

Tauroursodeoxycholic acid attenuates endoplasmic reticulum stress and protects the liver from chronic intermittent hypoxia induced injury

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Abstract. Obstructive sleep apnea that characterized by chronic intermittent hypoxia (CIH) has been reported to associate with chronic liver injury. Tauroursodeoxycholic acid (TUDCA) exerts liver-protective effects in various liver diseases. The purpose of this study was to test the hypothesis that TUDCA could protect liver against CIH injury. C57BL/6 mice were subjected to intermittent hypoxia for eight weeks and applied with TUDCA by intraperitoneal injection. The effect of TUDCA on liver histological changes, liver function, oxidative stress, inflammatory response, hepatocyte apoptosis and endoplasmic reticulum (ER) stress were investigated. The results showed that administration of TUDCA attenuated liver pathological changes, reduced serum alanine aminotransferase and aspartate aminotransferase level, suppressed reactive oxygen species activity, decreased tumor necrosis factor- α and interleukin-1 β level and inhibited hepatocyte apoptosis induced by CIH. TUDCA also inhibited CIH-induced ER stress in liver as evidenced by decreased expression of ER chaperone 78 kDa glucose-related protein, unfolded protein response transducers and ER proapoptotic proteins. Altogether, the present study described a liver-protective effect of TUDCA in CIH mice model, and this effect seems at least partly through the inhibition of ER stress.

Introduction

Obstructive sleep apnea (OSA) is a common disorder occurs in 2-14% of the general adult population (1,2). In OSA, upper airway obstruction usually associated with recurrent hypoxemia interspersed with periods of reoxygenation and typically terminated by brief arousals, that result in chronic intermittent hypoxia (CIH) and sleep fragmentation (3). The major health burden reported in patients with OSA is the strong risk of cardiovascular diseases (4-7) and brain injury (8-10). There is also emerging evidence showing that OSA is associated with chronic liver injury, such as hepatic oxidative stress (11), inflammatory reactions (12), abnormal lipid metabolism (13), hepatocytes apoptosis (14) and liver function injury (15).

Accumulation studies have proved that CIH could induce endoplasmic reticulum (ER) stress (16-18), which is closely linked to oxidative stress, inflammation and apoptosis (19). ER stress has been reported to be implicated in a variety of liver diseases, including alcohol-induced liver injury, nonalcoholic fatty liver disease, hepatic insulin resistance, ischemia-reperfusion injury and other acute hepatotoxins (20-22). Tauroursodeoxycholic acid (TUDCA), a hydrophilic nontoxic bile acid, is widely known as an ER stress inhibitor (23,24). TUDCA could be synthesized in the hydrophilic conjugation pathway of ursodeoxycholic acid, which has been effectively used for improving clinical and biochemical conditions in several liver diseases without major adverse reactions (25-27). Treatment with TUDCA abolished thapsigargin induced ER stress and apoptosis of the human liver-derived cell line Huh7 (28). Moreover, administration of TUDCA suppressed carcinogen-induced ER stress-mediated hepatocytes apoptosis and hepatic inflammation in a mouse hepatocellular carcinoma model (25), suggesting that TUDCA exerts beneficial effects on ER stress-induced liver disease. However, whether TUDCA is protectively in CIH-induced liver injury has not been studied.

In the present study, C57BL/6 mice was pre-treated with TUDCA and exposed to CIH for eight weeks, the liver histopathology, activity of liver enzymes, oxidative stress, inflammatory factor, hepatocyte apoptosis and ER stress of

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liver were analyzed to investigate the effect of TUDCA on CIH-induced liver injury.

Materials and methods

Animal model of CIH and experimental groups. A total of 24 male 8-week-old C57BL/6 mice (18–22 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China) and randomly divided into four groups, with 6 mice in each group. These were the following: Negative control (NC), TUDCA, CIH and CIH+TUDCA groups. Animals in NC group were kept in normal air (21% O₂), while the CIH model was established according to Lai *et al.* (29) with minor modification: Mice were housed in a sealed cabin, the air was controlled by an automated nitrogen/air switch pump. The concentration of O₂ was gradually reduced to 7%, stabilized for 60 sec, then increased to a peak of 21%, maintained for 60 sec, this cycle was repeated over 8 h (from 8:30 to 16:30) during the animals' diurnal sleep period. Mice in CIH+TUDCA group were treated with TUDCA 100 mg/kg/day by intraperitoneal injection, start from 2 days before CIH until the end of the experiment. Mice in TUDCA group were intraperitoneal injected with 100 mg/kg/day at the same schedule but did not undergo CIH treatment. Eight weeks later, the mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg; Chengdu Xiya Chemical Technology Co., Ltd., Chengdu, China), eyeballs were removed, blood samples were collected, animals were sacrificed by exsanguination as previously reported (30), and the livers were harvested. The care and disposal procedures of animal were strictly subject to the provisions of the Animal Ethics Committee of China Medical University (Shenyang, China).

Biochemical assays. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined by commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) following the user's instructions. Liver tissues were homogenized, centrifuged and total liver lysate were collected. Reactive oxygen species (ROS) activity was measured by ROS assay kit (Nanjing Jiancheng Bioengineering Institute). The level of tumor necrosis factor α (TNF- α) and interleukin-1 β (IL-1 β) in liver were determined by ELISA kits purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China).

Histology analysis. Liver tissues was fixed with formalin, embedded in paraffin and cut into 5- μ m sections. After dehydrated with grade alcohols, the sections were stained with hematoxylin and eosin terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using a commercial kit (KeyGEN, Nanjing, China) according to the manufacturer's instruction. Briefly, the sections was incubated with 1% Triton X-100 for 8 min at room temperature, then, endogenous peroxidase activity was blocked by 3% H₂O₂. Followed incubation with Proteinase K solution, the sections were stained with TUNEL reaction mixture at 37°C for 60 min. Then TUNEL positive cells were labeled with streptavidin-horse radish peroxidase (HRP) and developed with 3,3'-diaminobenzidine. Finally, the sections were counterstained with hematoxylin.

Western blot analysis. The liver tissues were dissolved by radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China), total protein was isolated and the protein concentration was determined using a bicinchoninic acid Method-Protein Quantitative Reagent kit (Beyotime). After that, an equal quantity of protein was fractionated using 8, 10 or 13% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Bedford, MA, USA). Then, the membranes were blocked by 5% (w/v) fat-free milk and immunoblotted with primary antibodies (dilution and suppliers were listed in Table I) 4°C overnight. After washing, the membranes was incubated with HRP-conjugated secondary antibodies (goat anti-rabbit IgG, 1:5,000; goat anti-mouse IgG, 1:5,000; Beyotime Institute of Biotechnology) at 37°C for 45 min. Next, the specific protein bands was visualized using enhanced chemiluminescence reagent (Qihai Biotec, Shanghai, China) and the gray density was analyzed using Gel-Pro Analyzer software version 3.0 (Media Cybernetics, Inc., Silver Spring, MD, USA).

Data analysis. Data are expressed as the mean \pm standard deviation. Differences between two groups were analyzed by Student's t-test. $P < 0.05$ was used to indicated a statistically significant difference. Statistical analysis was performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA).

Results

TUDCA protected liver from CIH-induced pathological and function injury. As shown in Fig. 1A, histology analysis of the liver tissues revealed normal hepatic architecture with no obvious lobular or portal inflammation, steatosis or necrosis in NC and TUDCA group. In contrast, mice exposed to CIH showed liver injury with discernible swelled, disordered hepatocytes and infiltrated inflammatory cells, however, these pathological changes were evidently ameliorated by the treatment of TUDCA. In order to evaluate the effect of TUDCA on CIH-induced liver function injury, the serum levels of liver enzymes ALT, AST and ALP were determined. As shown in Fig. 1B, the levels of ALT and AST in CIH group was significantly elevated when compare to the NC group (71.96 \pm 18.64 vs. 27.38 \pm 6.36 U/l, $P < 0.01$; 213.06 \pm 56.24 vs. 75.18 \pm 18.97 U/l, $P < 0.01$), but these were significantly decreased in CIH+TUDCA group when compared to CIH group (38.41 \pm 8.64 vs. 71.96 \pm 18.64 U/l, $P < 0.01$; 109.39 \pm 30.26 vs. 213.06 \pm 56.24 U/l, $P < 0.01$). There were no significant changes in the serum ALP level in CIH or CIH+TUDCA groups. The above results indicate that TUDCA alleviated CIH-induced liver pathological changes and protected liver function.

TUDCA suppressed CIH-induced liver oxidative stress and inflammatory reactions. The activity of ROS in liver was detected to evaluate the role of TUDCA on CIH-induced liver oxidative stress (Fig. 2A). Exposed to CIH-induced a 1.8-fold increase in ROS activity in the liver when compared to the NC group, while treatment with TUDCA decreased by 30% of the ROS activity when compared to CIH group. We further measured the levels of proinflammatory factors in livers to detect the anti-inflammatory effect of TUDCA, as shown in Fig. 2B and C, in the CIH group, there was

Table I. Details of antibodies.

Antibodies	Dilution	Catalogue number	Supplier
Cleaved caspase-3	1:1,000	ab2302	Abcam (Cambridge, MA, USA)
Cleaved caspase-9	1:200	sc-22182	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
GRP78	1:400	BA4293	Wuhan Boster Biological Technology, Ltd. (Wuhan, China)
PERK	1:500	bs-2469R	Bioss Antibodies (Beijing, China)
ATF6/cleaved ATF-6	1:400	ab11909	Abcam
p-IRE-1	1:500	bs-16698R	Bioss Antibodies
IRE-1	1:500	bs-8680R	Bioss Antibodies
CHOP	1:1,000	ab11419	Abcam
Cleaved caspase-12	1:500	ab18766	Abcam
p-eIF2 α	1:1,000	ab4837	Abcam
eIF2 α	1:1,000	ab26197	Abcam
p-JNK	1:500	bs-1640R	Bioss Antibodies
JNK	1:500	bs-10562R	Bioss Antibodies

GRP78, 78 kDa glucose-related protein; PERK, eukaryotic translation initiation factor 2 α kinase 3; ATF, activating transcription factor; JNK, janus kinase; IRE, inositol-requiring enzyme; CHOP, C/EBP homologous protein; eIF2 α , eukaryotic translation initiation factor 2 subunit α .

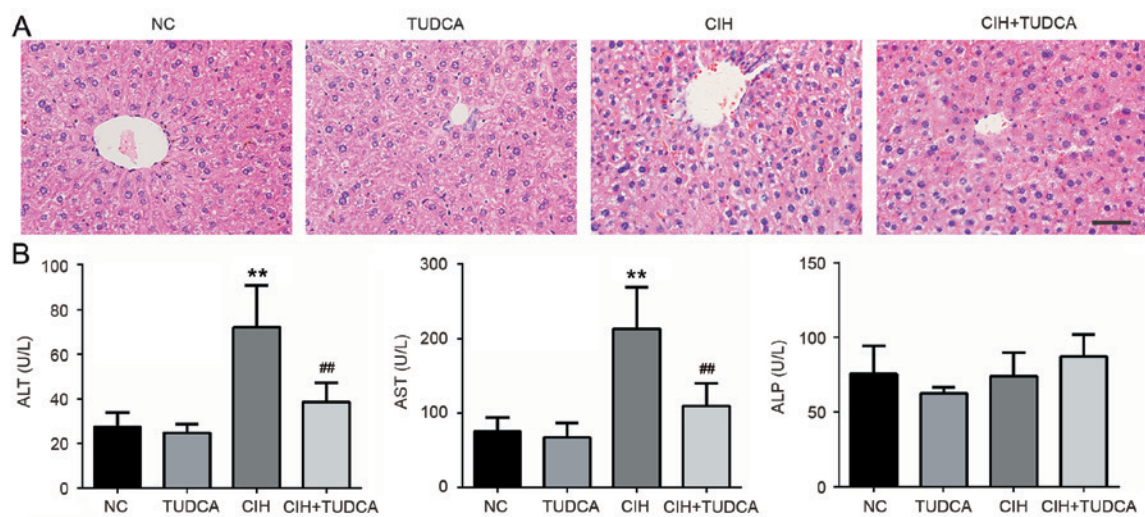


Figure 1. Liver histological changes and serum biochemical indicator levels. In total, 8 weeks after CIH, (A) the histological changes of liver tissues were analyzed by hematoxylin and eosin staining and (B) the biochemical indicators of liver function were detected by commercial kits. Scale bar, 50 μ m. ** P <0.01 vs. NC group, ## P <0.01 vs. CIH group. CIH, chronic intermittent hypoxia; NC, negative control; TUDCA, tauroursodeoxycholic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

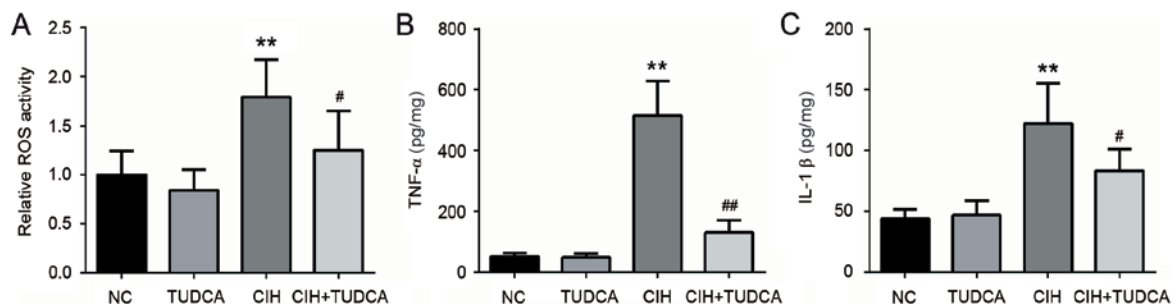


Figure 2. Liver ROS activity and proinflammatory cytokine level. Relative (A) ROS activity, (B) TNF- α and (C) IL-1 β level in liver tissues. ** P <0.01 vs. NC group, ## P <0.01, # P <0.05 vs. CIH group. CIH, chronic intermittent hypoxia; NC, negative control; TUDCA, tauroursodeoxycholic acid; ROS, reactive oxygen species; TNF, tumor necrosis factor; IL, interleukin.

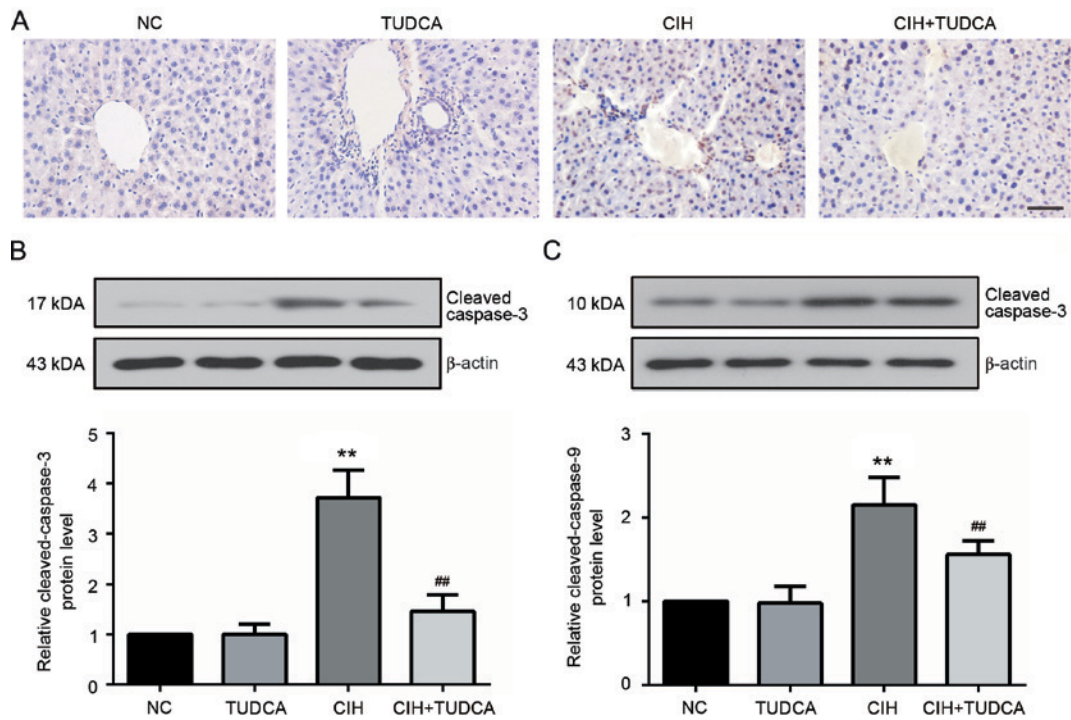


Figure 3. Apoptosis of hepatocytes. Apoptosis of hepatocytes were analyzed by (A) TUNEL, (B) the expression of cleaved caspase-3 and (C) cleaved caspase-9 in the liver were detected by western blotting. Scale bar, 50 μ m. ** P <0.01 vs. NC group, ## P <0.01 vs. CIH group. CIH, chronic intermittent hypoxia; NC, negative control; TUDCA, tauroursodeoxycholic acid.

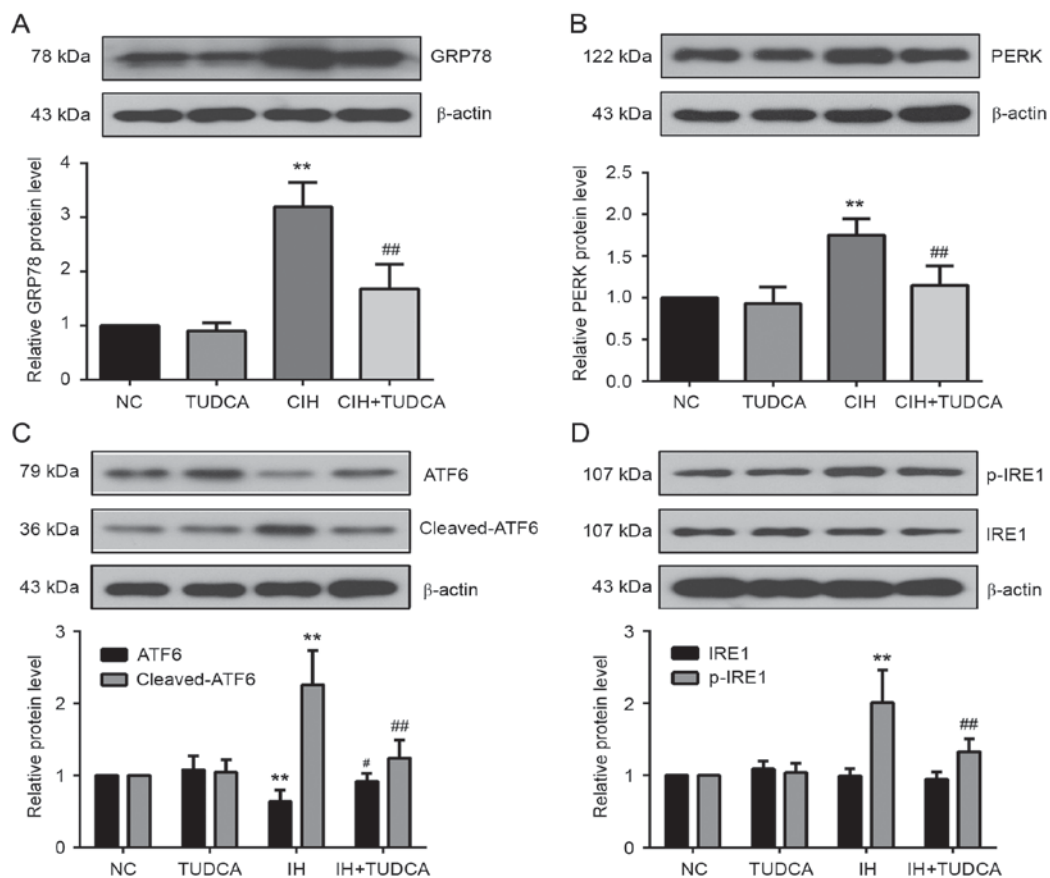


Figure 4. UPR activation in liver tissues. Expression of (A) GRP78 and three major transducers of UPR, (B) PERK, (C) ATF6 and (D) IRE1 were measured by western blotting. ** P <0.01 vs. NC group, ## P <0.01, # P <0.05 vs. CIH group. CIH, chronic intermittent hypoxia; NC, negative control; TUDCA, tauroursodeoxycholic acid; GRP78, 78 kDa glucose-related protein; PERK, eukaryotic translation initiation factor 2 α kinase 3; ATF, activating transcription factor; IRE, inositol-requiring enzyme; UPR, unfolded protein response.

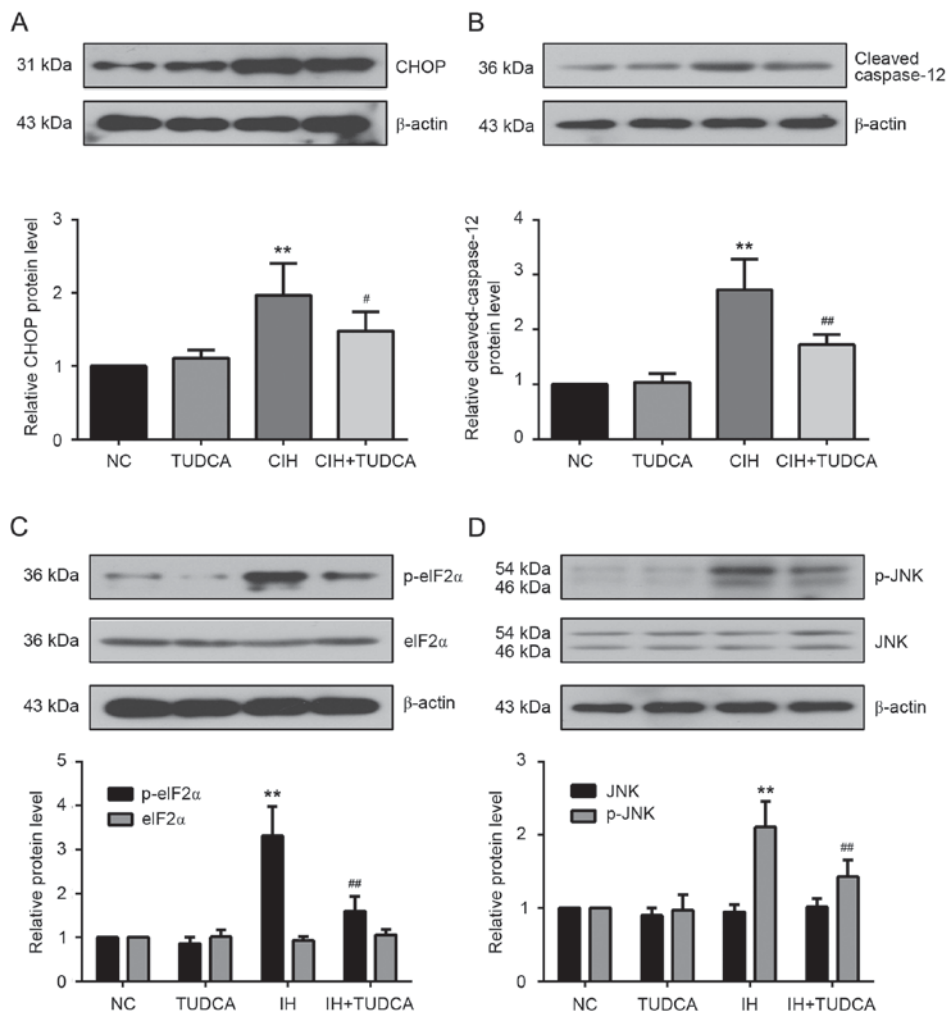


Figure 5. ER stress-associated apoptosis in the liver. Expression of ER proapoptotic molecules, (A) CHOP, (B) cleaved caspase-12, (C) p-eIF2 α and (D) p-JNK were detected by western blotting. **P<0.01 vs. NC group, ##P<0.01, #P<0.05 vs. CIH group. CIH, chronic intermittent hypoxia; NC, negative control; TUDCA, tauroursodeoxycholic acid; CHOP, C/EBP homologous protein; eIF2 α , eukaryotic translation initiation factor 2 subunit α ; JNK, janus kinase; ER, endoplasmic reticulum.

a significant elevation in both TNF- α (515.00 \pm 112.83 vs. 50.26 \pm 11.89 pg/mg, P<0.01) and IL-1 β (122.21 \pm 32.82 vs. 44.06 \pm 7.50 pg/mg, P<0.01) level when compared to the NC group. However, these levels were strongly decreased in the CIH+TUDCA group when compared to the CIH group (TNF- α , 130.75 \pm 38.80 vs. 515.00 \pm 112.83 pg/mg, P<0.01; IL-1 β , 83.60 \pm 17.58 vs. 122.21 \pm 32.82 pg/mg). Thus, treatment with TUDCA suppressed CIH-induced oxidative stress and inflammatory reactions in liver.

TUDCA protected liver from CIH-induced apoptosis. After eight weeks exposure to CIH, the presence of hepatocytes undergoing apoptosis was determined by TUNEL staining. As shown in Fig. 3A, TUNEL-positive apoptotic cells could obviously seen in the liver of CIH group, as expected, treatment with TUDCA distinctly reduced the apoptotic cell number. Additionally, the expression of two important biochemical markers of apoptosis, cleaved caspase-3 and cleaved caspase-9 were evaluated using western blot (Fig. 3B and C). Exposure to CIH significantly upregulated the protein levels of the two makers (P<0.01), while these changes were evidently restored by the treatment of TUDCA. These results

suggested that TUDCA protected the liver from CIH-induced apoptosis.

TUDCA suppressed CIH-induced ER stress and related proapoptotic pathway. To delineate the role of TUDCA on CIH-induced ER stress, western blot was used to evaluate the protein levels of the molecular chaperone 78 kDa glucose-related protein (GRP78) and three major transducers of unfolded protein response (UPR), eukaryotic translation initiation factor 2 α kinase 3 (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), that are known to be specifically induced by ER stress. As shown in Fig. 4, CIH increased the protein levels of GRP78, PERK, cleaved ATF6 and p-IRE1 in liver (P<0.01); however, these proteins were significantly reduced in the CIH+TUDCA group when compared to the CIH group (P<0.05). With the changed level of GRP78 and three UPR transducers, we further investigated the signal molecules of ER stress-associated apoptotic pathways (Fig. 5). There was an evident increase of C/EBP homologous protein (CHOP), cleaved caspase-12, p-eukaryotic translation initiation factor 2 subunit α (eIF2 α) and p-janus kinase (JNK) in CIH group when compared to the NC group

($P < 0.01$). However, these changes of protein levels were all significantly reversed by the treatment of TUDCA ($P < 0.05$). Therefore, the present findings demonstrated that TUDCA suppressed CIH-induced ER stress and ER stress-associated apoptosis of the liver.

Discussion

In the present study, mice were exposed to CIH and treatment with TUDCA, and it was observed that treatment with TUDCA attenuated liver pathological injury, improved liver function, suppressed ROS and proinflammatory factor production. Moreover, CIH-induced hepatocyte apoptosis and ER stress were also inhibited by the administration of TUDCA. To the best of our knowledge, this is the first report to demonstrate that TUDCA is protective in CIH-induced liver injury.

It is widely recognized that OSA and its associated CIH is implicated in the pathogenesis of various liver diseases (31-33). Different studies have proved over the previous decades that TUDCA could recovery liver function from variety of liver diseases (34-37); however, whether TUDCA has protective effects against CIH-induced liver injury remains unknown. The present results showed that intraperitoneal administration of TUDCA significantly protected liver from CIH-induced injury, as evidenced by improved liver pathologic changes and reduced serum ALT and AST levels. A double-blind randomized trial found that TUDCA at the daily dose of 750 mg could improve liver biochemical parameters and is safe to the patients of liver cirrhosis (38). Vandewynckel *et al* (25) have reported that administration of 300 mg/kg/day TUDCA significantly reduced ALT and AST levels of a diethylnitrosamine-induced mouse hepatocellular carcinoma model; however, the reduction in body weight and survival was also observed. Shi *et al* (39) found that intraperitoneal injection of 100 mg/kg/day TUDCA inhibited CIH-induced pulmonary cell apoptosis in a mouse model without any side effects. Thus, we also chose to administer 100 mg/kg/day TUDCA by intraperitoneal injection in the present experiment, and the results showed that this dosage of TUDCA could protect liver against CIH injury markedly.

Increasing evidence suggests that oxidative stress and inflammatory is involved in the pathophysiology of several chronic diseases associated with CIH. The high level of ROS generated by oxidative stress, could initiate the lipid peroxidation process and finally leading to DNA breakage and apoptosis (40). TNF- α and IL-1 β are proinflammatory cytokines that are known to be increased in livers treated with CIH (41). TUDCA has been shown to be a potent antioxidant and anti-inflammatory agent in cholestasis liver (42,43). The present data suggest that the upregulated TNF- α and IL-1 β levels and ROS activity induced by CIH were all markedly decreased by the treatment of TUDCA, further suggesting that TUDCA limits liver injury by reducing oxidative stress and inflammatory reactions.

Clinical data and animal models suggest that apoptosis is a pivotal mechanism of CIH-induced tissue or organ injuries (44-46). Hepatocellular apoptosis could be observed in the majority of types of human liver disease, including hepatic ischemia reperfusion injury, fibrosis, non-alcoholic

liver diseases, alcoholic liver disease and hepatocellular carcinoma (47). In particular, Zhen *et al* (14) reported that exposure to intermittent hypoxia significantly inhibited the proliferation and accelerated apoptosis of human liver cells. In the present study, the number of TUNEL-positive cells in the liver samples of the CIH group was obviously increased, along with the upregulation of two apoptosis markers, cleaved caspase-3 and cleaved caspase-9. In contrast, the increase in TUNEL-positive apoptotic cells and cleaved caspase-3 and cleaved caspase-9 expression was significantly attenuated by TUDCA treatment. These results are consistent with previous data showing that TUDCA could protect hepatocytes from bile-acid (48) or carcinogen (25) induced apoptosis.

ER stress may be activated by various disturbances, including CIH (18,49). In an ER stress state, the ER chaperone GRP78 was released and the transmembrane proteins, PERK, ATF6 and IRE-1 were activated to trigger three different UPR branches to protect ER from severe damage (50). When ER stress was excessive and prolonged, the downstream apoptotic proteins, CHOP, p-eIF2, p-JNK and cleaved caspase-12 were activated and turned to be executioners (51). In the present study, the activation of UPR and ER stress-associated apoptotic pathways induced by CIH were all significantly reduced by TUDCA treatment, suggesting that systemic administration of TUDCA could suppress CIH-induced ER stress in livers. In fact, TUDCA has been widely used as an ER stress inhibitor (23,24). Due to the relation between ER stress and oxidative stress and inflammatory response, we speculate that TUDCA may protect liver against CIH-induced injury at least partly by the suppression of ER stress. These were further supported by previous findings suggesting that TUDCA functions as a liver-protective agent by reducing ER stress (52).

Altogether, the present data demonstrated that TUDCA protected liver tissue from CIH-induced pathological injury, function impairment, oxidative stress, inflammatory response, hepatocyte apoptosis, as well as ER stress. These findings indicated a liver-protective effect of TUDCA in CIH-induced injury, and it is hypothesized that these effects are mediated at least partly through the inhibition of ER stress.

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