Peroxisome proliferator-activated receptor alpha acts as a mediator of endoplasmic reticulum stress-induced hepatocyte apoptosis in acute liver failure

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Summary statement: PPARα can ameliorate hepatic injury via inhibiting ER stress mediated hepatocyte apoptosis in a mouse model of D-GalN/LPS-induced ALF.

ABSTRACT

Peroxisome proliferator-activated receptor α (PPARα) is a key regulator to ameliorate liver injury in cases of acute liver failure (ALF). However, its regulatory mechanisms remain largely undetermined. Endoplasmic reticulum stress (ER stress) plays an important role in a number of liver diseases. This study aimed to investigate whether PPARα activation inhibit ER stress-induced hepatocyte apoptosis, thereby protecting against ALF. In a murine model of D-galactosamine (D-GalN) and lipopolysaccharide (LPS)-induced ALF, Wy-14643 was administered to activate PPARα, and 4-phenylbutyric acid (4-PBA) was administered to attenuate ER stress. PPARα activation ameliorated liver injury, because pre-administration of its specific inducer, Wy-14643, reduced the serum aminotransferase levels and preserved liver architecture compared with that of controls. The protective effect of PPARα activation resulted from the suppression of ER stress-induced hepatocyte apoptosis. Indeed, (1) PPARα activation decreased the expression of glucose-regulated protein 78 (Grp78), Grp94 and C/EBP-homologous
protein (CHOP) in vivo; (2) the liver protection by 4-PBA was due to the induction of PPARα expression, because 4-PBA pretreatment promoted up-regulation of PPARα, and inhibition of PPARα by small interfering RNA (siRNA) treatment reversed liver protection and increased hepatocyte apoptosis; (3) in vitro PPARα activation by Wy-14643 decreased the hepatocyte apoptosis induced by severe ER stress, and PPARα inhibition by siRNA treatment decreased the hepatocyte survival induced by mild ER stress. Here, we demonstrated that PPARα activation contributes to liver protection and decreases hepatocyte apoptosis in ALF, particularly through regulating ER stress. Therefore, targeting PPARα could be a potential therapeutic strategy to ameliorate ALF.
INTRODUCTION

Acute liver failure (ALF) is a clinical syndrome defined by the sudden onset of severe liver injury and is characterized by encephalopathy and coagulopathy in patients with previously normal liver function (Khan et al., 2006). The causes of ALF are diverse including toxins, infections, or metabolic and genetic diseases, but irrespective of etiology, ALF results from rapid and extensive hepatic apoptosis and necrosis (Riordan and Williams, 2003). Despite developments in treatment, orthotopic liver transplantation (OLT) is still considered the most effective therapy. Unfortunately, the feasibility of OLT is extremely limited by the rapid progression of the disease and the shortage of donor livers; therefore, the pathogenesis of ALF needs to be further explored.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-inducible transcription factors. To date, three subtypes of PPARs (α, β, γ) have been identified in many species, including humans (Desvergne and Wahli, 1999; Kota et al., 2005). PPARα has been reported to regulate lipid metabolism (Staels et al., 1998), inflammation (Devchand et al., 1996; Delerive et al., 1999), cell differentiation and apoptosis (Roberts et al., 2002). Studies have demonstrated that PPARα plays a different role in cancer cells than in normal cells. PPARα activation is commonly implicated in hepatocarcinogenesis protocols for rodents in which its anti-apoptotic action is assumed to play a critical role (Misra et al., 2013; Misra and Reddy, 2014); however, activation of PPARα by exogenous agonists reduces tumor cell growth in cell lines derived from colorectal cancer (Grau et al., 2006). In non-cancerous renal tubular cells, a lack of PPARα exacerbates gentamicin-induced apoptosis (Hsu et al., 2008).
Additionally, Wy-14643, a potent exogenous PPARα ligand and a selective PPARα agonist (Cuzzocrea et al., 2004; Briguglio et al., 2010), decreases the apoptosis of cardiomyocytes via reducing the nuclear translocation of nuclear factor-κB (NF-κB) and reducing caspase-3 activation, thus preserving myocardial function and maintaining cardiac contractility (Yeh et al., 2006). In a third normal cell system, PPARα agonist treatment has been shown to increase trefoil factor family-3 expression and attenuate apoptosis in the liver tissue of bile duct-ligated rats (Karakan et al., 2013). Our recent study has shown that PPARα activation protects the liver from acute injury by promoting the autophagy pathway in the D-galactosamine (D-GalN) and lipopolysaccharide (LPS)-induced ALF mouse model (Jiao et al., 2014). However, whether PPARα plays a protective role in the liver by inhibiting hepatocyte apoptosis is yet to be determined.

The endoplasmic reticulum (ER) is a vital cellular organelle for protein folding and trafficking, lipid synthesis and calcium homeostasis that are required for cell survival and functions. Endoplasmic reticulum stress (ER stress) is induced by physiological and/or pathological stress signals, leading to the accumulation of unfolded or misfolded proteins in the ER, and activates three ER-localized transmembrane protein sensors (Ron and Walter, 2007; Lin et al., 2008). The chaperone proteins glucose-regulated protein 78 (Grp78) and glucose-regulated protein 94 (Grp94) are master regulators of ER homeostasis and are hallmarks for ER stress responses (Little et al., 1994). The coordinated adaptive response is known as the unfolded protein response (UPR), and the pathological response is known as the ER stress response. The UPR signaling pathways act rapidly to mitigate the stressed state of the ER and enhance cell survival. However, if severe and prolonged
ER stress cannot be resolved, the signaling switches from a pro-survival to a pro-apoptotic ER stress response (Xu et al., 2005). Compelling evidence has suggested that C/EBP homologous protein (CHOP)/growth arrest and DNA damage-inducible protein 153 (GADD153) and caspase-12 in rodents (caspase-4 in humans) are activated and become involved in ER stress-induced cell apoptosis (Kim et al., 2008). As reported previously, PPARα protects HepG2 cells against H2O2-induced ER stress-mediates apoptosis through the down-regulation of CHOP (Tang et al., 2014). Additionally, activation of PPARα ameliorates hepatic insulin resistance to increased ER stress (Chan et al., 2013). However, a PPARα agonist has also been shown to induce apoptosis of triple-negative breast cancer cells via activation of the transcription factor NF-κB, which is connected with the ER stress response (Zhao et al., 2007). Thus, these studies have demonstrated that PPARα plays a complicated role in ER stress.

Although our studies have demonstrated that PPARα activation effectively protects mice from ALF, and severe ER stress promotes liver injury by inducing hepatocyte apoptosis in D-GalN/LPS treated mice (Jiao et al., 2014; Ren et al., 2015), the underlying mechanisms of the effects of PPARα and ER stress in vivo required further elucidation. Thus, this study sought to address the hypothesis that PPARα can protect mice from ALF by inhibiting ER stress-induced hepatocyte apoptosis. Indeed, we found that inhibition of ER stress enhanced the expression of PPARα, and PPARα activation attenuated ER stress-mediated hepatocyte apoptosis in the D-GalN/LPS-induced mouse model of ALF.
RESULTS

PPARα activation decreases hepatocyte apoptosis, thus protecting against ALF

We first evaluated whether PPARα activation could rescue liver injury by applying Wy-14643, a PPARα ligand activator. In the survival analysis (Fig. 1A), the mice in the D-GalN/LPS group began to die 6 hours after D-GalN/LPS administration, and the survival rate stabilized at 60% (6 of 10 mice) at 24 h; however, pretreatment with Wy-14643 before D-GalN/LPS administration reduced the mortality, and the survival rate was 90% (9 of 10 mice). With respect to liver damage, compared with the D-GalN/LPS administration group, the gross morphology of the liver appeared to be substantially better and the liver histopathological damages were ameliorated in the Wy-14643 treatment group (Fig. 1B); Liver function showed significantly lower alanine aminotransferase (ALT) and aspartic aminotransferase (AST) levels and lower total bilirubin (TBIL), alkaline phosphatase (ALP) and prothrombin time (PT) in the Wy-14643 pretreatment group compared with the D-GalN/LPS administration group (Fig. 1C, Supplementary figure and Supplementary table 2). To explore the potential protective mechanism of PPARα against ALF induced by D-GalN/LPS, we measured apoptotic cells in the three groups. As shown in Fig. 1D, in the D-GalN/LPS-treated group, a large number of TUNEL-positive cells were observed; however, the Wy-14643 pretreatment group displayed significantly fewer apoptotic hepatocytes. Moreover, consistently with the TUNEL data, the levels of cleaved caspase-3 (17 and 19 kDa) increased after D-GalN/LPS injection, but this increase was attenuated by Wy-14643 pretreatment (Fig. 1E). Thus, these results suggested that PPARα
activation significantly reduced apoptotic cells and thereby protected mice from ALF induced by D-GaIN/LPS.

**PPARα activation relieves ER stress in D-GaIN/LPS-induced ALF**

Our previous paper has shown that severe ER stress promotes liver injury in the D-GaIN/LPS-induced ALF mouse model (Ren et al., 2015). To examine the effects of PPARα on D-GaIN/LPS-induced ER stress in mice, we measured the levels of mRNA and protein for ER stress mediators. The expression of Grp78, Grp94 and CHOP, which are the classical ER stress markers, was increased significantly after D-GaIN/LPS administration but was significantly attenuated by pretreatment with Wy-14643 (Fig. 2A). These alterations were confirmed by western blot analyses (Fig. 2B). We also used siRNA to knock down the expression of PPARα in mice and found that, compared with D-GaIN/LPS-treatment, PPARα siRNA treatment further increased the levels of hepatocyte apoptosis (TUNEL) and promoted the cleavage of caspase-3 and the expression of CHOP in D-GaIN/LPS-treated ALF mice (Figure 2C, 2D). Furthermore, we further used siRNA to knockdown CHOP and analysis the hepatocyte apoptosis of liver. The results showed that, compared to the mice pretreated by PPARα siRNA, the intervention of CHOP siRNA decreased again the number of hepatocyte apoptosis (Fig. 2C). The results showed that PPARα activation suppressed ER stress during D-GaIN/LPS-induced ALF.

**Inhibition of ER stress increases the expression of PPARα in D-GaIN/LPS-induced ALF**

A small chemical chaperone, 4-phenylbutyric acid (4-PBA), has been shown to alleviate ER stress both *in vivo* and *in vitro* (Ozcan et al., 2006; Zode et al., 2011), and
inhibition of ER stress by 4-PBA protects mice from ALF induced by D-GaIN/LPS (Ren et al., 2015). Thus, we evaluated whether ER stress inhibition could promote the expression of PPARα in the context of ALF. The qRT-PCR and western blotting results showed that, compared with D-GalN/LPS treatment alone, pretreatment with 4-PBA promoted the expression of PPARα (Fig. 3A,B). Similar results were obtained by immunofluorescence staining of liver tissue. Moreover, our results also showed that the expression of PPARα was cytoplasmic rather than nuclear in the three groups (Fig. 3C). These results indicated that the expression of PPARα is promoted by 4-PBA pretreatment in D-GaIN/LPS-induced ALF.

Inhibition of ER stress protects mice from ALF through PPARα mechanisms

Next, we sought to confirm whether the inhibition of ER stress protects the liver from injury by inducing PPARα expression in mice. We used siRNA to knock down the expression of PPARα in mice. The specific inhibition of PPARα in the liver by siRNA in vivo was confirmed by the reduced levels of PPARα in mice (Fig. 4A). The results indicated that liver in mice receiving 4-PBA treatment suffered less liver injury and the hepatic protection was abolished by knockdown of PPARα, which was evidenced by the decreased survival rate (Fig. 4B), abnormal gross morphology and less preserved liver architecture as observed from histology (Fig. 4C) and the significantly higher levels of ALT, AST, TBIL and ALP (Fig. 4D, Supplementary figure and Supplementary table 2). Meanwhile, the knockdown of PPARα reversed the expression levels of Grp78, Grp94 and CHOP in 4-PBA-pretreatment ALF mice (Fig. 4E, F). Thus, these results demonstrated
that the mechanism of hepatoprotection by ER stress inhibition depends on PPARα activity.

**The expression profile of PPARα in the progression of ER stress-induced hepatocyte apoptosis in vitro**

Here, we further examined how PPARα is regulated in the progression of ER stress-induced primary hepatocyte apoptosis *in vitro*. The qRT-PCR and western blot results showed that the expression of PPARα was significantly up-regulated in the early stage of tunicamycin- (TM) or thapsigargin (TG)-induced ER stress and was significantly down-regulated in the later time points of TM or TG treatment compared with the control group (Fig. 5A-D). Moreover, there was a difference in responses at different doses of TM or TG, compared with the control group. The low dose of TM or TG markedly up-regulated PPARα expression, whereas the high dose of TM or TG reduced the expression of PPARα (Fig. 5E-H). Moreover, for the longer time and higher dose of TM or TG treatment, the cleavage of caspase-3 was increased (Fig. 5B,D,F,H). Therefore, these results showed that mild ER stress promotes the expression of PPARα, and severe ER stress reduces the expression of PPARα.

**The effect of PPARα regulation on ER stress-induced primary hepatocyte apoptosis in vitro**

PPARα had been shown to be differentially regulated in the progression of ER stress. Therefore, we further analyzed the impact of PPARα on the intrinsic potential of primary hepatocyte apoptosis triggered by ER stress *in vitro*. Under the conditions of mild ER stress, we used specific siRNA to knock down the expression of PPARα. TM or TG
treatment for 6 hours increased the release of lactate dehydrogenase (LDH) from the hepatocytes and decreased hepatocyte viability; down-regulation of PPARα by siRNA further increased the LDH levels from the hepatocytes and further decreased hepatocyte viability (Fig. 6A). To evaluate the role of CHOP in the ER stress-PPARα pathway, we used siRNA to knock down CHOP and analyzed the level of LDH release and MTT in the different groups. The results indicated that, compared with the combination of PPARα siRNA and TM or TG treatment, the silencing of CHOP with siRNA partially reversed the levels of LDH and cell viability (Fig. 6A). Western blot analysis revealed that PPARα siRNA increased the levels of CHOP and cleaved caspase-3 compared with TM or TG-treated cells (Fig. 6B). Under conditions of severe ER stress, we used Wy-14643 to activate PPARα. Compared with 24 hour treatment of hepatocytes with TM or TG, activation of PPARα by Wy-14643 significantly decreased the hepatocyte levels of LDH and increased hepatocyte viability (Fig. 6C). Western blot analysis also indicated that Wy-14643 decreased the levels of CHOP and cleaved caspase-3, as compared with TM or TG-treated cells (Fig. 6D). Therefore, the activation or expression of PPARα was a key point of balance between hepatocyte survival promoted by mild ER stress and hepatocyte apoptosis induced by severe ER stress.

The expression of CHOP and PPARα in the liver of ALF patients with HBV infection

To investigate whether CHOP and PPARα associate with the progression of ALF in patients with HBV infection, we quantified the expression of CHOP and PPARα in liver tissues of normal subjects, chronic hepatitis B (CHB) patients and HBV-related ALF patients. The qRT-PCR results revealed that CHOP gene expression increased significantly
in ALF patients compared to the normal subjects, but in the patients with CHB, no significant changes were observed. PPARα gene expression gradually decreased in the progression of CHB to ALF (Fig. 7A); similar results were observed for protein levels by western blot analysis (Fig. 7B). Interestingly, the immunofluorescence staining revealed that the expression level of CHOP was low in the hepatocytes in which PPARα was highly expressed (Fig. 7C). Thus, these results indicated that CHOP expression is up-regulated, and PPARα expression is decreased in patients with HBV-related ALF compared with normal livers.
DISCUSSION

In the present study, we demonstrated that PPARα activation significantly decreased hepatocellular apoptosis, thereby protecting mice from D-GalN/LPS-induced ALF. The protective mechanism of PPARα activation was that PPARα activation regulates ER stress and thus relieves liver injury caused by ALF in mice; moreover, PPARα could be a pivotal molecule that facilitates the transition from mild ER stress to progressively severe ER stress in ALF. Hence, the ER stress-PPARα pathway is necessary to the pathological mechanism of the ALF immune response cascade (depicted in Fig. 7D).

Acute liver failure (ALF) has a variety of etiologies including viral infection, acetaminophen damage, excessive alcohol, metabolic liver disease and uncertain causes. It is associated with massive hepatocellular death. The mode of hepatocyte death includes apoptosis or necrosis, but is still controversial. Traditionally, apoptosis or programmed cell death (PCD), is actively induced by specific signaling cascades, including the intrinsic and extrinsic apoptosis signaling pathways, and occurs in a highly controlled fashion. Necrosis is viewed as a largely unregulated consequence of physicochemical stress characterized by mitochondrial impairment, depletion of adenosine triphosphate (ATP), and subsequent failure of ATP-dependent ion pumps. Recent evidence has indicated that PCD can also trigger a specific form of necrosis, termed necroptosis (Heike et al., 2012, Tom et al., 2014). The regulated nature of multiple cell death modes not only affects our understanding of the underlying pathophysiology but also suggests the possibility of therapeutic treatment in diseases.

The first novel finding in this paper is that PPARα activation protects mice from liver
injury by inhibiting ER stress-induced hepatocyte apoptosis in ALF. Prolonged or severe ER stress triggers cell apoptosis. Several mediators of apoptosis are associated with ER stress-induced cell death. Some of the mediators are linked to the UPR sensors, but others are implicated in calcium and redox homeostasis. The transcription factor CHOP functions as the most well-characterized pro-apoptotic regulator. Previous studies have demonstrated that CHOP is significantly upregulated in GaIN/LPS induced ALF and is critical in mediating ER stress-induced apoptosis (Rao et al., 2015). Silencing of CHOP reduces hepatocyte apoptosis in alcohol induced liver disease (Ji et al., 2005; Tamaki et al., 2008). Our previous research has also shown that the expression levels of Grp78, Grp94 and CHOP are increased significantly in D-GaIN/LPS-induced ALF, demonstrating the critical role of ER stress-mediated hepatocyte apoptosis in the mechanisms of ALF (Chen et al., 2012). The studies have shown that PPARα plays a complex role in cell apoptosis. For example, PPARα shows duality in liver cancer: low amounts of PPARα activation increase cell apoptosis by changing the tumor microenvironment, and continued high levels of PPARα activation promote the growth of hepatoma carcinoma cells (Kimura et al., 2012). For normal cells, such as hepatocytes, vascular smooth muscle cells or kidney cells, PPARα activation suppresses apoptosis induced by various stimuli (Chung et al., 2012; Chen et al., 2013; Karakan et al., 2013). In the present study, we demonstrated that PPARα activation, through its agonist Wy-14643, down-regulated expression of Grp78, Grp94 and CHOP and reduced D-GaIN/LPS-induced ER stress-mediated cell apoptosis. Moreover, our results in vitro also indicated that knockdown of PPARα by siRNA or activation of PPARα by Wy-14643 promoted or inhibited ER stress-induced hepatocyte apoptosis,
respectively. Furthermore, inhibition of ER stress directly up-regulated the expression of PPARα in the ALF mouse model, and knockdown of PPARα reversed the protective effect of ER stress inhibition in the ALF mouse model. Together with the results reported here, these findings support a mechanism whereby severe ER stress promotes the progression of D-GaIN/LPS-induced ALF in mice by decreasing PPARα activation.

Another novel finding in this paper is that PPARα acts as a switch from mild ER stress to severe ER stress. ER stress and UPR have been linked to the pathophysiology of liver diseases. However, the UPR signaling pathways also play a crucial role in restoring ER homeostasis via PERK, IRE1, and ATF6. One set of effectors regulated by the UPR activates three adaptive signaling cascades to ameliorate ER stress. These adaptive mechanisms involve global attenuation of mRNA translation, which reduces the ER workload by blocking synthesis of new proteins; the upregulation of molecular chaperones, which expands the protein folding capacity of the ER; and the increase in ER-associated protein degradation (ERAD), which removes misfolded proteins from the ER. (Treglia et al., 2012). Under sustained or massive ER stress, the UPR switches from an adaptive program to a pro-apoptotic program. The CHOP protein is thought to be a critical mediator of ER stress-associated apoptosis (Kim et al., 2008). Therefore, the UPR activation elicits adaptive and pro-apoptotic effectors, and the UPR signaling serves as a binary switch between adaptation and death. What are the molecular mechanisms to govern this transition? Chan et al. have shown that JNK functions as a key factor that regulates β-cells fate (Chan et al., 2015). In this paper, our findings suggest that PPARα could be a pivotal molecule that facilitates the transition from mild ER stress-induced cell survival to
progressively severe ER stress-induced cell apoptosis. Our research has found that PPARα is expressed in normal hepatocytes and that mild ER stress upregulates the expression of PPARα, whereas severe ER stress downregulates the expression of PPARα. Knockdown of PPARα decreases the mild ER stress-promoted hepatocyte survival, whereas the activation of PPARα decreases the severe ER stress-induced hepatocyte apoptosis. Therefore, we believe that PPARα is a new mediator involved in the balance between adaptive and apoptotic factors regulated by the UPR.

In conclusion, we found that PPARα prevents ALF by suppressing ER stress-induced hepatocyte apoptosis. PPARα may be useful as a potential therapeutic agent to attenuate ALF. Further preclinical studies targeting PPARα agonists are warranted for the development of a clinically applicable treatment strategy to treat ALF.
MATERIALS AND METHODS

Animal experiments

Male C57BL/6 mice at the age of 8-12 weeks were purchased from the Capital Medical University (Beijing, China) and fed freely with a standard chow diet and water; they were housed under specific pathogen-free conditions for 1 week before the experiments. All animals received humane care according to the Capital Medical University Animal Care Committee guidelines.

The mice were intraperitoneally injected with D-GalN (700 mg/kg; Sigma, St. Louis, MO, USA) and LPS (10 μg/kg; InvivoGen, San Diego, CA, USA) to induce ALF or with saline in the control animals. The PPARα activator Wy-14643 (6 mg/kg; Sigma) was administered via injection into the tail vein 2 hours prior to D-GalN/LPS exposure. The downregulation of PPARα and CHOP were achieved by tail vein injection of specific siRNA (50 μM/kg; Jima, Suzhou). A chemical chaperone that relieves ER stress, 4-PBA (100 mg/kg; Sigma, St Luis, MO), was dissolved in PBS and administered intraperitoneally 6 hours prior to D-GalN/LPS exposure. The mice were sacrificed at 6 hours after D-GalN/LPS treatment, and liver and serum samples were collected for future analysis.

Human specimens.

Normal liver samples were collected from eight patients undergoing hepatic resection for liver transplantation. CHB samples were obtained from the livers of 12 patients undergoing liver puncture biopsy. ALF liver samples were obtained from the livers of 12 patients with HBV infection undergoing liver transplantation, which caused by acute
exacerbation of chronic hepatitis B. This study was conducted in compliance with the 1975 Declaration of Helsinki, and the study protocol was approved by the Medical Ethics Committee of the Beijing YouAn Hospital. Written informed consent was obtained from all patients or their families prior to enrollment. The clinical characteristics and details of the patients included in the study are shown in the supplementary material Table 1.

**Liver function tests and liver histological examination**

Liver injury was estimated by biochemical serum markers such as albumin (ALB), ALT, AST, TBIL, ALP and by coagulation index such as PT and by pathological examination. Blood biochemical indicators were measured by using a multi-parametric analyzer (AU 5400, Olympus, Japan), according to an automated procedure. PT was detected using fully Automatic Coagulometer (Ac.T 5diff AL, Beckman-Coulter Inc., Brea, CA, USA). Liver tissue was fixed with 10% neutral formaldehyde and then embedded in paraffin. The specimens were cut into 5 μm sections, which were then stained with hematoxylin and eosin (H&E) and observed under light microscopy.

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated from 50 mg of liver tissue with TRIzol reagent, following the manufacturer’s protocol. The RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Quantitative-PCR was performed using the DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). The reactions were set up in 20μl total volumes with 1x SuperMix (Platinum SYBR Green qPCR Kit; Invitrogen), cDNA (2 μl) and 0.5 μM of each primer. The PCR cycle was as follows: 50°C for 2 minutes and 95°C for 5 minutes,
followed by 50 cycles of 95℃ for 15 seconds and 60℃ for 30 seconds. The relative
mRNA levels were normalized to the level of hypoxanthine-guanine
phosphoribosyltransferase (HPRT) and calculated by using the $2^{-\Delta\Delta C_t}$ method. All samples
were run in duplicate to ensure amplification integrity.

**Western blot analyses**

Liver tissue samples were lysed in Radio Immunoprecipitation Assay (RIPA) buffer
containing phosphatase and protease inhibitors. After heat denaturation at 95℃ for 5
minutes, proteins in SDS-loading buffer were subjected to electrophoresis in an SDS-12%
polyacrylamide gel and subsequently transferred onto a PVDF membrane (Bio-Rad,
Hercules, CA, USA). Primary antibodies against PPARα (Abcam, Cambridge, MA, USA),
Grp78, Grp94, CHOP, caspase-3, cleaved caspase-3 and β-actin (Cell Signaling
Technology Inc., Santa Cruz, CA, USA) were used. The membranes were incubated with
primary antibodies (1:500-1:1000) in TBST with 5% skim milk at 4℃ overnight. The
membranes were washed with TBST three times and then were incubated with horseradish
peroxidase-conjugated secondary antibodies (1:2000) at room temperature for 1 hour. The
bands were visualized with SuperSignal West Pico chemiluminescent substrate (Thermo
Fisher Scientific, Rockford, IL, USA) and developed by exposure on an X-ray film.

**TUNEL assay**

Apoptosis in liver sections was detected by terminal deoxynucleotidyl
transferase-mediated dUTP nick-end labeling (TUNEL, red fluorescence) using the In Situ
Cell Death Detection Kit (Roche, Indianapolis, IN). Negative controls were prepared
through omission of the terminal transferase. Positive controls were generated by treatment
with DNase. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml; Shizebio, Shanghai, China) for 10 minutes. Images were obtained on a Nikon Eclipse E800 fluorescent microscope (Nikon Corp., Tokyo, Japan). After four fields were randomly selected from each section, 100 cells were successively counted for each field by an observer who did not identify the slides. The ratio of TUNEL-positive cell number to the total cell number is shown.

**Isolation and treatment of primary mouse hepatocytes**

The livers of 7-week old mice were perfused with collagenase-containing Hank’s solution, and viable hepatocytes were isolated by Percoll isodensity centrifugation as described (Klaunig et al., 1981). To study the effects of PPARα regulation on hepatocyte apoptosis induced by ER stress, the cells were treated with TM (10 μg/ml; Sigma) or TG (1 μg/ml; Sigma), which increases ER stress, and the indicated conditions including co-treatment with Wy-14643 (50μM), and/or PPARα siRNA (5 nM), and/or CHOP siRNA (5 nM). The MTT assay (Amersco, Solon, OH, USA) was used as a qualitative index of cell proliferation. Hepatocyte apoptosis was evaluated by western blotting for cleaved caspase-3 and by the LDH assay (Biochain Institute, Hayward, CA) of culture supernatants. The processing was conducted according to the manufacturer’s instructions.

**Immunofluorescence staining**

Paraffin sections were treated with xylene for 10 minutes three times. The sections were hydrated through a graded alcohol series and then rinsed three times with distilled water. After the sections were blocked for 20 minutes in 10% goat serum in PBS, they were incubated overnight at 4 °C with the PPARα-specific rabbit polyclonal antibody
(Abcam, Cambridge, MA, USA) and the CHOP-specific mouse monoclonal antibody (Cell Signaling Technology Inc., Santa Cruz, CA, USA). The slides were then incubated with Alexa Fluor® 488 goat anti-rabbit IgG or Alexa Fluor® 568 goat anti-mouse IgG (1:200; Invitrogen, Grand Island, NY, USA) for 45 minutes. After three washes with PBS, the nuclei were stained with DAPI (1 μg/ml; Shizebio, Shanghai, China) for 10 minutes. The images were examined on a Nikon Eclipse E800 fluorescent microscope (Nikon Corp., Tokyo, Japan).

**Statistical analyses**

The results are expressed as the means ± standard deviation (SD). Statistical analyses were performed using the unpaired Student’s t test or single-factor analysis of variance, and a value of P < 0.05 (two-tailed) was considered significant.
Competing interests

The authors declare no conflicts of interest.

Author contributions

C.Z. and Z.D. designed the experiments; L. Zhang and F.R. performed the experiments and wrote the manuscript; X.W. supervised the pathological observation; L. Zhang, F.R., X.Z., H.S. and L. Zhou prepared the samples and collected the data. L. Zhang, F.R., S.Z., Y.C., D.C. and L.L. performed statistical analyses. All authors have read and approved the submission of the manuscript.

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Fig. 1. Wy-14643 protects against D-GalN/LPS-induced liver injury and suppresses hepatocyte apoptosis. Male C57BL/6 mice were injected intraperitoneally with Wy-14643 (6 mg/kg) or vehicle (DMSO) 2 hours prior to D-GalN (700 mg/kg) and LPS (10 μg/kg) exposure (n=12/group). The control mice were pretreated with vehicle (DMSO) 2 hours before PBS injection (n=10). The mice were euthanized with chloral hydrate (1.0 g/kg) 6 hours after D-GalN/LPS treatment, and the liver and serum samples were collected for analysis. (A) The survival rate was analyzed in D-GalN/LPS-treated mice and Wy/D-GalN/LPS-treated mice up to 24 hours after D-GalN/LPS injection. (n = 10/group). (B) Representative livers and H&E staining of liver sections in the control mice, the D-GalN/LPS-treated mice, and the Wy/D-GalN/LPS-treated mice. (C) Serum levels of
ALT and AST from the different groups. (D) TUNEL staining images from the different groups. A representative experiment is shown. Original magnification 200x. (E) The levels of total caspase-3, cleaved caspase-3 and β-actin were measured by western blotting. A representative blot from two samples of every group is shown. Densitometry analysis of the protein levels was performed for each sample.
**Fig. 2.** Wy-14643 suppresses ER stress in D-GalN/LPS-induced ALF. Male C57BL/6 mice were injected with Wy-14643 (6 mg/kg) or DMSO 2 hours prior to D-GalN (700 mg/kg) and LPS (10 μg/kg) treatment (n=12/group). Mice were pretreated with PPARα siRNA (50 μM/kg) and/or CHOP siRNA (50 μM/kg) via tail vein injection 24 hours prior to D-GalN/LPS treatment (n=10/group). The control mice were injected with only PBS (n=10). The mice were euthanized 6 hours after D-GalN/LPS treatment, and the liver and serum samples were collected. (A) Relative hepatic mRNA expression levels of ER stress markers, including Grp78, Grp94, and CHOP were measured by qRT-PCR in the control mice, the D-GalN/LPS-treated mice, and the Wy/D-GalN/LPS-treated mice. (B) The
protein levels of Grp78, Grp94, CHOP and β-actin were measured by western blotting. A representative blot from two samples of every group is shown. Densitometry analysis of the proteins was performed for each sample. (C) TUNEL staining images from the control mice, the D-GalN/LPS-treated mice, the PPARα siRNA/D-GalN/LPS-treated mice, the PPARα siRNA/ control siRNA /D-GalN/LPS-treated mice and the PPARα siRNA/CHOP siRNA /D-GalN/LPS-treated mice. A representative experiment is shown. Original magnification 200x. (D) The levels of total caspase-3, cleaved caspase-3, CHOP and β-actin were measured by western blotting. A representative blot from two samples of every group is shown. Densitometry analysis of the protein levels was performed for each sample.
Fig. 3. Inhibition of the ER stress increases the expression of PPARα in D-GalN/LPS-induced ALF. Mice were pretreated with 4-PBA (100 mg/kg) or PBS by intraperitoneal injection 6 hours prior to the D-GaIN (700 mg/kg) /LPS (10μg/kg) treatment (n=14/group). The control mice were injected with only PBS (n=10). All mice were finally sacrificed with chloral hydrate (1.0 g/kg) 6 hours after D-GaIN/LPS injection.

(A) Relative hepatic PPARα mRNA expression was measured by qRT-PCR in the control mice, the D-GaIN/LPS-treated mice, and the 4-PBA/D-GaIN/LPS-treated mice. (B) The protein levels of PPARα and β-actin were measured by western blotting. A representative blot from two samples of every group is shown. Densitometry analysis of the proteins was
performed for each sample. (C) Immunofluorescence staining for PPARα (green) in liver tissues from the different groups. A representative experiment is shown. Original magnification 400x.
Fig. 4. 4-PBA protects against D-GaIN/LPS-induced ALF in mice by promoting PPARα activation. Mice were pretreated with PPARα siRNA (50 μM/kg) or control siRNA (50 μM/kg) via tail vein injection 24 hours prior to D-GalN (700 mg/kg)/LPS (10μg/kg) treatment and then injected with 4-PBA (100 mg/kg) or PBS 6 hours prior to D-GalN /LPS exposure (n=14/group). The control mice were injected with only PBS (n=10). The mice were euthanized 6 hours after D-GalN/LPS treatment, and the liver and serum samples were collected. (A) Protein levels of PPARα and β-actin were measured by western blotting in the control siRNA/D-GalN/LPS-treated mice and the PPARα siRNA/D-GalN/LPS-treated mice. A representative blot from two samples of every group.
is shown. Densitometry analysis of the proteins was performed for each sample. (B) The survival rate was analyzed in the D-GalN/LPS-treated mice, the 4-PBA/D-GalN/LPS-treated mice and the PPARα siRNA /4-PBA/D-GalN/LPS-treated mice up to 24 hours after D-GalN/LPS injection. (n=10/group). (C) Representative livers and H&E staining of liver sections from the control mice, the D-GalN/LPS-treated mice, the 4-PBA/D-GalN/LPS-treated mice, the control siRNA/4-PBA/D-GalN/LPS-treated mice, and the PPARα siRNA/4-PBA/D-GalN/LPS-treated mice. (D) Serum levels of ALT and AST from the different groups. (E) Relative hepatic mRNA expression levels of ER stress markers, including Grp78, Grp94, and CHOP were measured by qRT-PCR. (F) The protein levels of Grp78, Grp94, CHOP and β-actin were measured by western blotting. A representative blot from two samples of every group is shown. Densitometry analysis of the proteins was performed for each sample (#: p < 0.05).
Fig. 5. Expression of PPARα is increased by mild ER stress and decreased by severe ER stress. The primary hepatocytes were incubated with known ER stress inducers TM and TG for various times or at increasing doses. The primary hepatocytes were treated with only PBS as a control or 40 μg/ml TM or 1 μg/ml TG for 3, 6, 12 or 24 hours. Moreover, the primary hepatocytes were treated with increasing concentrations of TM (0, 2.5, 5, 10, 25, or 50 μg/ml) or TG (0, 0.25, 0.5, 1, 2.5, or 5 μg/ml) for 12 hours. (A, C, E, G) Relative PPARα mRNA expression was measured by qRT-PCR. (B, D, F, H) The protein levels of PPARα, CHOP, cleaved caspase-3 and β-actin were measured by western blotting. A representative blot from three independent experiments is shown. Densitometry analysis of the proteins was performed for each sample (compared with Control group, #: p < 0.05).
Fig. 6. PPARα can regulate ER stress-induced cell apoptosis *in vitro*. The primary hepatocytes were treated with only PBS as a control. (A, B) The primary hepatocytes were transfected with PPARα siRNA (5 nM), or control siRNA (5 nM), and/or CHOP siRNA (5 nM) for 24 hours, followed by TM (40 μg/ml) or TG (1 μg/ml) for 6 hours. Cell viability or apoptosis was measured by MTT assay or LDH activity assay, respectively, separately in different groups. The protein levels of PPARα, CHOP, cleaved caspase-3 and β-actin were measured by western blotting from the different groups. A representative blot from three independent experiments is shown. Densitometry analysis of the proteins was performed for each sample (#: p < 0.05). (C, D) The primary hepatocytes were incubated with
Wy-14643 (50 μM) or DMSO for 2 hours and then stimulated with TM (40 μg/ml) or TG (1 μg/ml) for 24 hours. Cell viability or apoptosis was measured by MTT assay or LDH activity assay, respectively, separately in different group. The protein levels of PPARα, CHOP, cleaved caspase-3 and β-actin were measured by western blotting from the different groups. A representative blot from three independent experiments is shown. Densitometry analysis of the proteins was performed for each sample (#: p < 0.05).
Fig. 7. PPARα is downregulated and CHOP is obviously increased in ALF patients with HBV infection. (A) Relative hepatic mRNA expression levels of PPARα and CHOP were measured by qRT-PCR in healthy controls (n=8), CHB patients (n=12) and ALF patients (n=12). (B) The protein levels of PPARα and CHOP were measured by western blotting. A representative blot from two samples of every group is shown. (C) Immunofluorescence staining for PPARα (green) and CHOP (red) in liver tissues from the different groups. A representative experiment is shown. Original magnification 400x. (D) In the progression of D-GalN/LPS-induced ALF in mice, mild ER stress is induced in the early phase of acute liver injury, which upregulates the expression of PPARα, but the
severe ER stress is induced in the late phase of ALF, which downregulates the expression of PPARα. In ALF, the decreased PPARα triggers CHOP activity, induces extensive hepatocyte apoptosis, and ultimately induces the development of ALF. Therefore, PPARα is a fulcrum in the regulation of ER stress-induced liver injury.