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Bip overexpression, but not CHOP inhibition, attenuates fatty-acid-induced endoplasmic reticulum stress and apoptosis in HepG2 liver cells

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ABSTRACT

Aims: In this study we investigated whether attenuation of endoplasmic reticulum stress (ER stress) could protect HepG2 cells from free fatty acid (FFA)-induced apoptosis.
Main methods: Human liver cell line HepG2 cells were exposed to Sodium Palmitate (Pa) or Sodium Oleate (Ol). Apoptosis and ER stress of HepG2 cells were analyzed with flow cytometry, real-time RT-PCR and Western Blotting. An expression plasmid encoding for the ER chaperone immunoglobulin heavy chain-binding protein (Bip) was transfected into HepG2 cells to attenuate ER stress. Small interfering RNA siCHOP was used to knockdown the expression of C/EBP Homologous Protein (CHOP) in HepG2.
Key findings: Pa led to cytotoxicity and apoptosis in HepG2 cells in a dose-dependent pattern and also induced ER stress indicated by increased phosphorylation of eIF2α, upregulation of IRE1α and CHOP. Bip expression levels were slightly down regulated after Pa treatment. The unsaturated fatty acid, Ol, induced neither apoptosis nor ER stress in HepG2 cells. Overexpression of Bip attenuated Pa-induced ER stress and led to a significant reduction in Pa-mediated apoptosis, indicating a requirement of ER stress for lipotoxicity in liver cells. siRNA-mediated reduction of CHOP did not protect against Pa-induced apoptosis.
Significance: While ER stress makes a necessary contribution to palmitate cytotoxicity, inhibition of CHOP alone is not sufficient to prevent palmitate-induced apoptosis. Our findings could advance the detailed understanding on the mechanism of high fatty acid (FFA)-induced apoptosis.

Introduction

The endoplasmic reticulum (ER) is a cytoplasmic organelle found in all eukaryotic cells that controls the most important steps in the folding and modification of secretory and membrane proteins. The ER is exquisitely sensitive to alterations in homeostasis, and to a number of biochemical and physiologic stimuli. The ER also serves important functions in calcium storage and signaling and lipid biosynthesis. The overloading of cholesterol can disrupt ER homeostasis, imposing stress to the ER (ER stress) and subsequently lead to accumulation of unfolded or misfolded proteins in the ER lumen (Zhang and Kaufman, 2006). The accumulation of unfolded proteins leads to the activation of the so called unfolded protein response (UPR), which includes adaptive mechanisms to reestablish normal ER functions (Xu et al., 2005). However, severe and prolonged ER stress triggers cell suicide, usually in the form of apoptosis (Laybutt et al., 2007; Wei et al., 2007).

It is well accepted that elevated free fatty acids (FFAs) lead to cell dysfunction and apoptosis in non-adipose tissues, a concept referred to as lipotoxicity. Deleterious effects on the cell viability of high FFAs,

Table 1
Primer sets used for real-time PCR.

Gene	Species	Size (bp)	Sequences
Rpl 19	Human	233	F: 5'-ATG TAT CAC AGC CTG TAC CTG-3' R: 5'-TTC TTG GTC TCT TCC TCC TTG-3'
CHOP	Human	201	F: 5'-ACC AAG GGA GAA CCA GGA AAC G-3' R: 5'-TCA CCA TTC GGT CAA TCA GAG C-3'
Bip	Human	211	F: 5'-CGG GCA AAG ATG TCA GGA AAG-3' R: 5'-TTC TGG ACG GGC TTC ATA GTA GAC-3'
IRE1α	Human	196	F: 5'-TGG GTA AAA AGC AGG ACA TCT GG-3' R: 5'-GCA TAG TCA AAG TAG GTG GCA TTC C-3'

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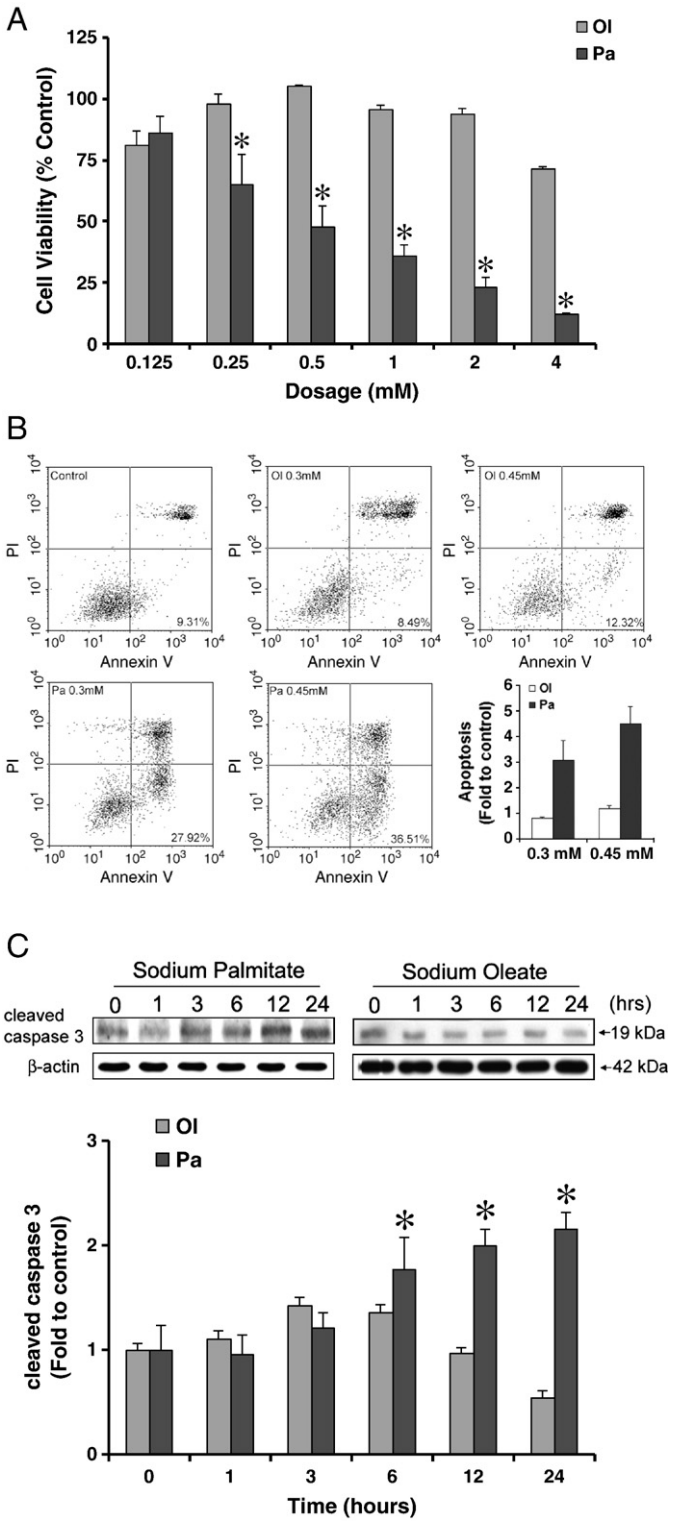

Fig. 1. Elevated palmitate-induced apoptosis in HepG2 cells. A. Viability of HepG2 cells exposed to Palmitate (Pa) or Oleate (Ol). HepG2 cells were treated with either BSA alone (set as control) or BSA coupled 0.125, 0.25, 1, 2 and 4 mM Pa or Ol for 24 hours. Cell viability was measured using MTT assay. Cell viability was calculated as percentage to controls. B. Apoptosis in HepG2 cells exposed to lipids. HepG2 cells were treated with either BSA alone (set as control) or BSA coupled 0.3 and 0.45 mM Pa or Ol. Representative flow cytometry results were shown and the numbers at lower right indicated the percentage of apoptotic cells. Apoptotic cells were then calculated as fold to control. C. Representative Western blotting results comparing time-course changes in cleaved caspase 3 in HepG2 cells treated with lipids (upper). β-actin served as a loading control. Bands were semi-quantified by densitometry and were expressed as fold-change compared with control (lower). Data are presented as means ± SE. * $p<0.05$ versus control.

particularly saturated fatty acids such as palmitate, have been reported in several cell types, including cardiac cells (Okere et al., 2006) (Miller et al., 2005), pancreatic beta cells (Laybutt et al., 2007), breast cancer cells (Liu et al., 2008), adipocytes (Guo et al., 2007) and hepatocytes (Wei et al., 2007). Recent studies have focused on ER stress as a potential mediator of high fatty acid (FFA)-induced apoptosis. However, the detailed mechnisms are not clear so far.

Hepatocytes are characterized by abundant and well-developed ER and thus serve as an established cellular model to study changes in ER homeostasis. Here in our present study, we examined the differential effects of saturated and unsaturated fatty acids on ER stress and apoptosis using human liver HepG2 cells in order to define the role of ER stress in lipotoxicity. Furthermore, we investigated the detailed mechanisms of lipotoxicity by modifying the signaling pathways in the ER stress.

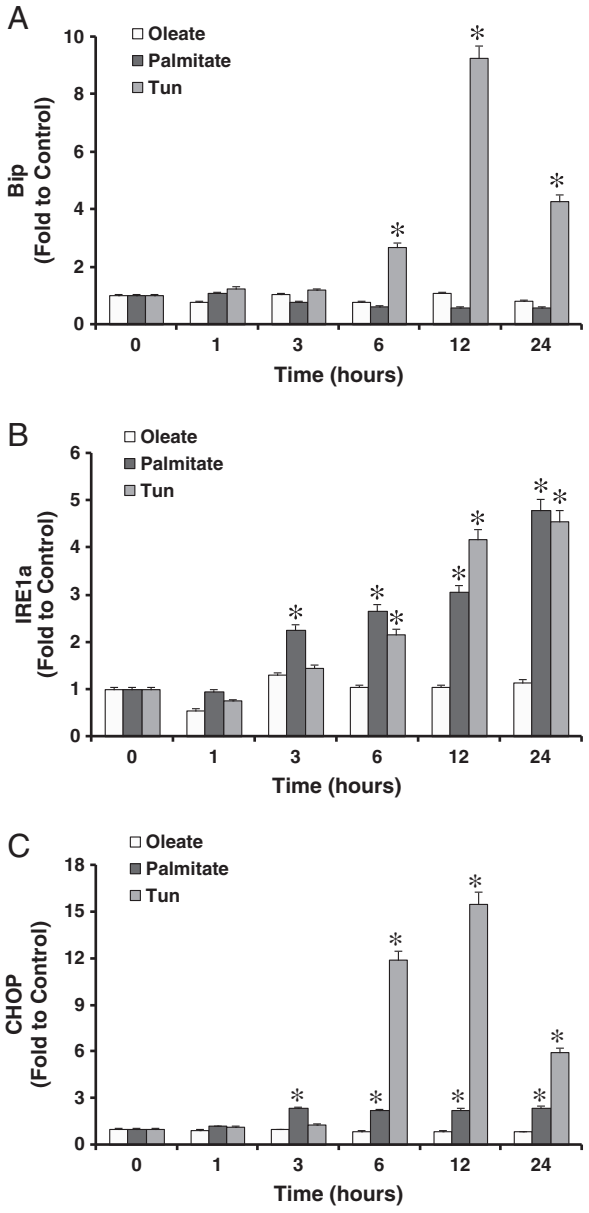

Fig. 2. Time-course changes of mRNA levels of genes involved in ER stress in lipid-treated HepG2 cells. HepG2 cells were treated with 0.45 mM of Ol, 0.45 mM Pa or 5 μg/ml of Tunicamycin (Tun) for 0, 1, 3, 6, 12 and 24 hours. The total RNA was extracted and analyzed by real-time RT-PCR. Results are presented as means ± SE and are expressed as fold-change compared with control. * $p<0.05$ versus control-treated HepG2 cells at the same time point.

Materials and methods

Research reagents

98% FA-free BSA, sodium palmitate, sodium oleate and all other biochemical reagents were from Sigma Aldrich. Cell culture medium and corresponding items were all purchased from Invitrogen. The anti-C/EBP Homologous Protein (CHOP) antibody was purchased from Santa Cruz Biotechnology and Cell Signaling Technology. All the other antibodies to phosphorylated eukaryotic translation initiation factor 2α (eIF2α, total eIF2α Immunoglobulin heavy chain-binding protein (Bip), Bcl-2, Bax, and cleaved caspase 3 were purchased from Cell Signaling Technology. Inositol requirement 1α (IRE1α was purchased from Abcam. Small interfering RNA siCHOP and scrambled control siRNA-A (siA) were purchased from Santa Cruz Biotechnology.

Cell culture

Human hepatocarcinoma HepG2 cells were grown in 75-cm^2 flasks with 10-ml growth medium, which was RPMI 1640 medium containing 10% FBS, 50 U/ml penicillin and 50-μg/ml streptomycin. Cells were seeded at either 2×10^5 in 1 ml of growth medium in a 12-well plate, or at 1.0×10^4 in 200 μl of growth medium in a 96-well plate. After 24 hours, the medium was replaced with fresh growth medium supplemented with either BSA alone or BSA coupled to palmitate. FA coupling was prepared as previously described (Busch

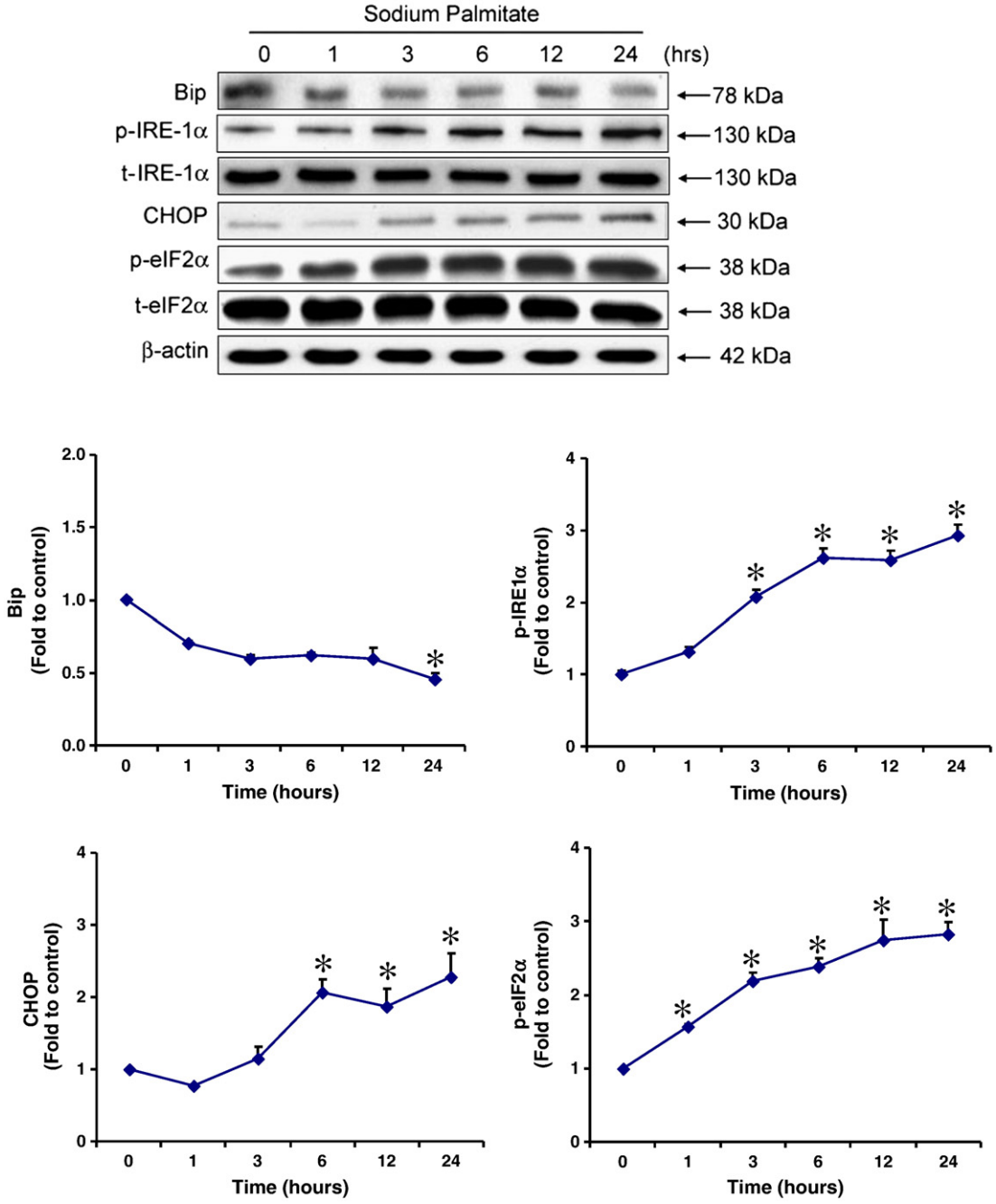

Fig. 3. Time-course changes of protein levels of markers involved in ER stress in lipid-treated HepG2 cells. HepG2 cells were treated with 0.45 mM Pa for 1, 3, 6, 12 and 24 hours. Representative Western blotting results were presented. Total eIF2α and β-actin served as loading controls. Bands were semi-quantified by densitometry and were expressed as fold-change compared with control. Data are presented as means ± SE. * $p<0.05$ versus control.

et al., 2002). Briefly, 18.4% BSA was dissolved in RPMI 1640 by gentle agitation at room temperature for 3 hours. Palmitate (8 mmol/l) was then added as Na^+ salts, and the mixture was agitated 6 hours or overnight at 37 °C. The pH was then adjusted to 7.4, followed by sterile filtering. The FA aliquots were stored at −20 °C.

MTT assay

The reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) was used to assess cell viability. To test the effects of FFAs, HepG2 cells were seeded on a 96-well plate at 1.0×10^4 cells/well. The next day, the medium was replaced with fresh medium containing BSA coupled palmitate or oleate at optimal concentrations or BSA only. Twenty-four hours later, the medium was changed to 1 mg/ml of MTT (Sigma) containing medium according to the manufacturer's instructions. Colorimetric measurements were made 4 h after addition of the MTT reagent at 570 nm with an ELISA plate reader. The background wavelength at 690 nm was subtracted from the 570-nm measurement.

Determination of apoptosis

Annexin V-FITC binding and propidium iodide staining were performed according to the manufacturer's protocol, and the cells were analyzed by flow cytometry. Apoptotic cells were defined as propidium iodide-negative (indicating an intact plasma membrane) and Annexin V-FITC-positive. Apoptotic cells were then calculated as fold change to BSA control.

Cell transfection

Plasmids that continuously express human Bip/GRP78, or EGFP, and their empty vector were generous gifts from Dr Ming-liang He. Cell transfection was performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. In brief, cells were seeded at 2×10^5 per well in 1 ml of growth medium without antibiotics in a 12-well plate. After 24 hours, the cells were transfected with 2-μg plasmid DNA and 4-μl lipofectAMINE 2000. For siCHOP, 3-μg DNA and 4-μl lipofectAMINE 2000 were used in one well in a 12-well plate. Six to eight hours later, the medium was changed to growth medium without antibiotics. Twenty-four hours after transfection, the cells were treated with either BSA or BSA coupled palmitate for another 24 hours.

RNA extraction, cDNA preparation and real-time PCR

The total RNA was extracted from HepG2 cells with Qiagen RNeasy Mini Kit (Qiagen) with DNase treatment following the manufacturer's instructions. Reverse transcription was performed with 0.2 μg of isolated total RNA using Taqman Reverse Transcription Reagent Kit with $OligodT_{16}$ (ABI). PCR reactions were performed in 384-well plates with the use of transcribed cDNA and Power SYBR Green Master Mix (ABI) (see Table 1 for primer sets). The specificity of products generated for each set of primers was examined for each fragment using a melting curve and gel electrophoresis. Reactions were run in triplicate and data calculated as the change in cycle threshold (ΔC_T) for the target gene relative to the ΔC_T for Rpl19, the internal control gene whose transcription is not regulated by ER stress (Lin et al., 2007)).

Western blotting

HepG2 cells were lysed in RIPA buffer (10-mM phosphate buffer pH 7.4, 150-mM NaCl, 2-mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100) containing 1-mM sodium orthovanadate and protease inhibitors. Protein concentrations were determined using Bio-Rad protein assay reagents. A Forty-μg of total protein was separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% free-fatty acid milk in 0.5% Triton X-100-TBS (TBST) for 1 h at room temperature and incubated overnight at 4 °C in blocking solution containing primary antibodies. Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Immunoreactive band signals were developed using ECL system (Millipore) and visualized on X-ray films (GE). The films were then scanned and the signal intensities were calibrated by software ImageJ 1.34 S (Wayne Rasband). The ratio of target proteins to β-actin was used to reflect the relative levels of the targeted proteins.

Biostatistic analysis

Data are presented as the means ± SE. Differences between two groups were evaluated using the Student's *t*-test and differences

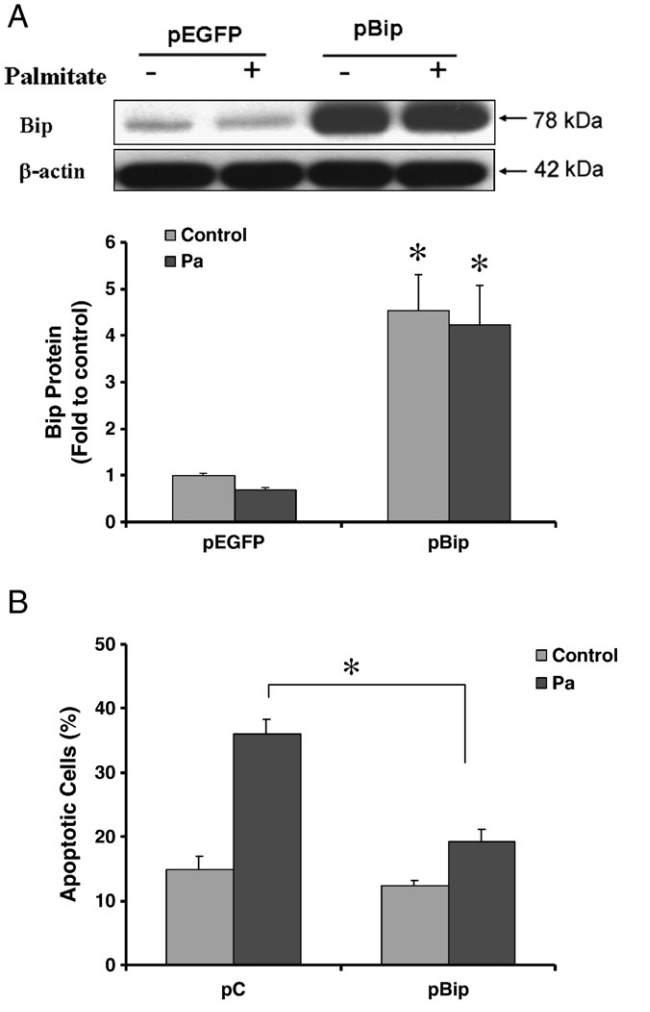

Fig. 4. Bip overproduction protected HepG2 cells from palmitate-induced apoptosis. A. Protein levels of Bip in EGFP- and Bip-overproducing HepG2 cells at the end of 24-hour treatment period (48 hours after transfection). HepG2 cells were transfected with an expression vector encoding for Bip (pBip) or EGFP (pEGFP) by LipofectAMIN2000. β-actin served as a loading control. B. Effects of Bip overproduction on palmitate-induced apoptosis in HepG2 cells. Twenty-four hours after transfection, cells were treated with either BSA or BSA-coupled palmitate for 24 hours. Apoptosis was measured based on Annexin-V and PI doubling staining using flow cytometry. Results are means ± SE. C. Changed expression of markers involved in ER stress and apoptosis in palmitate-treated Bip overproducing cells compared with palmitate-treated control (EGFP) cells. Representative Western blotting images are shown from three to four experiments. Data are presented as means ± SE. * $p<0.05$.

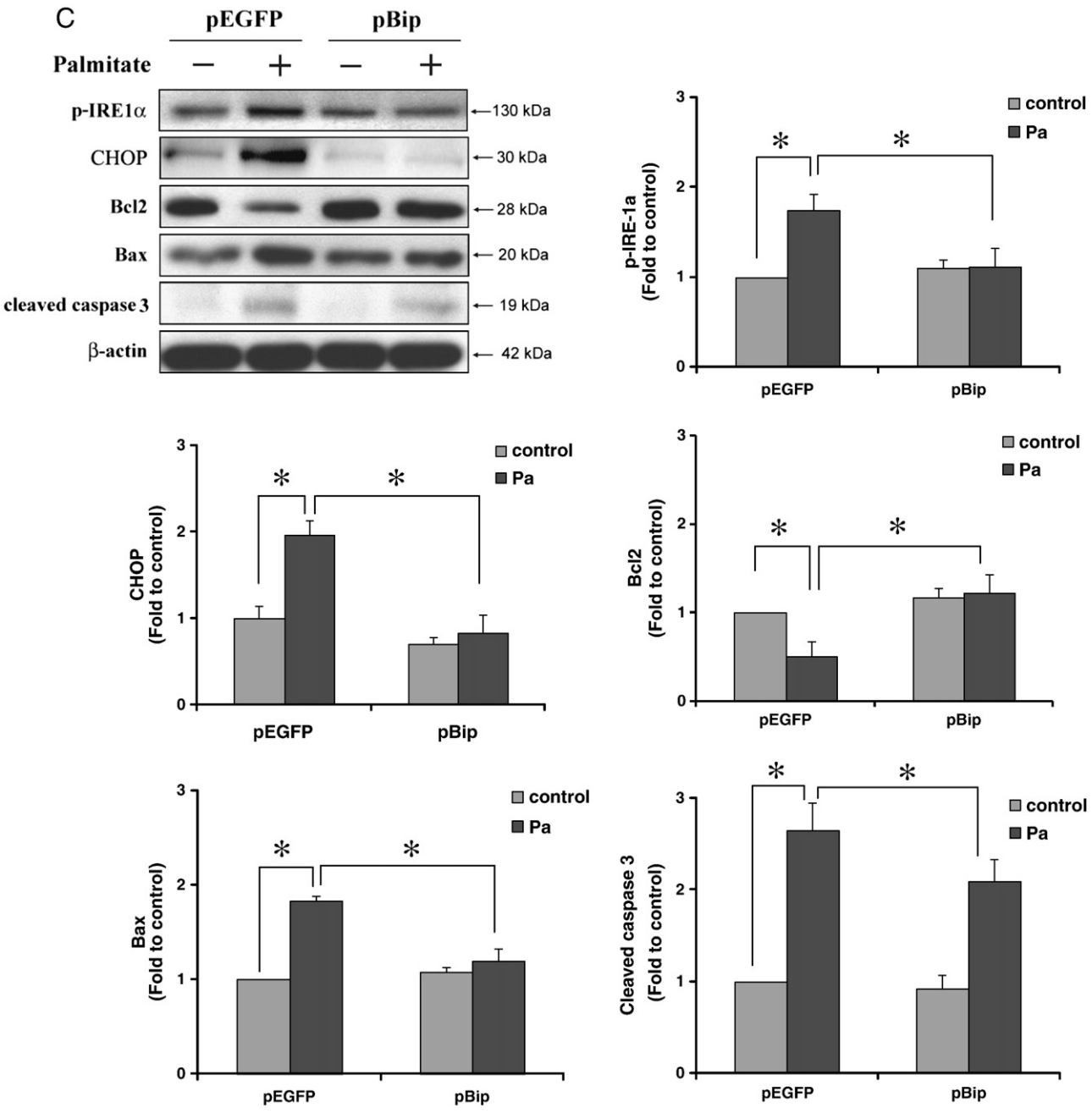

Fig. 4 (*continued*).

among more than two groups were carried out by ANOVA, followed by Duncan's post hoc test using SPSS 13.0 for windows. A P value of <0.05 was taken as evidence of statistical significance.

Results

Saturated fatty acid induces apoptosis of HepG2 cells

HepG2 cells were cultured with saturated fatty acids (sodium palmitate, Pa) or unsaturated fatty acids (sodium oleate, Ol) at serial diluted concentrations for 24 hours. MTT assays revealed that the saturated fatty acid Pa led to a significant dose-dependent reduction in cell viability of HepG2 cells (Fig. 1A). In contrast, the cell viability of HepG2 cells was not affected by exposure to the unsaturated fatty acid Ol (Fig. 1A). Since the 0.5-mM dose of Pa inhibited the growth of HepG2 cells by slightly more than 50%, we adopted a dosage of 0.45 mM Pa for the following study.

Next we determined the process of cell death induced by Pa. The incubation of HepG2 cells with Pa for 24 hours significantly increased the incidence of apoptosis in a concentration dependent manner; apoptosis was increased by more than 4-fold in HepG2 cells incubated with 0.45 mM Pa compared to control cells (Fig. 1B). In contrast, Ol did not significantly affect apoptosis in HepG2 cells (Fig. 1B).

Caspase-3 (also named CPP32, apopain, YAMA) has been identified as a key mediator of apoptosis in mammalian cells. We assessed changes of active caspase 3 (cleaved caspase 3) in order to confirm the presence of apoptosis at a molecular level after Pa treatment. The protein expression level of active caspase 3 was increased after 6 hours of Pa treatment and the increased expression persisted up to 24 hours (Fig. 1C). In contrast, active caspase 3 was slightly up-regulated 3 to 6 hours after Ol treatment and decreased after 12 to

24 hours. However, these changes had no statistical significance (Fig. 1C). These data indicate cellular apoptosis in lipotoxicity induced by Pa but not by Ol.

Saturated fatty acid induces ER stress in HepG2 cells

To examine whether FFAs trigger ER stress in HepG2 cells, we examined the expression patterns of several molecular indicators of the UPR. Tunicamycin (5 μg/ml), which inhibits protein glycosylation, was used as positive control to induce ER stress (Lin et al., 2007). The ER chaperon Bip, also named Glucose Regulated 78-kDa Protein (GRP78), is a central regulator of the UPR (Oyadomari and Mori, 2004). The treatment of HepG2 cells with Tunicamycin significantly increased the mRNA levels of Bip, which reached a peak after 12 hours (Fig. 2A) and slightly decreased 12 hours after treatment with Pa (Fig. 2A). In contrast, Bip mRNA levels were unchanged in Ol-treated HepG2 cells. IRE1 is one of the type I transmembrane serine/threonine protein kinase receptors that is activated in response to unfolded proteins in the ER to signal adaptive responses, transcriptional induction, and translational attenuation, respectively (Liu et al., 2002). The mRNA levels of IRE1α were progressively increased in Pa-treated HepG2 cells, showing a pattern of time-dependent upregulation that was similar to the changes in HepG2 cells after Tunicamycin treatment (Fig. 2B). The mRNA levels of the transcription factor CHOP were also significantly increased 3 to 24 hours after Pa treatment (Fig. 2C). In contrast, the mRNA levels of these three ER stress genes were unchanged in HepG2 cells treated with Ol (Fig. 2A–C).

To examine that changes observed in mRNA levels were associated with alterations in protein abundance, protein expression levels of ER stress markers were examined using Western blotting. Consistently, the protein expression levels of Bip in Pa-treated HepG2 cells were slightly downregulated, with statistically significant decrease found at 24 hours (Fig. 3). IRE-1α expression was induced within 3 hours of Pa treatment and increased thereafter in a time-dependent manner (Fig. 3). CHOP was significantly upregulated by 2-fold in HepG2 cells at 6, 12 and 24 hours of Pa treatment (Fig. 3). The phosphorylation of eIF2α, another important marker of ER stress downstream of activated pancreatic endoplasmic reticulum eIF2alpha kinase (PERK) (Wiseman and Balch, 2005), was upregulated in HepG2 cells after Pa treatment, to similar or even higher levels as those induced by Tunicamycin (Supple. Fig. 1B). After Ol treatment, protein expression of these four ER stress markers was not significantly altered, with the exception of CHOP expression, which was reduced at 24 hours (Supple. Fig. 1A). All these data demonstrate that ER stress is activated in a time-dependent and selective manner by saturated but not by unsaturated fatty acids in HepG2 liver cells.

Overexpression of Bip protects HepG2 cells from saturated fatty-acid-induced apoptosis

The overproduction of the ER stress chaperon Bip attenuates ER stress, both by enhancing protein folding and by helping to maintain IRE1, activating transcription factor 6 (ATF6) and PERK in their inactive states (Bertolotti et al., 2000; Laybutt et al., 2007). HepG2 cells were therefore transfected with an expression vector encoding for Bip (pBip) or enhanced green fluorescent protein (pEGFP) prior to fatty acid exposure for 24 hours. After transfection with Bip, the expression levels of Bip were dramatically upregulated in HepG2 cells with or without treatment of Pa (Fig. 4A).

Annexin V-FITC and PI double staining with flow cytometry was used to detect apoptosis in HepG2 cells. Because EGFP fluorescence interferes with Annexin V signals, an empty vector without EGFP (pC) was used as the transfection control. We found that Pa-induced apoptosis was significantly reduced in Bip-overexpressing versus control HepG2 cells (Fig. 4B). The overproduction of Bip was accompanied by the reduced expression of IRE-1α (Fig. 4C), which indicated the attenuation of ER stress in HepG2 cells (Laybutt et al., 2007). The overexpression of Bip was shown to attenuate the induction of CHOP, to reduce the expression levels of pro-apoptotic protein Bax and active/cleaved caspase 3, and to restore the expression level of anti-apoptotic protein Bcl-2 (Fig. 4C). These data imply that ER stress is required for the Pa-induced apoptosis in HepG2 liver cells and the overexpression of Bip could attenuate the induction of ER stress and apoptosis by palmitate.

Knockdown of CHOP does not protect HepG2 cells from saturated fatty-acid-induced apoptosis

Next we tested whether CHOP was also required for the Pa-induced apoptosis in HepG2 cells. Small interfering RNA siCHOP was

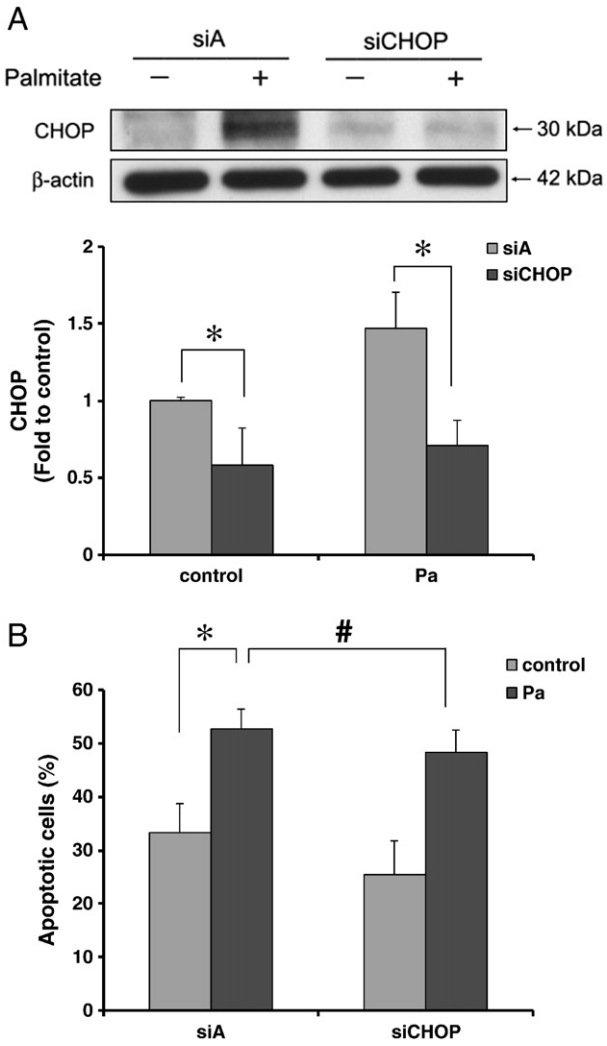

Fig. 5. Knockdown of CHOP did not protect HepG2 cells from palmitate-induced apoptosis. A. Representative Western blotting results showing protein expression levels of CHOP (upper) in CHOP knocked down HepG2 cells at the end of the 24-hour treatment period (48 hours after transfection). HepG2 cells were transfected with small interference RNA siCHOP and scramble siRNA (siA) by LipofectAMIN2000. β-actin served as a loading control. Protein expression levels of CHOP were quantitated by densitometry and are expressed as fold-change compared with control (lower). B. Effects of CHOP knockdown on palmitate-induced apoptosis in HepG2 cells. Twenty-four hours after transfection, cells were treated with either BSA or BSA-coupled palmitate for 24 hours. Apoptosis was measured based on Annexin-V and PI doubling staining using flow cytometry. Results are means ± SE. C. Changed expression of markers involved in ER stress and apoptosis in palmitate-treated CHOP knocked down HepG2 cells compared with palmitate-treated control (siA) cells. Representative Western blotting images are shown from three to four experiments. * $p<0.05$; # $p>0.05$.

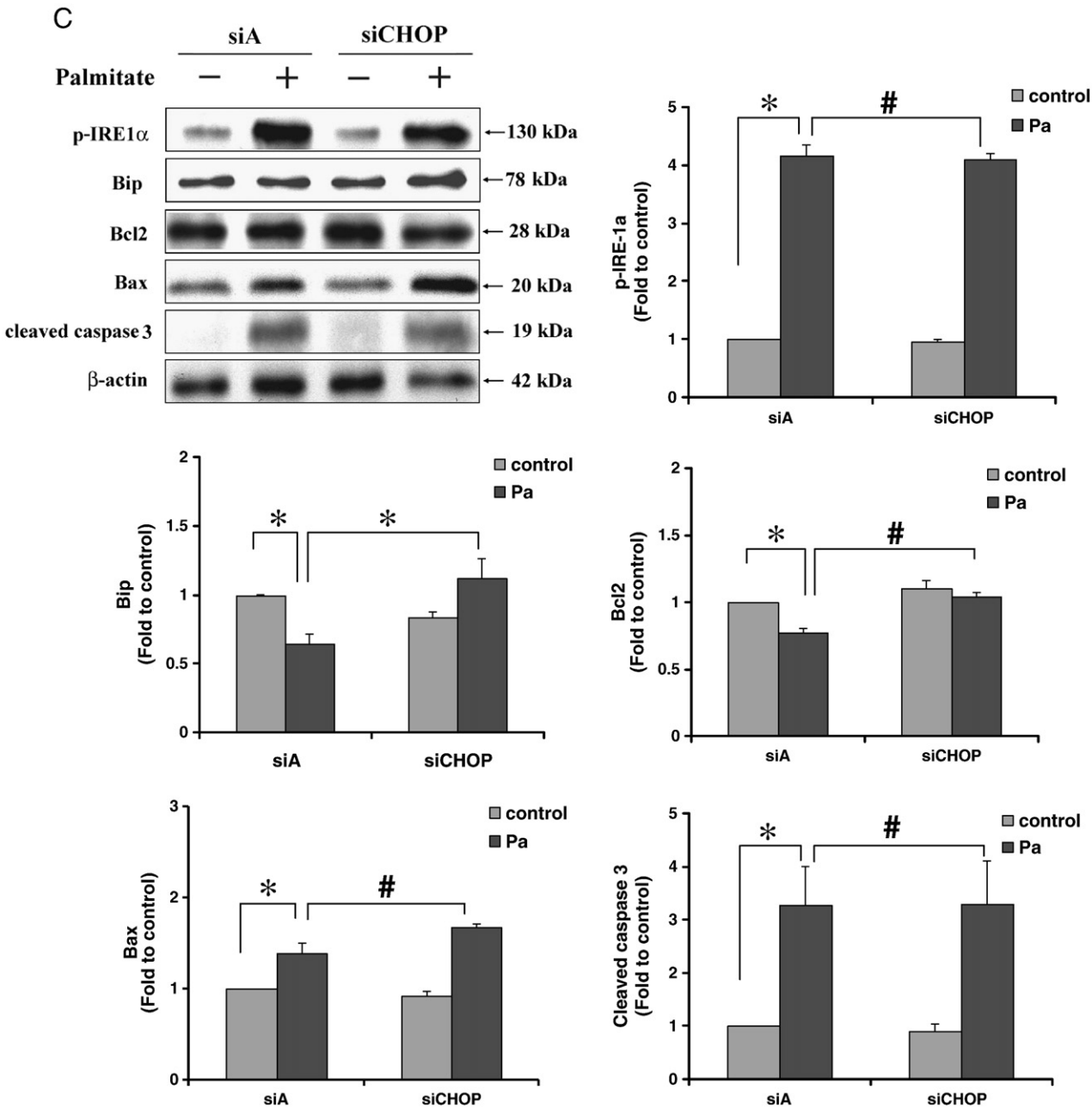

Fig. 5 (*continued*).

used to knock down the expression of CHOP in HepG2 cells and scramble RNA siA was used as control. Transfected HepG2 cells were treated with Pa for additional 24 hours. In HepG2 cells transfected with siCHOP, Pa treatment failed to induce CHOP expression (Fig. 5A), confirming successful knockdown of CHOP.

Treatment with Pa increased the incidence of apoptosis in control HepG2 cells. However, this increased apoptosis was not improved in HepG2 cells with siRNA-mediated knockdown of CHOP (Fig. 5B). Furthermore, the pro-apoptosis markers Bax and active/cleaved caspase 3 remained induced and Bcl2 expression remained suppressed after Pa treatment in siCHOP-transfected HepG2 cells (Fig. 5C). The knockdown of CHOP did not affect ER stress as the expression levels of IRE1α were similarly upregulated in control and siCHOP-transfected cells with or without Pa treatment. To our surprise, CHOP-siRNA slightly upregulated the expression of Bip in Palmitate-treated HepG2 cells but not in non-treated control ones. These data demonstrate that the knockdown of CHOP alone is not sufficient enough to affect the Pa-induced ER stress and apoptosis in HepG2 cells.

Discussion

In the present study, we provide new insights into the effects of elevated exogenous fatty acids with evidence of an important role of ER stress in lipotoxicity. The saturated fatty acid Pa disrupts ER homeostasis and induces apoptosis through ER stress signaling independent from CHOP pathway.

Hepatocytes are characterized by abundant and well-developed ER and thus serve as an established cellular model to study changes in ER homeostasis. It has been reported that ER stress is induced in the liver under many disease conditions such as HBV or HCV infection,

hepatocellular carcinoma (HCC), alcohol abuse, NAFLD, obesity and diabetes (Ji and Kaplowitz, 2006). In a rat hepatic steatosis model elevated fatty acid levels are accompanied by ER stress and increased caspase-3 activity (Wang et al., 2006). Our data, consistent with recent studies (Laybutt et al., 2007; Wei et al., 2007; Wei et al., 2006), have also shown that elevated lipids, especially long-chain saturated fatty acids, can induce ER stress and subsequent apoptosis in liver cells.

We showed that Pa-induced apoptosis was associated with a persistent upregulation of IRE1α, phosphorylated eIF2α and CHOP after Pa treatment in HepG2 cells. The early ER stress marker phospho-eIF2α was detected within 1 hour and activation of IRE1a and CHOP occurred after 3 hours. More importantly, this change preceded the induction of cleaved caspase 3, which was apparent after 6 hours. When using the overexpression of Bip to attenuate ER stress, Pa-induced apoptosis was significantly improved. These findings raise the possibility that ER stress and UPR dysfunction may be upstream components in lipotoxicity. However, the mechanism by which Pa induces cellular stress is not clear. We speculate that Pa may modify the ER membrane lipid environment, making it unfavorable for proper protein folding, as implied by studies in other cell types (Thumser and Storch, 2007).

Bip/GRP78 is known to bind the luminal domains of the three ER stress transducer proteins PERK, IRE1, and ATF6 to keep them inactive (Zhao and Ackerman, 2006). When HepG2 cells were treated with ER stress inducer Tunicamycin, Bip expression levels were dramatically upregulated. However, we were surprised to find that Bip expression was slightly but significantly downregulated after Pa treatment in HepG2 cells. This is consistent with findings in pancreatic beta cells that the major ER chaperone proteins such as Grp78/Bip and Grp94 were not upregulated by chronic Ol or Pa treatment despite the presence of ER stress (Karaskov et al., 2006). Hence, we hypothesize that the absence of upregulated Bip levels with exposure to elevated saturated fatty acids may sensitize liver cells and beta cells to ER stress. This is supported by findings that the overexpression of Bip at least partially protects HepG2 cells from ER stress and apoptosis induced by Pa. In addition, the overexpression of Bip has been shown to attenuate the induction of CHOP in ER stress and to reduce ER stress-induced apoptosis (Oyadomari and Mori, 2004), which was consistently confirmed in our data (Fig. 4C). Recent studies also demonstrated that Bip overexpression in the livers of ob/ob mice improves insulin sensitivity and reduces hepatic steatosis (Kammoun et al., 2009). These data provide definitive evidence that ER stress is actually required for lipotoxicity in hepatocytes and that the sensitivity of liver cells to ER stress is highly dependent on Bip expression.

Finally, we found that Pa-induced apoptosis and ER stress was independent of the upregulation of CHOP expression alone. CHOP is one of the highest inducible genes during ER stress (Oyadomari and Mori, 2004), the expression level of which is induced by the ATF6 and PERK pathway. It was shown in our data that CHOP-siRNA slightly upregulated the expression of Bip in Palmitate-treated HepG2 cells but not in non-treated control ones. However the knockdown of CHOP could not attenuate lipid-induced apoptosis in HepG2 cells. This suggests that alternative and/or additional ER stress signaling pathways, such as JNK or caspase 12 pathway, are required for lipid-induced apoptosis in HepG2 cells (Rasheva and Domingos, 2009). It has been shown that chronic treatment with saturated fatty acids causes numerous other cellular alterations that can initiate apoptosis including generation of reactive oxygen species (Srivastava and Chan, 2007), mitochondrial perturbations (Maestre et al., 2003) and generation of ceramide (Wei et al., 2006). Recently double-stranded RNA-dependent protein kinase (PKR) and Bcl-2 pathway was reported to play an important role in Pa-induced apoptosis in HepG2 cells (Yang and Chan, 2009). CHOP-induced GADD34 transcription and dephosphorylates eIF2a (Lee and Glimcher, 2009), the excessive phosphorylation of these proteins may potentiate fatty-acid-induced ER stress and apoptosis (Cnop et al., 2007). Therefore, knockdown of CHOP might result in the excessive phosphorylation of eIF2a to inhibit protein synthesis and activate ATF4-induced apoptosis in cells (Lange et al., 2008; Ord et al., 2007).

Above all, in the present study, as shown in Fig. 6, we found that elevated exogenous saturated fatty acids Pa disrupted ER homeostasis by reducing the expression of Bip. The shortage of Bip induced apoptosis through ER stress signaling. The overexpression of Bip attenuated ER stress, reduced CHOP expression and protected HepG2 cells from Pa-induced apoptosis, while the knockdown of CHOP could not attenuate lipid-induced apoptosis in HepG2 cells.

Conclusion

Our results support the hypothesis that the saturated fatty acid, palmitate, induces ER stress and apoptosis in HepG2 cells, whereas the unsaturated fatty acid, oleate, does not. Although the ER stress may be involved in the palmitate cytotoxicity, the inhibition of CHOP alone is not sufficient to prevent the palmitate-induced apoptosis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

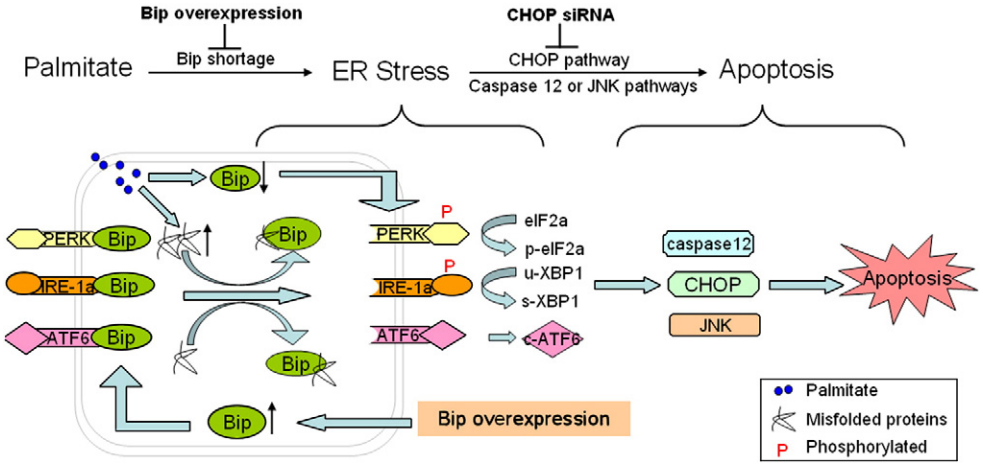

Fig. 6. Schematic diagram on the possible mechanism underlying fatty-acid-induced ER stress and apoptosis in HepG2 liver cells. ATF6: activating transcription factor 6; c-ATF6: cleaved ATF6; Bip: immunoglobulin heavy chain-binding protein; CHOP: C/EBP homologous protein; IRE1α: inositol requirement 1α; JNK: c-Jun N-terminal kinase; PERK: pancreatic endoplasmic reticulum eIF2alpha kinase; u-XBP1: unspliced X-box binding protein 1; XBP-1: spliced X-box binding protein 1.

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Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.lfs.2010.10.012.

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