

Saturated Free Fatty Acid Sodium Palmitate-Induced Lipoapoptosis by Targeting Glycogen Synthase Kinase-3 β Activation in Human Liver Cells

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Abstract

Background Elevated serum saturated fatty acid levels and hepatocyte lipoapoptosis are features of nonalcoholic fatty liver disease (NAFLD).

Aim The purpose of this study was to investigate saturated fatty acid induction of lipoapoptosis in human liver cells and the underlying mechanisms.

Methods Human liver L02 and HepG2 cells were treated with sodium palmitate, a saturated fatty acid, for up to 48 h with or without lithium chloride, a glycogen synthase kinase-3 β (GSK-3 β) inhibitor, or GSK-3 β shRNA transfection. Transmission electron microscopy was used to

detect morphological changes, flow cytometry was used to detect apoptosis, a colorimetric assay was used to detect caspase-3 activity, and western blot analysis was used to detect protein expression.

Results The data showed that sodium palmitate was able to induce lipoapoptosis in L02 and HepG2 cells. Western blot analysis showed that sodium palmitate activated GSK-3 β protein, which was indicated by dephosphorylation of GSK-3 β at Ser-9. However, inhibition of GSK-3 β activity with lithium chloride treatment or knockdown of GSK-3 β expression with shRNA suppressed sodium palmitate-induced lipoapoptosis in L02 and HepG2 cells. On a molecular level, inhibition of GSK-3 β expression or activity suppressed sodium palmitate-induced c-Jun-N-terminal kinase (JNK) phosphorylation and Bax upregulation, whereas GSK-3 β inhibition did not affect endoplasmic reticulum stress-induced activation of unfolded protein response.

Conclusions The present data demonstrated that saturated fatty acid sodium palmitate-induced lipoapoptosis in human liver L02 and HepG2 cells was regulated by GSK-3 β activation, which led to JNK activation and Bax upregulation. This finding indicates that GSK-3 β inhibition may be a potential therapeutic target to control NAFLD.

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Saturated fatty acid · Lipoapoptosis · Glycogen
synthase kinase-3 β · Endoplasmic reticulum stress

Abbreviations

NAFLD Nonalcoholic fatty liver disease
NASH Nonalcoholic steatohepatitis
FFA Free fatty acid
ER Endoplasmic reticulum
UPR Unfolded protein response

PERK	Protein kinase RNA-like ER kinase
IRE1	Inositol-requiring protein 1
GRP78	Glucose-regulated protein 78
GSK-3	Glycogen synthase kinase-3
JNK	c-Jun-N-terminal kinase
Bax	Bcl-2-associated X protein
BSA	Bovine serum albumin
7-AAD	7-amino actinomycin D
ATF6	Activating transcription factor 6
ATF4	Activating transcription factor 4
CHOP	C/EBP-homologous protein
XBP-1	X-box-binding protein 1
shRNA	Short hairpin RNA
PI3K	Phosphatidylinositide-3-OH kinase

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder characterized by fat accumulation in the liver, which is not due to excessive alcohol consumption. Clinically, NAFLD encompasses a broad spectrum of hepatic derangements from steatosis to nonalcoholic steatohepatitis (NASH). The latter is characterized by hepatic fat accumulation coincident with inflammation, reduced liver function, fibrosis, and eventually liver cirrhosis [1–4]. Most patients with NAFLD have few or no symptoms. Thus, NAFLD has emerged as a major public health issue throughout the world. However, to date, the pathogenesis of NAFLD is still poorly understood, and its treatments remain ineffective because most treatments seem to improve only alanine transaminase (ALT) levels but do not reverse histological abnormalities or improve clinical endpoints.

The initial NAFLD studies have shown elevated liver enzyme levels (such as ALT) or aberrant liver ultrasound images (such as steatosis). Morphologically, in NAFLD, triglycerides are accumulated in liver cells to form lipid droplets. Although triglyceride production is a normal function of liver cells, NAFLD is related to insulin resistance and metabolic syndrome but is not due to excessive alcohol use, resulting in a fatty liver. Mechanistically, in NAFLD, a large portion of elevated hepatic triglyceride stores appears to arise from reesterification of circulating free fatty acids (FFAs) released from adipose tissue [5, 6]. FFAs are taken up by the liver, where they are esterified into neutral triglycerides, but excess saturated FFAs overwhelm the capacity of the liver to esterify FFAs and thus may induce lipotoxicity, which includes the promotion of apoptosis, a phenomenon termed lipoapoptosis. The latter is a prominent feature of NASH and is associated with severity of the disease [7, 8].

The molecular mechanism responsible for saturated FFA-induced hepatocyte lipoapoptosis remains undefined.

Recently, several studies have linked saturated FFA-induced dysfunction of the endoplasmic reticulum (ER) with lipoapoptosis in hepatocytes [9–11]. The ER is a highly dynamic organelle that synthesizes and processes secretory and transmembrane proteins. Perturbation of ER homeostasis interferes with protein folding and causes aberrant accumulation of unfolded or misfolded proteins in the ER lumen, triggering an evolutionarily conserved response, termed the unfolded protein response (UPR). This condition has been designated as ER stress. Proximal sensors of UPR include protein kinase RNA-like ER kinase (PERK), inositol-requiring protein 1 (IRE1), and activating transcription factor 6 (ATF6). The UPR serves to overcome the stress stimulus; however, prolonged ER stress will promote cell apoptosis [12–15]. Indeed, in a nutritional murine model of steatohepatitis, ER stress was able to induce X-box-binding protein 1 (XBP-1) mRNA splicing and C/EBP-homologous protein (CHOP) expression, which is associated with the severity of apoptosis [11]. Moreover, in tissue samples from patients with NAFLD, eukaryotic initiation factor-2 α is strongly activated [16]. Our own study [17] demonstrated that saturated FFA sodium palmitate-induced ER stress and lipoapoptosis in human liver cell lines are enacted through the PERK/ATF4/CHOP signaling pathway. These data suggest that UPR is the major injurious pathway in NAFLD.

Furthermore, glycogen synthase kinase-3 (GSK-3) is a multi-functional serine/threonine kinase that is a key effector for numerous signaling pathways by phosphorylating a serine or threonine residue on its target substrate and is involved with critical cellular functions, such as cell cycle progression, gene expression, cytoskeletal integrity, and apoptosis. There are two GSK-3 isoforms encoded by distinct genes: GSK-3 α and GSK-3 β . These kinases share 84 % overall identity and 98 % homology in their catalytic domains. Despite the fact that GSK-3 α and GSK-3 β share common substrates, their expression patterns, substrate preferences, regulation, and cellular functions are not identical. GSK-3 α is inactivated by phosphorylation at Ser-21, and phosphorylation at Ser-9 inactivates GSK-3 β [18, 19]. In humans, only GSK-3 α is deactivated by insulin during physiological conditions [20], whereas supraphysiological insulin injection in the rat leads to deactivation of both GSK-3 α and GSK-3 β [20, 21]. In addition, recent studies have shown that GSK-3 β is an important regulator of apoptosis [22] by promoting mitochondria-mediated apoptotic signaling in a variety of liver diseases [18], while inhibition of GSK-3 β suppresses caspase-3 activation and subsequent cell death induced by thapsigargin [23]. GSK-3 β is also able to regulate the expression of Bcl-2-associated X protein (Bax), a proapoptotic Bcl-2 family member [24], and activate c-Jun-N-terminal kinase (JNK) protein, which contributes to acetaminophen-induced liver injury [25].

In this study, we hypothesized that GSK-3 β might play a role in saturated FFA sodium palmitate-mediated hepatocyte lipoapoptosis under ER stress. Therefore, we determined the pharmacological and genetic inhibition of GSK-3 β activity and expression, respectively, on saturated FFA sodium palmitate-induced cell lipoapoptosis in human liver cell lines. In addition, on a molecular level, the effect of inhibition of GSK-3 β activity or expression on JNK-mediated Bax upregulation was also determined.

Experimental Procedures

Cell lines, Culture, and Gene Transfection

The normal human hepatic cell line L02 and the hepatocellular carcinoma cell line HepG2 were obtained from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). These cells were cultured in RPMI-1640 medium and Dulbecco's modified Eagle's medium (DMEM), respectively, as described previously [17]. For gene transfection, L02 and HepG2 cells were seeded at 1×10^6 per well in 1 mL of complete medium with serum and antibiotics in a 6-well plate. After 18–24 h, the cells were transfected with 1 μ g of GSK-3 β short hairpin RNA (shRNA) or negative control shRNA (Invitrogen, Shanghai, China) and 3 μ L of Poly JetTM (Sigma Gene Laboratories, Rockville, MD, USA) according to the manufacturer's instructions. Twelve to 18 h after transfection, the medium was changed to regular medium, and the cells were treated with sodium palmitate as described above.

Preparation of FFA Solution and Cell Treatment

The FFA with bovine serum albumin (BSA) solution was prepared as described previously [17]. Briefly, 18.4 % fatty acid-free BSA (Sigma, St. Louis, MO, USA) was dissolved in RPMI-1640 through gentle agitation at room temperature for 3 h. Sodium palmitate (9 mM) was added to the BSA-containing cell culture medium, and then the mixture was agitated for 6 h or overnight at 37 °C. The pH value of the medium was adjusted to 7.4, followed by sterile filtration. Lithium chloride, a GSK-3 β inhibitor (Sigma-Aldrich), was dissolved in RPMI-1640 or DMEM at a concentration of 20 mM. Cells were grown until 80–85 % confluency and were treated with the FFA/BSA mixture for up to 48 h in the presence or absence of lithium chloride.

Annexin V-Flow Cytometry Assay

After treatment with sodium palmitate, the cells were subjected to annexin V-PE binding and 7-amino-actinomycin D

(7-AAD) staining according to the manufacturer's protocol (Kaiji, Nanjing, China). Next, they were analyzed by flow cytometry within 1 h. Cells negative for both 7-AAD and annexin V-PE staining were considered alive cells, annexin V-PE-positive cells were early apoptotic, and cells positive for both 7-AAD and annexin V-PE were in the late stage of apoptosis. Apoptotic cells were then calculated as fold changes, relative to the BSA controls.

Transmission Electron Microscopy

After treatment, cells were collected by trypsinization, pelleted by centrifugation, and fixed with 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Cells were then post-fixed with 1 % osmium tetroxide for 30 min, dehydrated in a graded ethanol series (50–100 %), and embedded in Epon 812 resin. Next, ultra-thin sections were cut using an ultramicrotome (Leica Ultracut UCT, Vienna, Austria), stained with uranyl acetate and lead citrate, and reviewed under a transmission electron microscope (JEM-1200 EX II; Jeol, Tokyo, Japan).

Detection of Caspase-3 Activity

After treatment, cells were harvested, resuspended in a cell lysis buffer, and subjected to three freeze–thaw cycles at -70 °C. Cell lysates were then incubated on ice for 15 min, the supernatant fractions were collected, and the protein concentration was determined by the bicinchoninic acid assay. Caspase-3 activity was analyzed in these protein samples (100–200 μ g each) using a Caspase-3 Colorimetric Assay Kit (Kaiji, Nanjing, China) according to the manufacturer's protocol. The activity was measured with an ELISA reader at 405 nm (BioTek).

Protein Extraction and Western Blotting

Whole cell lysates were prepared and subjected to immunoblot analysis as described previously [17]. Samples containing 110 μ g of protein were resolved by 8–15 % SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and incubated with primary antibodies at a dilution of 1:1000. Membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:5000. Protein bands were developed using chemiluminescence detection reagents and then visualized and captured with the ChemiDoc imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

The density of protein bands was quantified with the software Quantity One (Bio-Rad Laboratories, Hercules, CA, USA). Expression of the target proteins was normalized to β -actin (Santa Cruz Biotechnology). Rabbit anti-glucose-regulated

protein 78 (GRP78) was purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-phospho-JNK (Thr183/Tyr185) antibody was purchased from Santa Cruz Biotechnology. Rabbit anti-total GSK-3 β and rabbit anti-phosphorylated GSK-3 β (Ser9) antibodies were purchased from Epitomics (Burlingame, CA, USA). Rabbit anti-phospho-IRE1 (Ser724) and rabbit anti-phospho-PERK (Thr981) antibodies were purchased from Abcam (Cambridge, England). Mouse anti-Bax antibody was from BD Biosciences (San Jose, CA, USA).

Statistical Analysis

The experimental results were summarized as mean \pm standard error (SE). Differences between two groups were evaluated using the Student's *t* test, and differences in more than two groups were analyzed using analysis of variance (ANOVA) with SPSS software (version 13.0, SPSS, Chicago, IL, USA). A probability (*P*) value < 0.05 was considered statistically significant.

Results

Induction of Lipoapoptosis by Saturated Fatty Acid Sodium Palmitate in L02 and HepG2 Cells

In our previous study, we showed that the saturated fatty acid sodium palmitate significantly reduced cell viability and increased intracellular triglyceride content in a time- and dose-dependent manner in human liver cell lines [17]. In this study, we determined the effects of sodium palmitate on lipoapoptosis in L02 and HepG2 cells. After incubation for 48 h with 108 μ M sodium palmitate, the number of apoptotic cells was significantly increased to 13.38 \pm 1.41 % in L02 and 16.75 \pm 1.48 % in HepG2 cells (Fig. 1A). On a molecular level, caspase-3 activity was increased by 2.53- and 3.19-fold in L02 cells and 3.27- and 4.83-fold in HepG2 cells at 24 and 48 h, respectively, as compared to the untreated cells (Fig. 1B). Electron microscopic data showed that L02 and HepG2 cells treated with sodium palmitate for 48 h displayed the ragged edge of chromatin condensation and the formation of tiny apoptotic bodies and vacuoles. Moreover, cellular volume was reduced as compared with control cells, and cytoplasmic organelles remained intact (Fig. 2A, B).

GSK-3 β Inhibition Decreased Saturated Fatty Acid Sodium Palmitate-Induced Lipoapoptosis in L02 and HepG2 Cells

Next, we determined whether GSK-3 β is involved in sodium palmitate-induced lipoapoptosis in L02 and HepG2

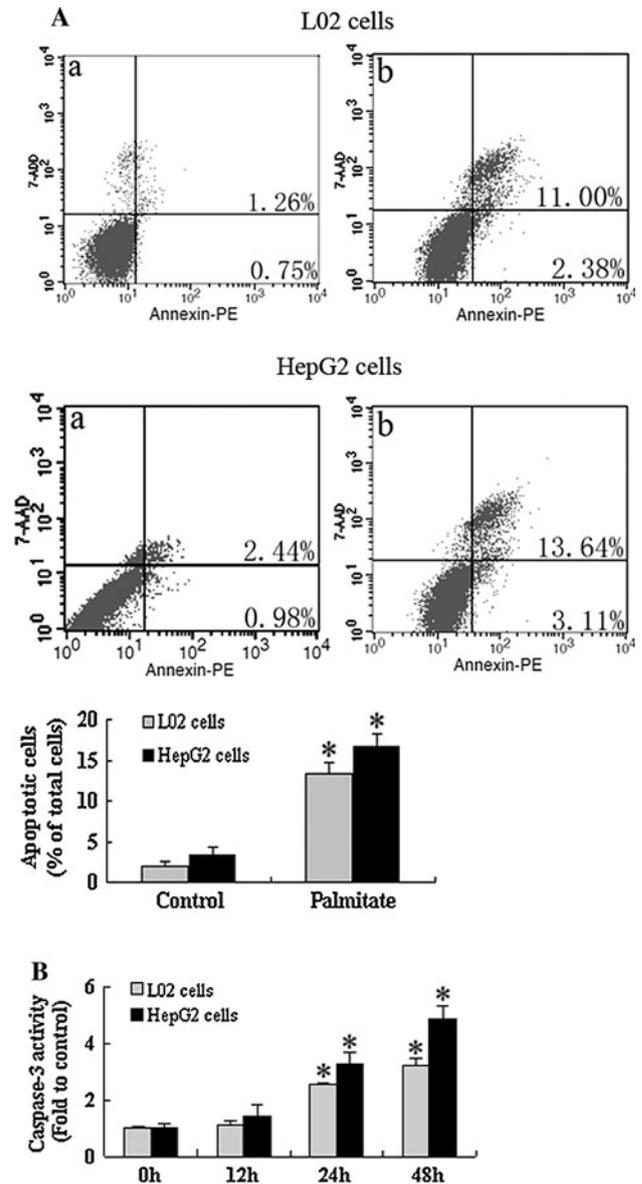
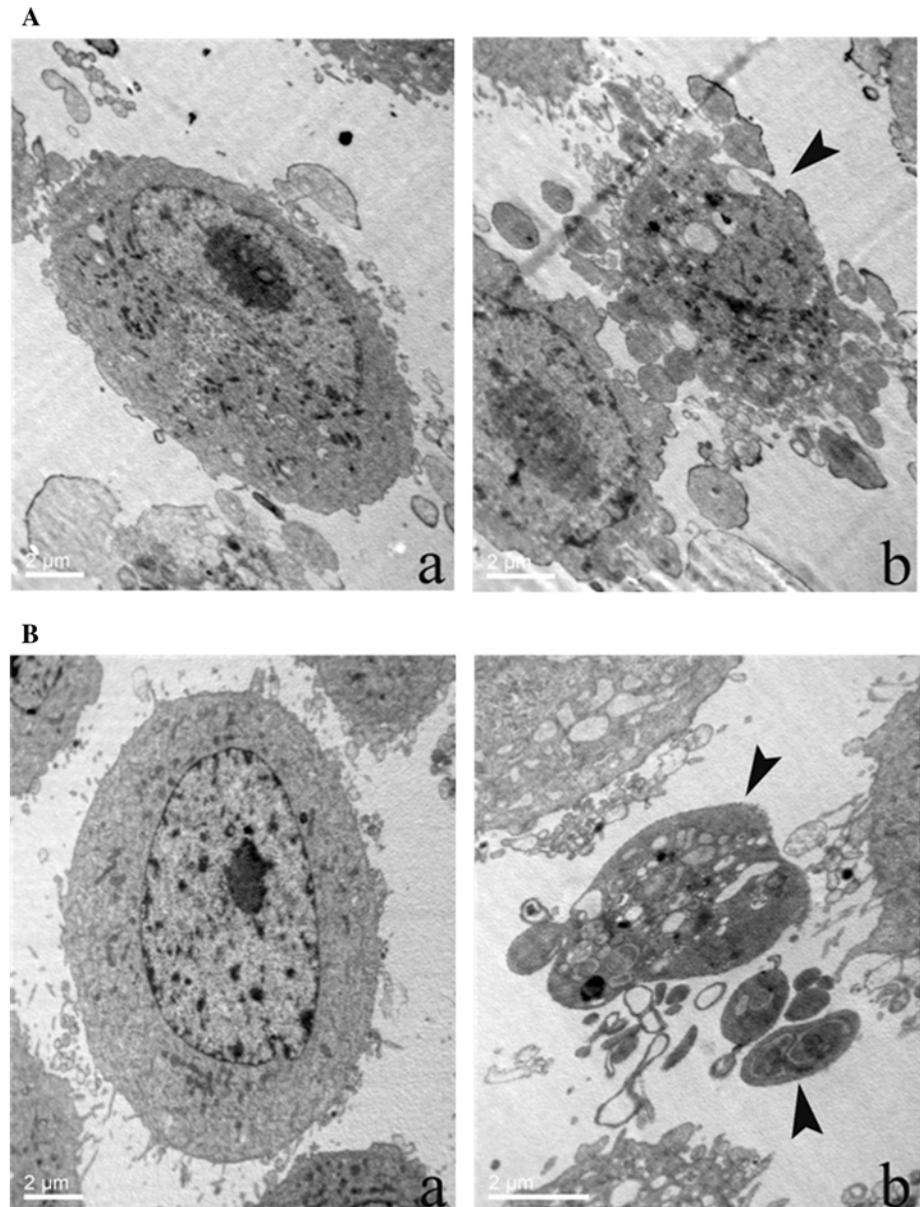


Fig. 1 Induction of lipoapoptosis by saturated fatty acids (e.g., sodium palmitate) in human liver cell lines. **A** Apoptosis assay. L02 and HepG2 cells were treated with 108 μ M sodium palmitate for 48 h and then subjected to annexin V-PE binding, 7-AAD staining, and flow cytometry. *a* Control; *b* sodium palmitate. The numbers at the *lower* or *upper right* indicate the percentage of early or late apoptotic cells, respectively. **B** Caspase-3 activity. Similarly treated cells were subjected to caspase-3 activity measurements using a caspase-3 colorimetric assay kit. The experiments were repeated three times. Data are summarized as mean \pm standard error (SE). **P* < 0.05 , palmitate-treated cells versus control cells

cells. First, we measured the GSK-3 β activity using western blot analysis, and our data showed that sodium palmitate did induce the dephosphorylation of GSK-3 β at Ser-9 in a time-dependent manner, while total GSK-3 β , used as a control protein, was constantly expressed (Fig. 3A).

Fig. 2 Morphological changes in apoptosis after treatment with sodium palmitate for 48 h. L02 (A) and HepG2 (B) cells were treated with 108 μ M sodium palmitate for 48 h and analyzed by transmission electron microscopy. *a* Control; *b* sodium palmitate. Chromatin condensation and formation of apoptotic bodies were observed at 48 h after treatment with sodium palmitate and are indicated by the *arrows*



Furthermore, we inhibited GSK-3 β protein activity in L02 and HepG2 cells using lithium chloride (20 mM) and found that lithium chloride inhibited GSK-3 β activity by induction of phosphorylation of GSK-3 β at Ser-9 (Fig. 3A). In addition, lithium chloride reduced sodium palmitate-induced lipoapoptosis by 44 % in L02 (Fig. 3B) and 37 % in HepG2 cells (Fig. 3C). Lithium chloride treatment also reduced caspase-3 activation by more than 25 % in L02 and more than 31 % in HepG2 cells (Fig. 3D).

In addition, we transiently transfected GSK-3 β shRNA into L02 and HepG2 cells, and our data showed that GSK-3 β shRNA significantly suppressed GSK-3 β protein expression in L02 and HepG2 cells, as compared to the

negative control cells (Fig. 4A). Negative control shRNA lacks complementary sequences in the human genome. A BLAST search was carried out to avoid unintentional silencing of non-target host cell genes (www.ncbi.nlm.nih.gov). In these GSK-3 β -knocked down L02 and HepG2 cells, sodium palmitate-induced apoptosis was reduced by 57 and 43 %, respectively, compared to the control shRNA (Fig. 4B, C). Similarly, caspase-3 activation induced by sodium palmitate treatment was also inhibited by GSK-3 β shRNA transfection (Fig. 4D). These data indicate that GSK-3 β protein is required for sodium palmitate-induced lipoapoptosis in L02 and HepG2 cells.

Fig. 3 Inhibition of GSK-3 β activity reduced sodium palmitate-induced lipoapoptosis in L02 and HepG2 cells.

A Western blot. L02 and HepG2 cells were pretreated with or without 20 mM lithium chloride for 1 h, followed by treatment with 108 μ M sodium palmitate for up to 48 h, and then subjected to protein extraction and western blot analyses. The target protein bands were semi-quantified using densitometry and displayed as fold-changes relative to the control. **B** and **C** apoptosis assay. L02 (**B**) and HepG2 (**C**) cells were pretreated with or without 20 mM lithium chloride for 1 h, followed by treatment with 108 μ M sodium palmitate for 48 h, and then subjected to annexin V-PE binding, 7-AAD staining, and flow cytometry. *a* Control; *b* sodium palmitate; *c* sodium palmitate plus lithium chloride. The numbers at the *lower* or *upper right* indicate the percentage of early or late apoptotic cells, respectively. **D** Caspase-3 activity. Similarly treated cells were subjected to caspase-3 activity measurements using a caspase-3 colorimetric assay kit. The experiments were repeated three times. Data are summarized as mean \pm standard error (SE). * $P < 0.05$, palmitate-treated cells versus control cells; # $P < 0.05$, palmitate plus lithium-treated cells versus palmitate-treated cells at the same time point

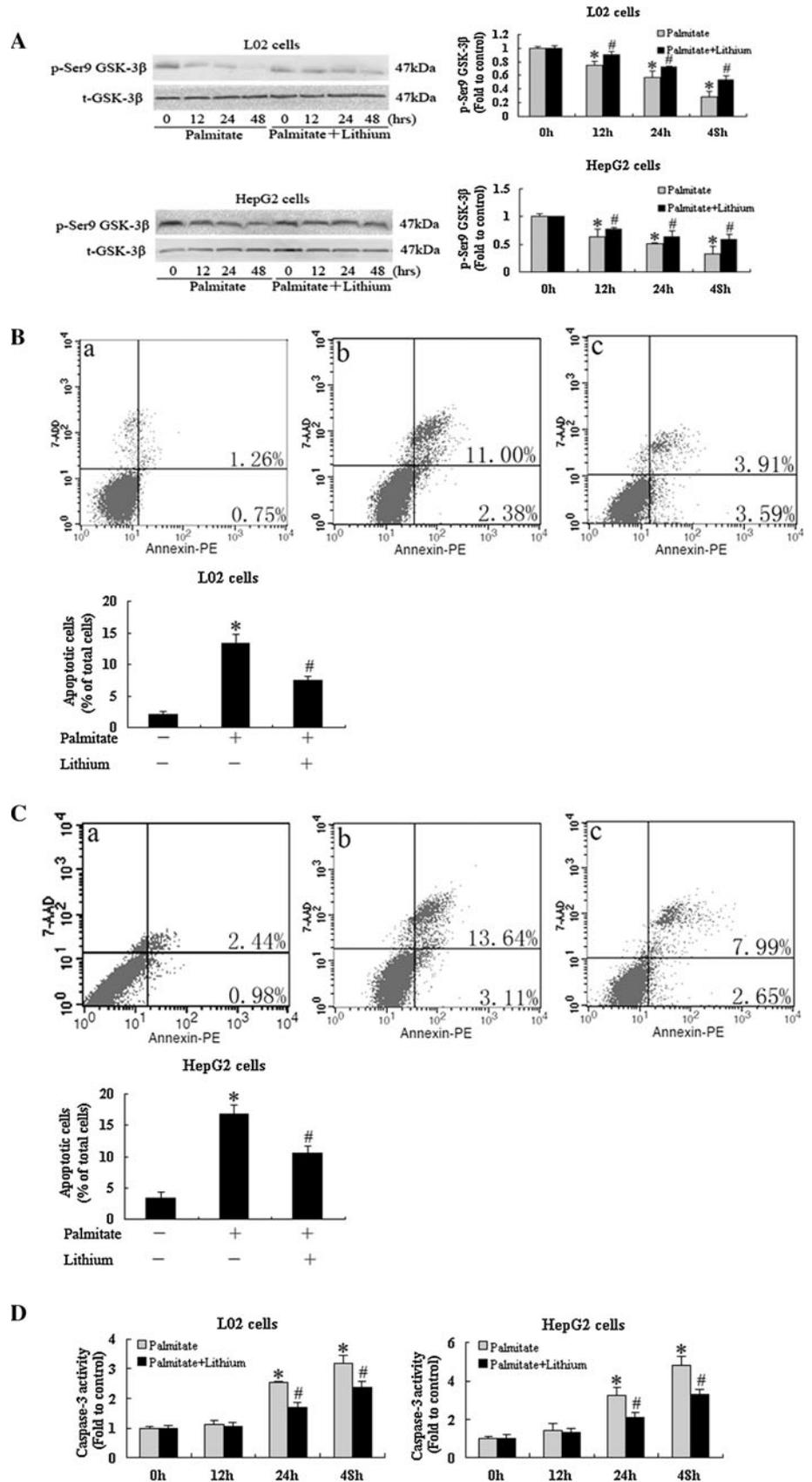
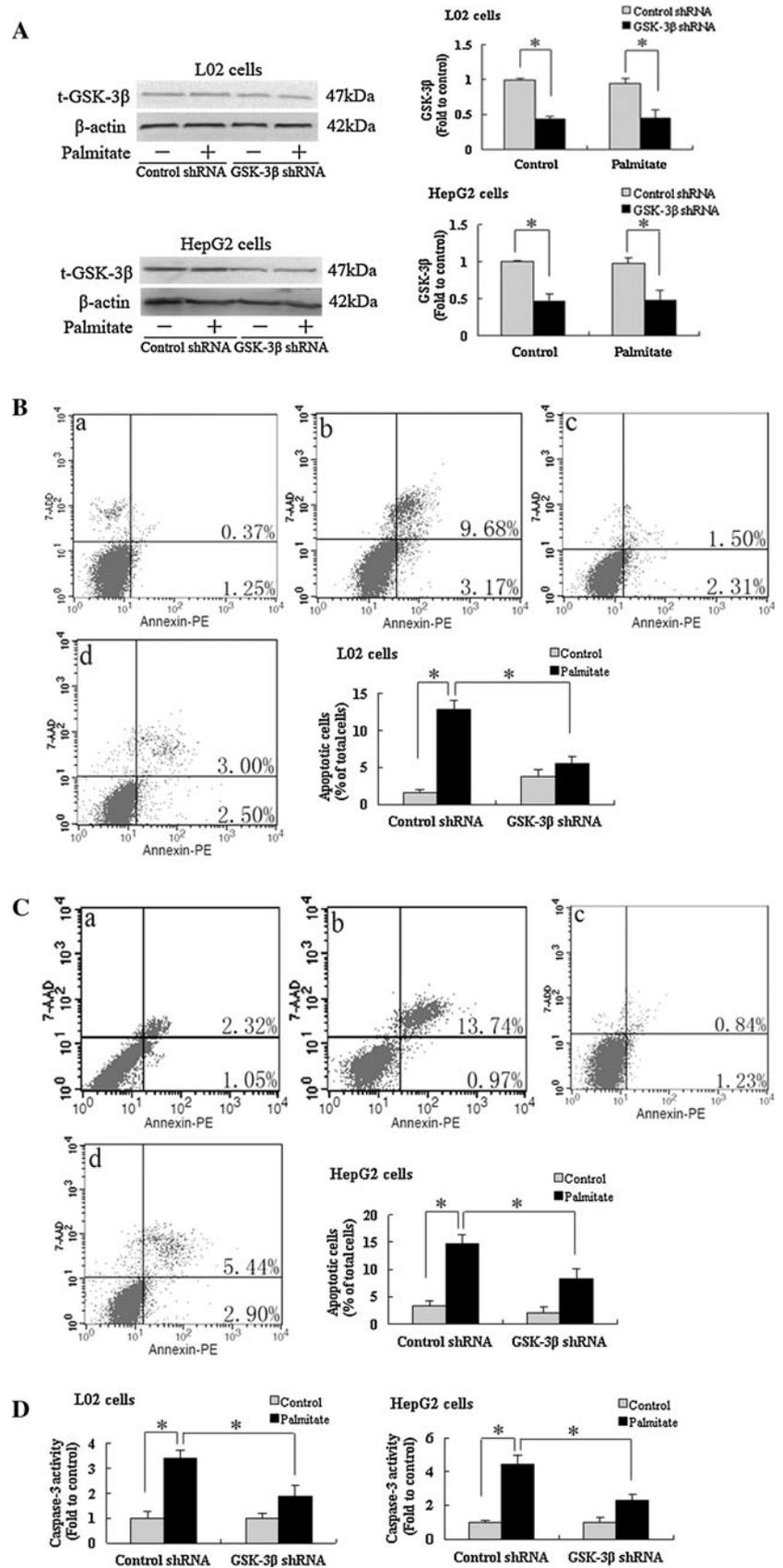


Fig. 4 Effects of GSK-3 β -targeted shRNA on the reduction of sodium palmitate-induced lipoapoptosis in L02 and HepG2 cells. **A** Western blot. The cells were grown and transfected with GSK-3 β shRNA or negative control shRNA for 12 h, then treated with 108 μ M sodium palmitate for an additional 48 h, and subjected to protein extraction and western blot analyses. **B** and **C**, apoptosis assay. Similarly treated L02 (**B**) and HepG2 (**C**) cells were subjected to annexin-PE and 7AAD double staining and flow cytometry. *a* negative control shRNA; *b* negative control shRNA plus sodium palmitate; *c* GSK-3 β shRNA; *d* GSK-3 β shRNA plus sodium palmitate. **D** Caspase-3 activity. Similarly treated cells were subjected to caspase-3 activity measurements with a caspase-3 colorimetric assay kit. The experiments were repeated three times. Data are summarized as mean \pm standard error (SE). * $P < 0.05$ versus the control



GSK-3 β Inhibition Decreased Sodium Palmitate-Induced JNK Activation and Bax Upregulation in L02 and HepG2 Cells

To further investigate how GSK-3 β inhibition mediated suppression of sodium palmitate-induced lipoapoptosis, we analyzed phosphorylated JNK and Bax expression levels after sodium palmitate treatment in L02 and HepG2 cells. Our data showed that phosphorylated JNK levels progressively increased in sodium palmitate-treated L02 and HepG2 cells (Fig. 5A), while Bax, a pro-apoptotic effector downstream of JNK, was also significantly upregulated at 24–48 h after sodium palmitate treatment (Fig. 5A). In contrast, sodium palmitate-induced JNK phosphorylation was significantly inhibited by lithium chloride treatment (Fig. 5A). Consistent with the inhibition of GSK-3 β activity, shRNA-silenced GSK-3 β expression also attenuated sodium palmitate-induced JNK activation (Fig. 5B). Moreover, inhibition of GSK-3 β expression or activity effectively reduced sodium palmitate-induced levels of Bax protein expression (Fig. 5A, B).

No Effects of GSK-3 β Inhibition on the Regulation of ER Stress-Induced Activation of UPR

Our previous data showed that blockage of the PERK/ATF4/CHOP signaling pathway was able to suppress sodium palmitate-induced apoptosis in human liver cell lines [17]. In this study, we further investigated whether inhibition of GSK-3 β modulated sodium palmitate-induced UPR in L02 and HepG2 cells. Our data showed that treatment of L02 and HepG2 cells with sodium palmitate promoted a significant increase in GRP78 expression, but inhibition of GSK-3 β expression using GSK-3 β shRNA transfection did not affect GRP78 expression or sodium palmitate-induced phosphorylation of PERK and IRE1 (Fig. 6). Taken together, this study suggests that inhibition of GSK-3 β expression or activity attenuates sodium palmitate-induced lipoapoptosis through inhibition of JNK activation and Bax expression, but not through UPR signaling.

Discussion

In the clinic, NAFLD is strongly associated with increased serum FFA levels [26]. Saturated FFAs are substantially cytotoxic compared to unsaturated FFAs because the former is able to induce ER stress and subsequently activate the mitochondrial pathway to induce lipoapoptosis in liver cells. Indeed, lipoapoptosis is a key pathogenic process in NAFLD, and it correlates with progressive inflammation and fibrosis. In the present study, we determined that

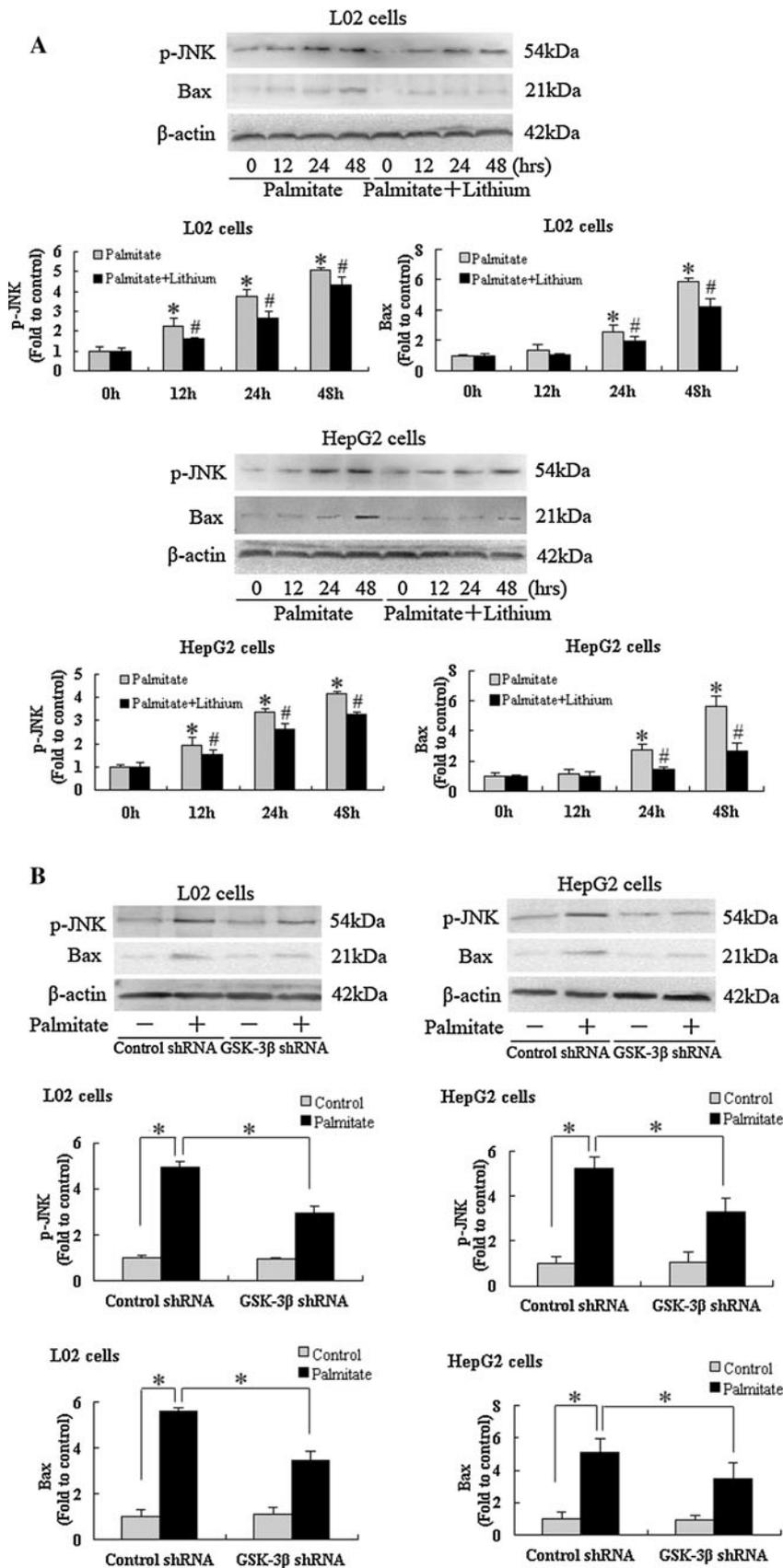
sodium palmitate treatment induced lipoapoptosis in human liver cell lines and then found the pro-apoptotic effects of GSK-3 β on mediating saturated FFA (e.g., sodium palmitate)-induced lipoapoptosis in these cell lines. At a molecular level, sodium palmitate activated GSK-3 β and its target genes, whereas inhibition of GSK-3 β expression or activity suppressed sodium palmitate-induced JNK activation and Bax upregulation. Therefore, sodium palmitate-induced lipoapoptosis was attenuated. However, GSK-3 β inhibition did not affect ER stress-induced activation of UPR. Based on these observations, our present study provides novel insight into the effect of GSK-3 β on mediating the lipotoxicity of elevated exogenous saturated FFAs (e.g., sodium palmitate) in human liver cell lines.

Previous studies have provided evidence that saturated FFAs (e.g., palmitic acid and stearic acid) are able to induce lipoapoptosis in human granulosa cells [27], rat testicular Leydig cells [28], and rat liver cells [29]. This study corroborated these previous findings and further provided a molecular link between sodium palmitate-induced lipoapoptosis and GSK-3 β activation in these two liver cell lines (a normal liver cell line and a hepatoma line). These two cell lines were chosen because they have been frequently used in the literature for establishing the *in vitro* model of fatty liver, e.g., NAFLD [17, 30, 31]. However, due to the fact that they are normal and tumor cells, respectively, their reactions to sodium palmitate treatment were slightly different, which is understandable.

GSK-3 β has multiple functions in the human body, such as homeostasis and disease formation. GSK-3 β can function as a pro-apoptotic kinase [32], and previous studies have demonstrated that overexpression of GSK-3 β induces PC12, Rat-1, and C3H10T1/2 cells to undergo apoptosis [22, 33] by activation of caspase-3 and decreases Bcl-2 expression [33]. However, the mechanism by which GSK-3 β regulates saturated FFA-induced lipoapoptosis remains to be determined, although GSK-3 β has been recently shown to be a key regulator of cell fate [23, 34, 35]. In this study, we took this challenge, and our data showed that sodium palmitate treatment induced the dephosphorylation of GSK-3 β at Ser-9 in a time-dependent manner; whereas lithium chloride, a selective GSK-3 β inhibitor, suppressed sodium palmitate-induced cell apoptosis and caspase-3 activation. Moreover, knockdown of GSK-3 β gene expression using GSK-3 β shRNA antagonized the effect of sodium palmitate on induction of apoptosis in L02 and HepG2 cells. This finding indicates that GSK-3 β is an important mediator in the initiation of lipoapoptosis caused by sodium palmitate treatment.

Furthermore, JNK belongs to a family of intracellular mitogen-activated protein kinases. There are three known JNK genes, while JNK1 and JNK2 are expressed in the

Fig. 5 Inhibition of GSK-3 β activity or expression on the reduction of sodium palmitate-induced JNK phosphorylation and Bax upregulation in L02 and HepG2 cells. **A** Western blot. L02 and HepG2 cells were pretreated with or without 20 mM lithium chloride for 1 h, followed by treatment with 108 μ M sodium palmitate for up to 48 h, and then subjected to protein extraction and western blot analysis. **B** Western blot. L02 and HepG2 cells were grown and transfected with GSK-3 β shRNA or negative control shRNA for 12 h, then treated with 108 μ M sodium palmitate for an additional 48 h, and subjected to protein extraction and western blot analyses. The target protein bands were semi-quantified by using densitometry and are displayed as fold-changes relative to the control. The experiments were repeated three times. Data are presented as mean \pm standard error (SE). * $P < 0.05$, palmitate-treated cells versus control cells; # $P < 0.05$, palmitate plus lithium-treated cells versus palmitate-treated cells at the same time point



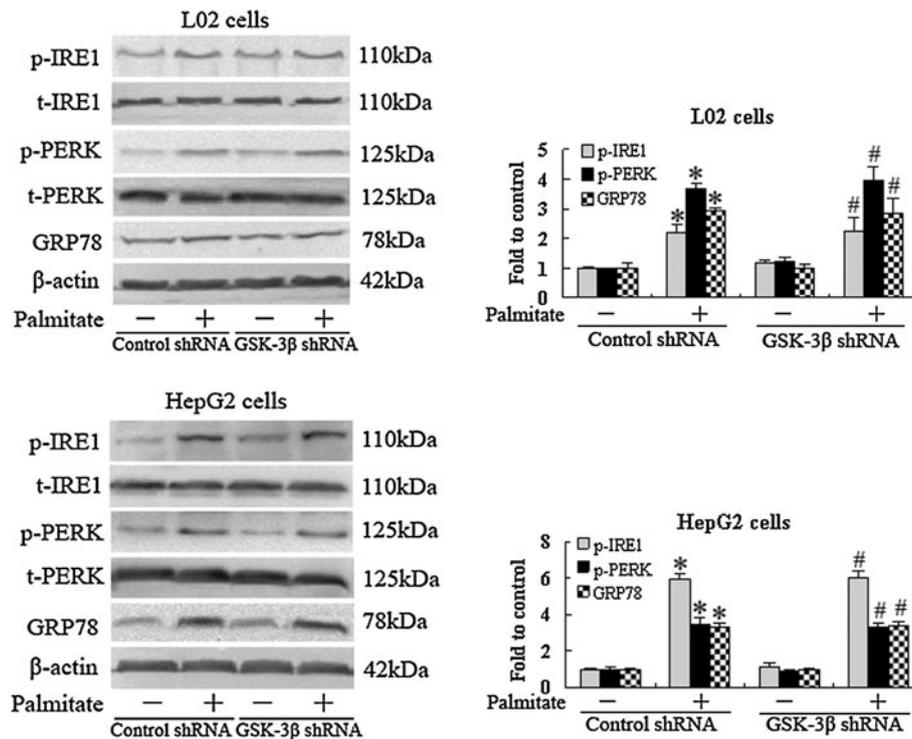


Fig. 6 No effects of GSK-3 β inhibition on the regulation of ER stress-induced activation of UPR. L02 and HepG2 cells were grown and transfected with GSK-3 β shRNA or negative control shRNA for 12 h, then treated with 108 μ M sodium palmitate for an additional 48 h, and subjected to protein extraction and western blot analyses. The target protein bands were semi-quantified by using densitometry and are displayed as fold-changes relative to the control. The

liver [36]. JNK activation is pivotal in both the metabolic syndrome accompanying NAFLD and cellular apoptosis. For example, JNK has been shown to be activated in experimental murine dietary and genetic models of NASH [37–39] and also in human NASH [16, 40]. In addition, saturated FFAs have been shown to induce hepatocyte lipoapoptosis by a JNK-dependent mechanism [41]. In the present study, we confirmed that GSK-3 β physically interacted with and activated upstream kinases to trigger JNK activation [42, 43] and that GSK-3 β activated JNK in the initial phase of acetaminophen-induced liver injury [25]. In addition, JNK may also induce apoptosis by activating proapoptotic members of the Bcl-2 family, such as Bim and Bax, or by inactivating antiapoptotic members of this family, such as Bcl-2 and Bcl-xL [44, 45]. In a previous study, Harmeet et al. [41] showed that saturated FFAs induce JNK-dependent hepatocyte lipoapoptosis by stimulating Bax expression and triggering the mitochondrial apoptosis pathway. In our present study, we demonstrated that levels of phosphorylated JNK increased in a time-dependent manner after L02 and HepG2 cells were treated with sodium palmitate. Bax expression was also

experiments were repeated three times. Data are presented as mean \pm standard error (SE). * P < 0.05, negative control shRNA plus sodium palmitate (+)-treated cells versus negative control shRNA plus sodium palmitate (-)-treated cells; # P > 0.05, GSK-3 β shRNA plus sodium palmitate (+)-treated cells versus negative control shRNA plus sodium palmitate (+)-treated cells

significantly upregulated by sodium palmitate treatment, and caspase-3 was activated afterwards. In contrast, inhibition of GSK-3 β expression or activity suppressed sodium palmitate-induced expression of phosphorylated JNK and Bax in human liver cell lines. These data suggest that the GSK-3 β /JNK/Bax pathway takes precedence in sodium palmitate-treated L02 and HepG2 cells.

In addition, ER stress can cause the apoptotic signaling cascade, and the role of perturbations in the ER in NAFLD have become a subject of considerable interest in recent years based on studies in rodent models and humans [11, 16, 46]. We previously showed that sodium palmitate-induced ER stress and lipoapoptosis in human liver cell lines are enacted through the PERK/ATF4/CHOP signaling pathway [17]. GSK-3 inhibition is able to protect cells from ER stress-induced apoptosis following exposure of a variety of cell types to several conditions that induce ER stress [34, 35, 47, 48]. However, the underlying mechanisms by which GSK-3 promotes apoptotic signaling induced by ER stress remain unclear. Meares et al. [49] recently revealed that GSK-3 β regulates the outcome of tunicamycin-induced UPR signaling by promoting CHOP expression to

balance UPR-associated survival pathways with apoptotic signaling in SH-SY5Y human neuroblastoma cells. In the present study, we found that knockdown of GSK-3 β expression had no effect on regulation of sodium palmitate-induced GRP78 expression and expression of UPR transducers, such as PERK and IRE1 protein. Therefore, these data are not consistent with the previous findings [49]. Our data imply that GSK-3 β activation may not regulate expression of all other agent-induced UPR proteins in various cells because GSK-3 β inhibition likely attenuates lipoapoptosis without the influences of UPR signaling pathways in human liver cell lines. This mechanism likely determines the role of GSK-3 β in ER stress-induced apoptosis. How GSK-3 β is activated by sodium palmitate is not clear. Pap et al. [22] recently reported that the phosphatidylinositol-3-OH kinase (PI3K)/Akt signaling pathway is a signaling system implicated in the survival of PC12 and Rat-1 cells that leads to the inhibition of GSK-3 by increasing Ser9 phosphorylation. Moreover, PI3K/Akt was activated on short-term exposure to ER stress but downregulated on long-term exposure to ER stress in primary cultured glial cells [50]. It is unknown whether PI3K/Akt is responsible for GSK-3 β activation stimulated by sodium palmitate in hepatocytes during lipoapoptosis. It will be interesting to investigate this mechanism in future studies.

In summary, the present data provide mechanistic insight into sodium palmitate-induced lipoapoptosis and link it to GSK-3 β activation. This study suggests that inhibition of GSK-3 β expression or activity could attenuate saturated FFA (e.g., sodium palmitate)-induced lipoapoptosis in human liver cell lines, which may further be translated into future clinical treatment of NAFLD, pending further confirmation studies.

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Conflict of interest None.

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