partly reduced by PBA*. The rate of cell apoptosis in the control cells was 1.1±0.6%, and this rate increased to 12.9±1.4% in the LDL group and to 21.3±2.4% in the LDL+58035 group (Fig. 5). However, the UPR inhibitor PBA lowered the rate of cell apoptosis caused by cholesterol overloading. When PBA (3 mmol/l) was added to the L02 cells of the LDL and LDL+58035 groups, the cell apoptosis rate decreased to 8.8±1.1 and 14.9±1.6%, respectively (Fig. 5).

*Active caspase-3 expression is increased by cholesterol overloading in L02 cells, which is also partly abrogated by PBA*. The active caspase-3 protein expression in the LDL and LDL+58035 groups was increased 4.8±0.21- and 8.4±0.46-fold compared to that in the controls, but decreased 2.5±0.13- and 5.7±0.35-fold after using PBA, respectively (Fig. 6).

## Discussion

The content of cholesterol in hepatocytes is controlled in two ways: the cholesterol uptake from LDL and synthesis, and cholesterol export through bile salt synthesis and esterification (23). A high concentration of LDL increases the source of cholesterol (24), and 58035 can inhibit ACAT2 which is located in the ER of hepatocytes (25) to block the export of cholesterol esterification (26). Both can result in cell cholesterol loading. Thus, we established a model of free cholesterol loading by using L02 cells incubated with LDL in the presence or absence of 58035 in order to investigate whether cholesterol loading in hepatocytes causes ERS/UPR to induce apoptotic injury.

Three independent response pathways of ERS/UPR are mediated by ER stress sensors including type-1 ER transmembrane protein kinase (IRE1), ATF6 and the RNA-dependent protein kinase (PKR)-like ER kinase (PERK). Without ER stress, the sensors are usually associated with or bound to intralumenal calcium-dependent chaperone BiP. With stress, BiP engaged to unfolded proteins in the ER dissociates from IRE1, ATF6, and PERK liberating these signal transducers to promote a compensatory defense response (1). Activated IRE1 can activate transcription factor XBP1 by alternative splicing to form sXBP1 mRNA. sXBP1 mRNA which loses a 26-bp intron is used to evaluate the activation of the transcriptional arm of UPR (27-29). Both sXBP1 and activated ATF6 act on ER stress response elements (ERSE) to promote expression of the chaperone (22). Thus, there is increased BiP expression when UPR is activated. ATF4 and CHOP are the downstream molecules of PERK, and activated PERK activates both.

The markers of ERS/UPR were induced in FC-overloaded L02 cells, resulting from incubation with a high concentration of LDL in the presence of inhibitor 58035. In the LDL group, BiP, sXBP1 and CHOP mRNA was up-regulated, and BiP protein expression was increased, but there was no significant change in ATF6 and ATF4 expression. However, in the LDL+58035 group, expression of BiP and ATF6 was dramatically increased, and the sXBP1, ATF4, and CHOP mRNA was markedly up-regulated. These results demonstrate that cholesterol loading in L02 cells caused by LDL incubation alone induces UPR and is involved in the activation of the IRE1-XBP1 and PERK-CHOP pathways. Free cholesterol overloading by LDL and the inhibitor of ACAT (58035) can cause more severe UPR, which activates the IRE1-XBP1, ATF6 and PERK-CHOP pathways. Although the mechanism of UPR caused by cholesterol overloading in hepatocytes is unclear, Feng *et al* (6) reported that FC loading in macrophages results in cytotoxicity to the ER membrane to disturb the function of ER and depletes ER calcium stores which can induce UPR activation.

Upon observation of apoptosis using Annexin-FITC and PI staining, we found that the rate of cell death was increased significantly with cholesterol overloading. An approximate 13- and 20-fold increase in the number of apoptotic cells was found in the LDL and LDL+58035 groups, respectively, and active caspase-3 expression was obviously increased as well. Typically, under normal physiological conditions, ERS/UPR initiates cellular protection to enhance the survival rate of cells. However, prolonged or severe ER stress activates the apoptotic pathway of UPR such as PERK-CHOP and specific pro-apoptotic factor caspase 12 leading to cell death (30-32). In our experiments, free cholesterol overloading in L02 cells induced CHOP and active caspase-3 expression. It has been reported that overexpression of CHOP induces apoptosis via down-regulation of the Bcl-2 protein and translocation of the Bax protein from the cytosol to the mitochondria and disturbance of the cellular redox state by depletion of cellular glutathione (33).

However, there are several mechanisms related to apoptosis (34). For example, macrophages with cholesterol loading can activate the Fas/FasL pathway, or p38 or JNK to induce cell death (35,36). Marí *et al* (18) found that free cholesterol loading of hepatocyte mitochondria easily caused oxidative stress, depletion of reduced glutathione, and promoted cytochrome c release and caspase activation. It also sensitized hepatocytes to tumor necrosis factor and Fas which can induce apoptosis and necrosis. In order to confirm the correlation between apoptosis and the activation of ERS/UPR caused by cholesterol loading in hepatic L02 cells, UPR inhibitor PBA was used to treat cells and then the effects on the apoptotic cell death by cholesterol overloading was observed. PBA, a low-molecule-weight chaperone, stabilizes protein conformation, improves ER folding capacity, and inhibits lipid-induced ERS (37,38), but does not affect cellular lipid contents (13). Moreover, data indicate that the chemical chaperone PBA prevents cell death caused by hypoxia-induced ERS while decreasing CHOP expression (37,39). Thus, PBA typically acts as an inhibitor of UPR. In the LDL or LDL+58035 groups with PBA, expression of active caspase-3 and the rate of cell apoptosis significantly decreased. These data indicate that UPR mediated the apoptosis of FC-loaded hepatocytes to some degree.

In conclusion, our study found that the long-term free cholesterol overloading of hepatic L02 cells induced ERS/UPR, up-regulated expression of BiP, ATF6, sXBP1, ATF4 and CHOP, and increased active caspase-3 protein and induced apoptosis; while the UPR inhibitor PBA decreased the activation of caspase-3 and the rate of apoptotic cell death caused by FC loading. These results suggest that the primary mechanism of hepatic injury in cholesterol-overloaded hepatocytes is related to the activation of the unfolded protein response.

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