

Differential Activation of ER Stress Signal Pathways Contributes to Palmitate-Induced Hepatocyte Lipoapoptosis

Xiaofang Zhao, Fuli Yao, Chunyan Zhang, Ying Cheng, Bin Xiao, Yuxiao Li, Chunyan Duan, Youping Liu, Hong Li, Rongyang Dai*

Department of Biochemistry and Molecular Biology, Southwest Medical University, Luzhou, Sichuan, China

Email address:

zhaoxiaofang2099@163.com (Xiaofang Zhao), fuliyao@163.com (Fuli Yao), zcy_2004_116@163.com (Chunyan Zhang), 123964977@qq.com (Ying Cheng), xbwin@163.com (Bin Xiao), lyx452x@hotmail.com (Yuxiao Li), swallow0213@163.com (Chunyan Duan), lyp2507622@163.com (Youping Liu), lihong7188@163.com (Hong Li), dryrun2502@163.com (Rongyang Dai)

*Corresponding author

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Abstract: Saturated free fatty acids-induced hepatocyte lipoapoptosis plays a pivotal role in non-alcoholic steatohepatitis. The activation of endoplasmic reticulum (ER) stress is involved in hepatocyte lipoapoptosis induced by the saturated free fatty acid palmitate (PA). However, the underlying mechanisms of the role of ER stress in hepatocyte lipoapoptosis remain largely unclear. In this study, we showed that PA and tunicamycin (Tun), a classic ER stress inducer, resulted in differential activation of ER stress pathways. Our data revealed that PA induced chronic and persistent ER stress response, but Tun induced acute and transient ER stress response. Compared with Tun treatment, PA induced much lower glucose-regulated protein 78 (GRP78), a central regulator of ER homeostasis, accumulation. It is noteworthy that GRP78 over-expression not only inhibited PA-induced ER stress but also decreased PA-induced apoptosis. Taken together, our data suggest that the differential activation of ER stress signal plays an important role in PA-induced hepatocyte lipoapoptosis. More detailed studies on the mechanisms of PA in repressing the accumulation of GRP78 will contribute to the understanding of molecular mechanisms of lipoapoptosis.

Keywords: ER Stress, Palmitate, Hepatocyte Lipoapoptosis, GRP78

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a public health problem associated with the metabolic syndrome, such as obesity and insulin resistance [1]. Up to 30% of the population is afflicted by NAFLD and 5–10% of these patients develop non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis and hepatocellular carcinoma [2, 3]. Both obesity and insulin resistance are major risk factors for NASH and are characterized by an increase in circulating free fatty acids (FFAs) [4-6]. The excess serum FFAs in the context of insulin resistance are transported into hepatocyte by the fatty acid transporter. FFAs are esterified into neutral triglycerides

in hepatocyte. However, surfeit of FFA is deleterious for hepatocyte. Saturated FFAs can induce hepatocyte apoptosis, a phenomenon termed lipoapoptosis [7-9]. Indeed, the lipoapoptosis of hepatocyte is a pathologic feature of NAFLD and correlates with disease severity [10-12]. Thus, the cellular and molecular mechanisms of hepatocyte lipoapoptosis are of biomedical interest.

The endoplasmic reticulum (ER) provides a contained environment for the synthesis and modification of membrane proteins and proteins destined to be secreted. Upon a number of biochemical, physiologic to pathologic stimuli, the ER protein processing system can be disrupted by disordered calcium (Ca^{2+}) homeostasis and redox status, which subsequently cause the accumulation of unfolded or

misfolded proteins, a condition defined as ER stress. Conditions of ER stress lead to the onset of the unfolded protein response (UPR) [13-16]. The canonical UPR signaling pathways are mediated by three UPR transmembrane sensors: protein kinase RNA-like ER kinase (PERK), transcription factor activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) [17, 18]. In non-stressed situations, glucose-regulated protein 78 (GRP78) acts as a master regulator of the UPR through direct interaction with ATF6, PERK, and IRE1 and maintains them in an inactive form. Upon ER stress, GRP78 is titrated away by unfolded proteins, releasing the UPR sensors, which subsequently activates these sensors and initiates the activation and transduction of UPR signals. The UPR is a coordinated response to reduce the accumulation of misfolded proteins and restore ER function. If the efforts of UPR fail to restore ER homeostasis, the UPR will induce cell apoptosis to eliminate the stressed cells [16, 18].

Intracellular FFAs are esterified within the ER, and inundation of hepatocyte with FFA disturbs ER function, which resulting in ER stress response [19, 20]. The activation of UPR signal pathways have been well documented in NAFLD [19]. It has been postulated that ER may sense metabolic stresses associated with obesity and transmit signals that ultimately influence both pancreatic insulin secretion and hepatic insulin action. Since it has been reported that ER stress and inability of the UPR to reestablish ER homeostasis may be upstream components of lipotoxicity [19, 21], the mechanisms of ER stress in lipotoxicity are poorly defined.

In this study, we investigated the roles of ER stress in palmitate (PA, a 16 carbon FFA with no carbon-carbon double bonds, indicated 16:0)-induced hepatocyte lipoapoptosis. We found that PA caused ER stress as evidenced by increased X-box-binding protein-1 (XBP-1) mRNA splicing, increased expression of C/EBP homologous protein (CHOP) and GRP78. Interestingly, the pattern of PA-initiated ER stress is different from N-glycosylation inhibitor tunicamycin (Tun)-induced ER stress, and the differential activation of ER stress signal pathways plays an important role in hepatocyte lipoapoptosis.

2. Materials and Methods

2.1. Chemicals and Antibodies

Palmitate (PA), ER stress inducer tunicamycin (Tun, inhibitor of N-linked glycosylation), and ER stress inhibitor PBA were purchased from Sigma. Antibodies against phosphor-eIF2 α (Ser-51), PARP, and Myc-tag were purchased from Cell Signaling Technology. Antibodies against GRP78, and β -actin were purchased from Santa Cruz Biotechnology. GRP78 plasmid was purchased from Addgene.

2.2. Cell Culture and Transfection

Human hepatocellular carcinoma cell line SMMC-7721 was cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics. Cells were maintained at

37°C in a humidified incubator containing 5% CO₂ and 95% air. Transfection of vectors for the expression of GFP and GRP78 were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manuals.

2.3. Fatty Acid Treatment

PA was dissolved in isopropanol at a concentration of 200 mM. PA was added to DMEM containing 1% bovine serum albumin to obtain a physiologic ratio between bound and unbound FFA in the media. The concentration of PA used in the experiments was 500 μ M and was similar to the fasting total FFA plasma concentrations observed in human with non-alcoholic steatohepatitis.

2.4. Western Blot Analysis

The procedure for Western blot has been described in detail previously [22-24]. Briefly, cells were lysed in Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM sodium fluoride, 5 mg/ml of aprotinin, 20 mM leupeptin, and 1 mM sodium orthovanadate) and centrifuged at 12,000 g for 15 minutes. Protein concentrations were measured using the BCA assay. Protein samples were denatured with 4 \times SDS-loading buffer (200 mM Tris, pH 6.8, 8% SDS, 400 mM DTT, 0.4% bromophenol blue, 40% glycerol) at 100 °C for 5 minutes and subjected to standard SDS-PAGE and Western blot analysis.

2.5. Apoptosis Analysis

Apoptosis was detected using the annexin V-FITC apoptosis detection kit (Invitrogen) according to the manufacturer's manual. The experiments were repeated three times.

2.6. Reverse Transcription Reaction

Total RNA was prepared as detailed previously [22, 24, 25]. Reverse transcription was performed with 0.2 μ g of isolated total RNA using the M-MLV reverse transcriptase kit (Promega). 18S was used as a normalization control. PCR products were run on 2-4% agarose gel. The gel images were digitally captured with a SynGene gel documentation system and analyzed with the Genetools analysis software (Syngene, Frederick, MD, USA) as previously described [22, 24, 25].

The primers used in this study:

18S-forward GGGAGGTAGTGACGAAAAAT,
18S-reverse ACCAACAAAATAGAACCGCG;
XBP1-forward CCTTGTAAGTTGAGAACCAGG,
XBP1-reverse GGGGCTTGGTATATATGTGG;
GRP78-forward ATCACGCCGTCCTATGTCCG,
GRP78-reverse TCTCCCCCTCCCTCTTATCC;
GADD153-forward AGTCATTGCCTTTCTCTTCG,
GADD153-reverse GGTGCAGATTCACCATTCGG.

2.7. Statistical Analysis

Results were expressed as the mean \pm S.D. Statistical analysis was performed using Student's t test. $p < 0.05$ was considered statistically significant.

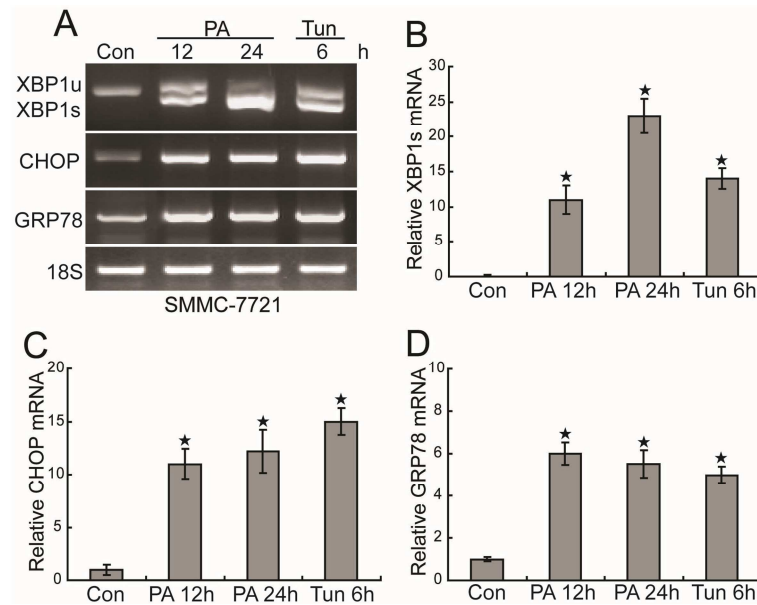


Figure 1. PA induces ER stress. (A) SMMC-7721 cells were treated with palmitate (PA, 500 μ M) or tunicamycin (Tun, 2.5mg/ml) for indicated periods and total RNA were analyzed by RT-PCR. (B-D) The mRNA values of spliced XBP1 (XBP1s, B), CHOP (C) and GRP78 (D) were normalized to 18S rRNA content. Data are presented as mean values \pm SD of three measurements; bars, SE. *Significantly different from control value.

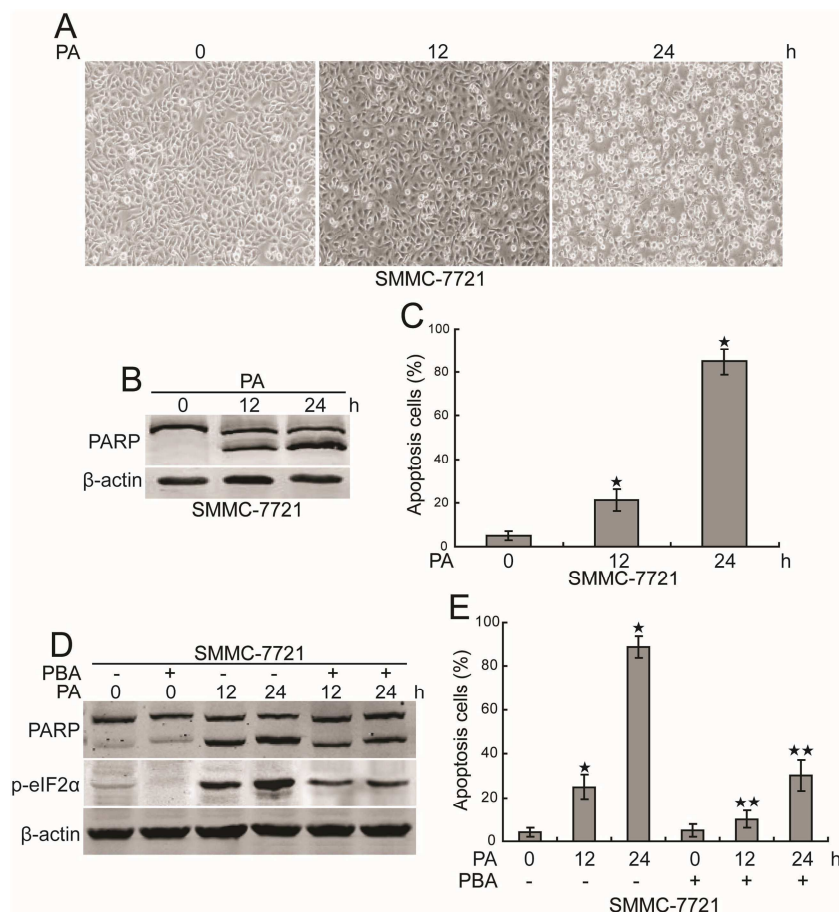


Figure 2. ER stress is involved in PA-induced apoptosis. (A-C) SMMC-7721 cells were treated with palmitate (PA, 500 μ M) for indicated periods and apoptosis was detected by morphological examination (A), Western blot analysis (B) and annexin V-FITC apoptosis detection kit (C). Data are presented as mean values \pm SD of three measurements; bars, SE. *Significantly different from control value. (D, E) SMMC-7721 cells were treated with palmitate (PA, 500 μ M) for indicated periods with or without ER stress inhibitor PBA (1 mM) pretreatment for 1 hour and apoptosis was detected by Western blot analysis (D) and annexin V-FITC apoptosis detection kit (E). Data are presented as mean values \pm SD of three measurements; bars, SE. *Significantly different from control value. **significantly different from *value.

3. Results

3.1. PA Initiates ER Stress

To investigate the potential role of PA in the induction of ER stress in hepatocyte, we first examined the ER stress induction effects of PA in SMMC-7721 cells. The mRNA levels of ER stress markers, including XBP1, CHOP, and GRP78, were evaluated by RT-PCR from PA treated SMMC-7721 cells. We found that PA treatment markedly elevated both the mRNA levels of GRP78 and CHOP, and induced XBP1 mRNA splicing, an indicator of UPR activation, in SMMC-7721 cells (Figure 1A). Tun-treated SMMC-7721 cells were used as positive control. For quantitative analysis, the mRNA levels of spliced XBP1, GRP78, and CHOP were normalized to the corresponding 18S rRNA levels (Figure 1B-D). These data suggest that PA can initiate ER stress response in hepatocyte.

3.2. ER Stress Is Involved in PA-initiated Apoptosis

To investigate the potential role of PA in the induction of apoptosis in hepatocyte, we investigated the cytotoxicity effects of PA in SMMC-7721 cells. The results showed that PA treatment exerts obviously cytotoxicity in SMMC-7721 cells in a time-dependent manner (Figure 2A). In order to confirm the role of PA in inducing SMMC-7721 cells apoptosis, cleavage of poly (ADP-ribose) polymerase (PARP) was detected by Western blot analysis. Western blot analysis revealed that PA treatment induced the cleavage of PARP in SMMC-7721 cells in a time-dependent manner (Figure 2B). Furthermore, Annexin V-FITC staining (Figure 2C) confirmed that PA treatment can induce SMMC-7721 cells apoptosis in a time-dependent manner. Thus, these data suggest that PA can initiate apoptosis of hepatocyte.

To make sure whether ER stress is involved in PA-mediated apoptosis, ER stress inhibitor PBA was used to block the activation of ER stress. The results showed that PBA pre-incubation not only inhibited the phosphorylation of eIF2 α , an indicator of UPR activation, but also obviously decreased the apoptosis of SMMC-7721 cells upon PA treatment (Figure 2D & E). Taken together, these data suggest that ER stress plays an important role in PA-induced SMMC-7721 cells apoptosis.

Considering that ER stress plays a pivotal role in PA-induced SMMC-7721 cells apoptosis, we examined whether ER stress can induce apoptosis in SMMC-7721 cells upon Tun treatment. The data showed that SMMC-7721 cells were relatively resistant to ER stress-induced apoptosis triggered by Tun treatment for a relatively long time (<15% apoptotic cells at 36 h) (Figure 3A & B). However, PA treatment efficiently killed SMMC-7721 cells by induction of apoptosis at 24 h (Figure 3A & B).

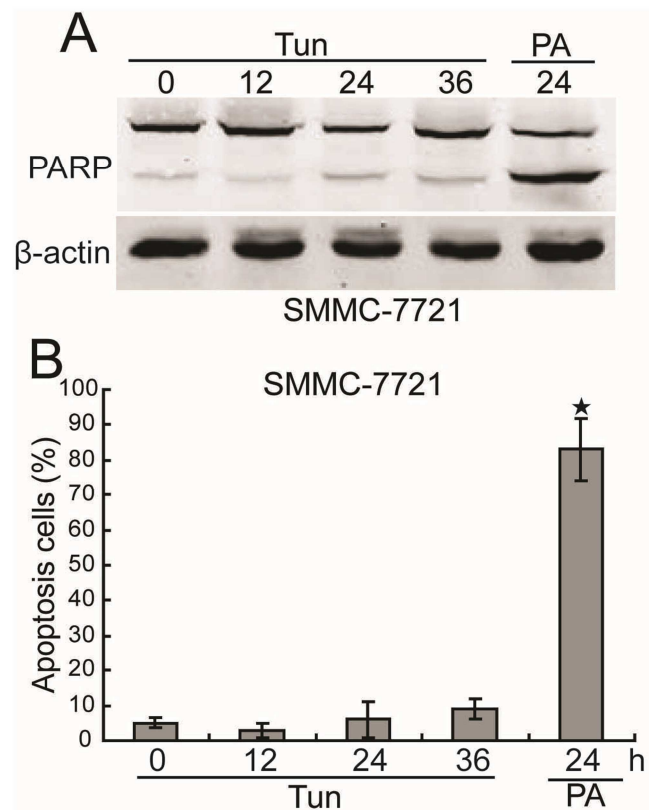


Figure 3. SMMC-7721 cells are resistant to ER stress-induced apoptosis. (A, B) SMMC-7721 cells were treated with tunicamycin (Tun, 2.5mg/ml) or palmitate (PA, 500 μ M) for indicated periods and apoptosis was detected by Western blot analysis (A) and annexin V-FITC apoptosis detection kit (B). Data are presented as mean values \pm SD of three measurements; bars, SE. *Significantly different from control value.

3.3. PA Differently Activates ER Stress Signal Pathways

As ER stress induced by PA and Tun plays different roles in the induction of apoptosis, we investigated whether PA and Tun can differently activate ER stress signal pathways. The results showed that the mRNA levels of spliced XBP1 began accumulating within 3 h and decreased after 12 h in SMMC-7721 cells, when exposed to Tun (Figure 4A). In contrast, the up-regulating of spliced XBP1 mRNA levels can't be observed within 6 h in SMMC-7721 cells upon PA treatment (Figure 4A). However, PA-induced up-regulating of spliced XBP1 mRNA levels sustained more than 24 h (Figure 4A). Upon PA and Tun treatment, no obvious difference of CHOP mRNA levels was observed in SMMC-7721 cells (Figure 4A). Moreover, Tun-induced up-regulating of GRP78 mRNA levels are not only higher but also earlier than that of PA in SMMC-7721 cells (Figure 4A). For quantitative analysis, the mRNA levels of spliced XBP1, CHOP, and GRP78 were normalized to the corresponding 18S rRNA levels (Figure 4B-D). Together, these data indicate that PA and Tun initiate ER stress pathways activation with different kinetics specifically.

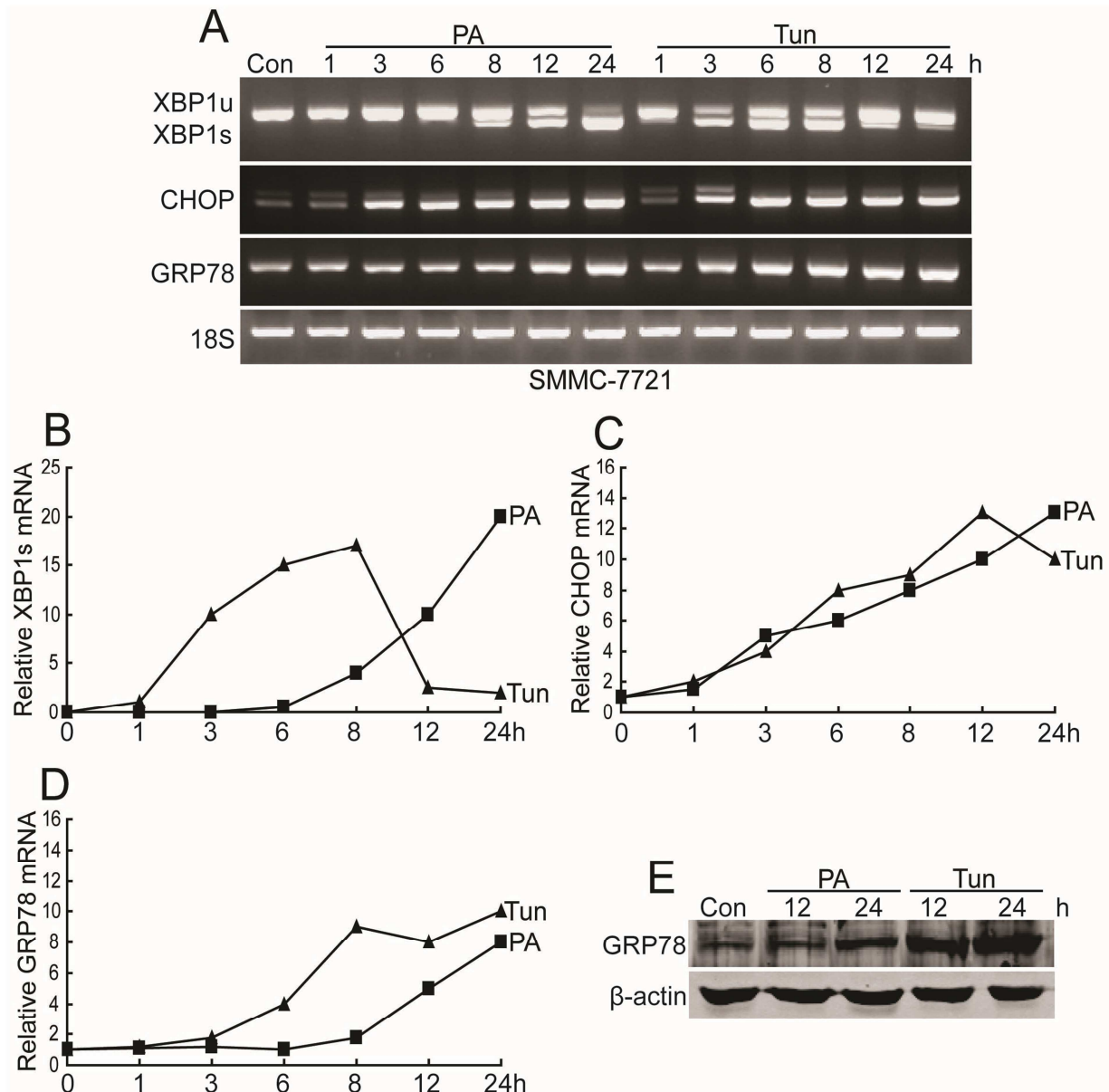


Figure 4. PA and Tun activate ER stress pathways with different kinetics. (A) SMMC-7721 cells were treated with palmitate (PA, 500 μ M) or tunicamycin (Tun, 2.5mg/ml) for indicated periods and total RNA were analyzed by RT-PCR. (B-D) The mRNA values of spliced XBP1 (XBP1s, B), CHOP(C), GRP78 (D) were normalized to 18S rRNA content. Data are presented as mean values \pm SD of three measurements. (E) SMMC-7721 cells were treated with palmitate (PA, 500 μ M) or tunicamycin (Tun, 2.5mg/ml) for indicated periods and cell lysates were analyzed for the levels of indicated proteins.

3.4. GRP78 Inhibits PA-induced Apoptosis

Since the accumulation of GRP78 is inhibited in SMMC-7721 cells upon PA treatment, we therefore investigated whether GRP78 over-expression can protect SMMC-7721 cells from PA-induced apoptosis. The Western blot data showed that GRP78 over-expression obviously inhibited PA-mediated SMMC-7721 cells apoptosis (Figure 5A). The effects of GRP78 on apoptosis inhibition in PA-treated SMMC-7721 cells were reconfirmed by annexin V-FITC apoptosis analysis (Figure 5B). To evaluate whether GRP78 over-expression can decrease PA-induced ER stress response, spliced XBP1 mRNA levels were measured in GRP78 over-expressed SMMC-7721 cells and the control

cells. As shown in Figure 5C, GRP78 over-expression decreased the induction of spliced XBP1 mRNA in PA-treated SMMC-7721 cells. Taken together, these data indicate that GRP78 deficiency plays an important role in PA-mediated apoptosis.

Since GRP78 plays a pivotal role in protecting cells from ER stress-induced apoptosis, we therefore examined the induction of GRP78 protein upon PA and Tun treatment in SMMC-7721 cells. As shown in Figure 4E, GRP78 protein levels induced by PA were much lower than that of Tun in SMMC-7721 cells. Thus, it is suggested that differential GRP78 accumulation might be a reason for the different roles of PA and Tun-induced ER stress in apoptosis inducing.

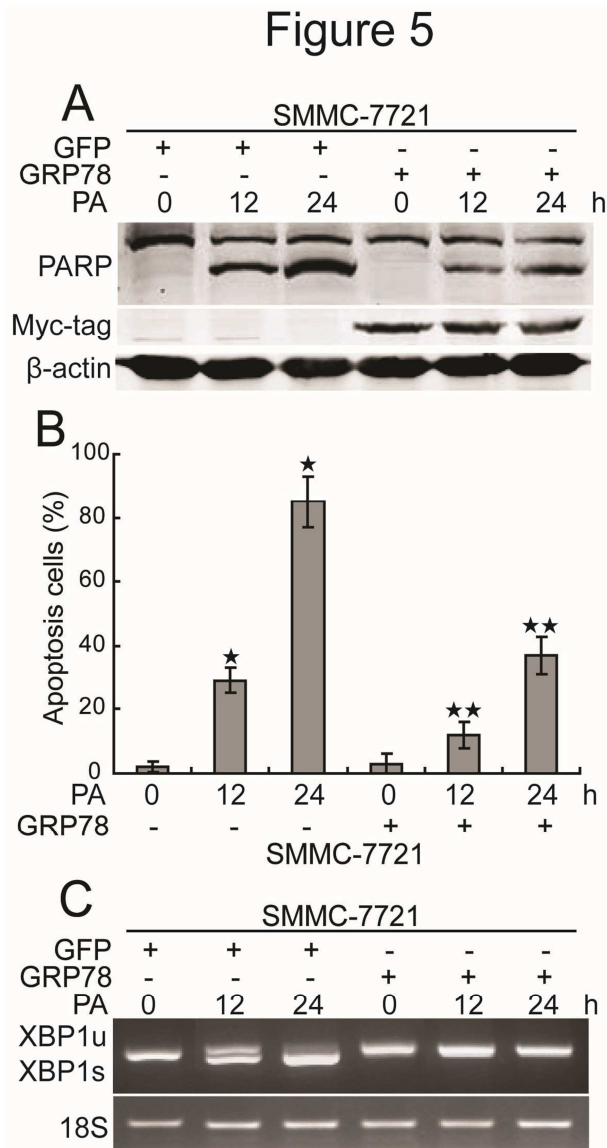


Figure 5. GRP78 inhibits PA-induced ER stress and apoptosis. (A, B) After transient transfection with GFP or Myc-GRP78 constructs for 24 h, SMMC-7721 cells were treated with palmitate (PA, 500 μ M) for indicated periods and apoptosis was detected by Western blot analysis (A) and annexin V-FITC apoptosis detection kit (B). Data are presented as mean values \pm SD of three measurements; bars, SE. *Significantly different from control value. **Significantly different from *value. (C) After transient transfection with GFP or Myc-GRP78 constructs for 24 h, SMMC-7721 cells were treated with palmitate (PA, 500 μ M) for indicated periods and total RNA were analyzed by RT-PCR.

4. Discussion

PA is one of the most common fatty acids in human diets as well as in animal and human fat tissue. PA is also the end product of de novo fatty acid synthesis and is generated in large quantity in subjects on a low-fat diet. The toxic effects of PA on cell viability have been reported in several cell types [26-30]. Although PA is implicated in the induction of apoptosis, it remains largely unclear how PA exerts its pro-apoptotic action. The results of the present study provide mechanistic insights regarding the pro-apoptotic effects of ER

stress during PA-induced lipoapoptosis. Our data reveal that PA initiates ER stress and induces apoptosis in SMMC-7721 cells in part through decreasing the induction of GRP78, which results in persistent ER stress activation.

It has been reported that PA can initiate ER stress response in various cell types [29-32]. We firstly detected the potential role of PA in the induction of ER stress in SMMC-7721 cells. In agreement with these reports, we found that PA initiates ER stress in SMMC-7721 cells, evidenced by increased mRNA levels of spliced XBP1, CHOP and GRP78. Our data indicate that at least two of the three major UPR signaling pathways, IRE1/XBP1 and PERK/CHOP were altered by PA. It has been suggested that ER stress is involved in PA-induced apoptosis [12, 19, 20, 31]. Based on the data that blocking ER stress inhibited PA-induced apoptosis in SMMC-7721 cells, we suggest that ER stress promotes PA-induced apoptosis in hepatocyte.

The UPR is an adaptive response that increases cell survival under ER stress conditions [16, 17]. However, if ER stress is persistent and the stress can't be resolved, the UPR signaling switches from pro-survival to pro-apoptotic mode and the cells are destroyed by apoptosis [22, 24, 33, 34]. Since SMMC-7721 cells are more resistant to ER stress-induced apoptosis than that of PA, we suggest that the activation pattern of PA-induced ER stress signal might be different from classic ER stress inducer Tun-induced ER stress signal. This hypothesis is supported by our data, which demonstrated PA and Tun activated UPR pathways with different kinetics. In the present study, we found that Tun-induced spliced XBP1 mRNA accumulation decreased within 12 h. However, the PA-induced the accumulation of spliced XBP1 mRNA sustained high levels more than 24 h. As the induction of spliced XBP1 mRNA is a potential biomarker of the degree of ER stress response [35, 36], it is reasonable to suggest that PA-induced ER stress is persistent, but Tun-induced ER stress is transient. The difference of lasting time between PA and Tun-induced ER stress response might be a major reason for the different effects of PA and Tun-induced ER stress in apoptosis induction.

How does PA induce persistent ER stress? As a central regulator of ER homeostasis and the UPR, GRP78 plays multiple roles in protein folding, protein quality control, maintaining ER homeostasis, and controlling of the activation of transmembrane ER stress sensors [37-39]. Considering that PA-induced up-regulation of GRP78 mRNA is lower than that of Tun, it seems that PA can't efficiently induce the accumulation of GRP78. This speculation was demonstrated by the findings that the accumulation of GRP78 protein was much lower than that of Tun. In our case, it is reasonable to speculate that PA-induced ER stress is persistent, and the stressed cells can't recover from ER stress due to the deficient induction of GRP78. It's notable that GRP78 over-expression not only decreased the degree of PA-induced ER stress, but also inhibited PA-mediated apoptosis. These data reconfirm that the deficient induction of GRP78 exerts the pro-apoptotic effect of PA-induced ER stress.

5. Conclusion

The current study demonstrates that ER stress plays an essential role in PA-induced hepatocyte apoptosis. Our data provide mechanistic insights regarding the link between ER stress activation and FFA-mediated lipotoxicity. The ability of PA to inhibit GRP78 induction exerts the pro-apoptotic effect of PA-induced ER stress. More detailed studies on the mechanisms of PA in repressing the accumulation of GRP78 will contribute to the understanding of molecular mechanisms of lipoapoptosis.

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