

Activated farnesoid X receptor attenuates apoptosis and liver injury in autoimmune hepatitis

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Abstract. Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease associated with interface hepatitis, the presence of autoantibodies, regulatory T-cell dysfunction and raised plasma liver enzyme levels. The present study assessed the hepatoprotective and antiapoptotic role of farnesoid X receptor (FXR) in AIH. A mouse model of AIH was induced by treatment with concanavalin A (ConA). The FXR agonist, chenodeoxycholic acid (CDCA), was administered to mice exhibiting ConA-induced liver injury and a normal control. Blood samples were obtained to detect the levels of aminotransferases and inflammatory cytokines. Liver specimens were collected, and hematoxylin-eosin staining was used for histopathological examination and detection. Apoptosis was evaluated using the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) method. The expression levels of apoptosis-associated genes and proteins were determined by reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. The results demonstrated that FXR was downregulated at the mRNA and protein level in the liver specimens of mice induced with ConA-induced hepatitis. Increased levels of aminotransferases and inflammatory cytokines, including interferon- γ , tumor necrosis factor- α , interleukin (IL)-4 and IL-2, were detected in ConA-treated mice. The mice pretreated with the FXR agonist, CDCA, were more resistant to ConA hepatitis, as indicated by reduced levels of alanine transaminase/aspartate aminotransferase and aminotransferases. The activation of FXR ameliorated hepatocyte apoptosis, as demonstrated by TUNEL analysis and downregulation of the Fas/Fas ligand, tumor necrosis factor-related apoptosis-inducing ligand and caspase-3. Taken together, FXR activation ameliorated

liver injury and suppressed inflammatory cytokines in ConA-induced hepatitis. FXR, therefore, exerts a protective role against ConA-induced apoptosis.

Introduction

Autoimmune hepatitis (AIH) is a chronic liver disorder characterized by increased transaminase levels, interface hepatitis, hypergammaglobulinemia and the production of autoantibodies (1-3). The immunological mechanism of AIH remains to be elucidated. A lack of response to treatment may lead to end-stage liver disease (4-6).

The farnesoid X receptor (FXR, or nuclear receptor subfamily 1, group H, member 4), a member of the ligand-activated nuclear receptor superfamily, is highly expressed in the liver, bile duct and gastrointestinal tract (7,8). FXR is a bile acid sensor essential in bile acid, cholesterol and triglyceride metabolism (9). Previous studies suggested that FXR is important in the regulation of immune signaling (10,11). FXR-deficient mice tended to lose the maintenance of homeostasis in their innate immunity responses (12). FXR^{-/-} mice exhibited an enhanced susceptibility to T cell-mediated hepatitis and developed immune-mediated liver damage (13). The activation of FXR reduced the expression of inflammatory cytokines and attenuated animal model colitis (13). The expression level of FXR is increased in Barrett's esophagus and an FXR agonist induced the immune response of Barrett's esophagus (14). Concanavalin A (ConA)-induced hepatitis is a well-established T cell-mediated murine model mimicking human AIH. It is characterized by liver injury, granulocyte infiltration and proinflammatory cytokine overproduction (15,16). In the present study, the underlying mechanism by which the expression of FXR affected ConA-induced AIH was assessed.

Materials and methods

Mice and treatments. Sixty female C57BL/6 mice (age, 8-12 weeks; weight, 20-24 g) were obtained from Shanghai Slac Laboratory Animal, Co., Ltd. (Shanghai, China) and were housed in the animal facility under controlled conditions of 22-24°C and 55% humidity, with a 12 h day/night cycle and free access to food and water. All experiments were approved by the Animal Experiment Committee of

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Sun Yat-sen University, following the Guide for the Care and Use of Laboratory Animals (17). To induce hepatitis, ConA (Sigma-Aldrich, St. Louis, MO, USA) was injected through the tail vein [20 mg/kg in 100 μ l pyrogen-free phosphate-buffered saline (PBS)]. Negative controls received the identical dose of PBS (18,19). To investigate the effect of FXR on ConA-induced hepatitis, experimental animals were injected with ConA in the presence or absence of the FXR agonist, chenodeoxycholic acid [CDCA; oral administration for 14 days prior to ConA injection and a chow diet with 1% (w/w) CDCA; Chemos GmbH, Regenstauf, Germany]. Blood samples were obtained after 0, 4, 8, 12, 16 and 24 h for the detection of serum markers. All serum markers were assessed in duplicate.

Measurement of alanine transaminase (ALT) and aspartate aminotransferase (AST). Serum ALT and AST levels were analyzed using an ALT/AST R1 and R2 kit (Sekisui Chemical Co., Ltd., Osaka, Japan) with an Automated Chemical Analyzer (7600; Hitachi, Tokyo, Japan), according to the manufacturer's instructions. The concentrations of ALT and AST were expressed as U/liter.

Cytokine detection. The cytokines, interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), interleukin (IL)-4 and IL-2 in the serum were analyzed by fluorimetry using a Bio-Plex Suspension Array system (cytokine assay), according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was extracted from mouse liver samples and first-strand DNA was synthesized using the following hexamer primers: Murine FRX forward, 5'-TCCAGGGTTTCAG-ACACTGG-3', and reverse, 5'-GCCGAACGAAGAAACATGG-3' (Guangzhou RiboBio Co., Ltd., Guangzhou, China). and SuperScript II Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). qPCR was performed using the SYBR[®] Green PCR Master mix (Applied Biosystems, Applied Biosystems GmbH, Darmstadt, Germany) in a thermocycler ABI PRISM TM7700. The PCR reaction conditions used for all assays were as follows: 95°C for 30 sec, followed by 40 cycles of amplification at 95°C for 5 sec, 59°C for 30 sec and 72°C for 30 sec. The primer sequence for FXR was designed, as previously described (20,21). RT-qPCR was used to detect the mRNA expression levels of FXR and the apoptotic markers, Fas, Fas ligand (FasL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), Bcl-2 and caspase-3. β -actin was used as an endogenous control.

Western blot analysis. The total protein was extracted from the mouse liver. An equal quantity of protein from each sample was separated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The membranes were then incubated with 5% non-fat milk and 0.1% Tris-buffered saline with Tween 20 blocking solution for 2 h at room temperature. Western blot analysis was performed using rabbit monoclonal anti-mouse FXR (cat. no. NBP2-16550; 1:1,000; Cell Signaling Technology, Frankfurt, Germany) and rabbit monoclonal anti-mouse caspase-3 (cat. no. 9664; 1:1,000;

Cell Signaling Technology) antibodies. To control the sample loading, the membranes were stripped with Western Blot stripping buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and re-probed with mouse monoclonal anti-mouse β -actin antibody (cat. no. A2228; 1:5,000; Sigma-Aldrich). The blots were incubated with enhanced chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA) and the signals were quantified using a Bio-Rad 2000 gel imaging system with Quantity One software (Bio-Rad Laboratories).

Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay. A TUNEL assay was performed using an *in situ* cell death detection kit (Roche, Penzberg, Germany) to observe the apoptotic cells. According to the manufacturer's instructions, paraffin-embedded liver sections were dewaxed by heating the sections to 600°C prior to washing them with xylene (Shanghai Baoman Biotechnology Co., Ltd., Shanghai, China), and rehydrated through a graded series of ethanol (100, 95, 90, 80 and 70%) and double distilled water. Following permeabilization and PBS washing, the sections were incubated in 50 μ l TUNEL reaction mixture for 1 h at 37°C. The slides were stained with 3,3'-diaminobenzidine following sample quality evaluation. The percentage of TUNEL-positive cells was quantified in randomly selected fields (at least 1,000 liver cells; 5 fields per slide). A total of 1% hematoxylin (Sigma-Aldrich) was used as a counterstain. The cells were visualized using an optical microscope (BX51; Olympus, Tokyo, Japan), and the index was calculated according to a previous report (22): No. apoptotic cells / (no. apoptotic cells + no. negative cells).

Histology and immunohistochemistry. The liver tissues were fixed with 4% paraformaldehyde (Shanghai Standard Co., Ltd., Shanghai, China) for 24 h at 40°C, and embedded in paraffin. The 5 μ m slices were subsequently stained with hematoxylin-eosin and evaluated for pathological changes under a BX51 optical microscope (Olympus). Immunohistochemical staining was performed on the 4 μ m paraffin-embedded sections. The slices were incubated with the previously mentioned primary antibody against FXR (1:100 dilution; Cell Signaling Technology). Following incubation with goat anti-rabbit immunoglobulin (Ig) G horseradish peroxidase-conjugated (cat. no. sc-2004; 1:1,000; Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG horseradish peroxidase-conjugated (cat. no. AP308P; 1:2,000; Sigma-Aldrich) secondary antibodies, the images were visualized using a BX51 optical microscope (Olympus). The positively stained tissue was counted using Image Pro Plus software, version 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Statistical analyses. The data were expressed as the mean \pm standard deviation, and all statistical analyses were performed using SPSS 19.0 software (SPSS, Inc., Armonk, NY, USA). The differences in measurements were compared using one-way analysis of variance to compare the mean of the results between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ConA induced hepatitis and the expression of FXR. Hepatic histopathology was assessed, as indicated above. Liver injury

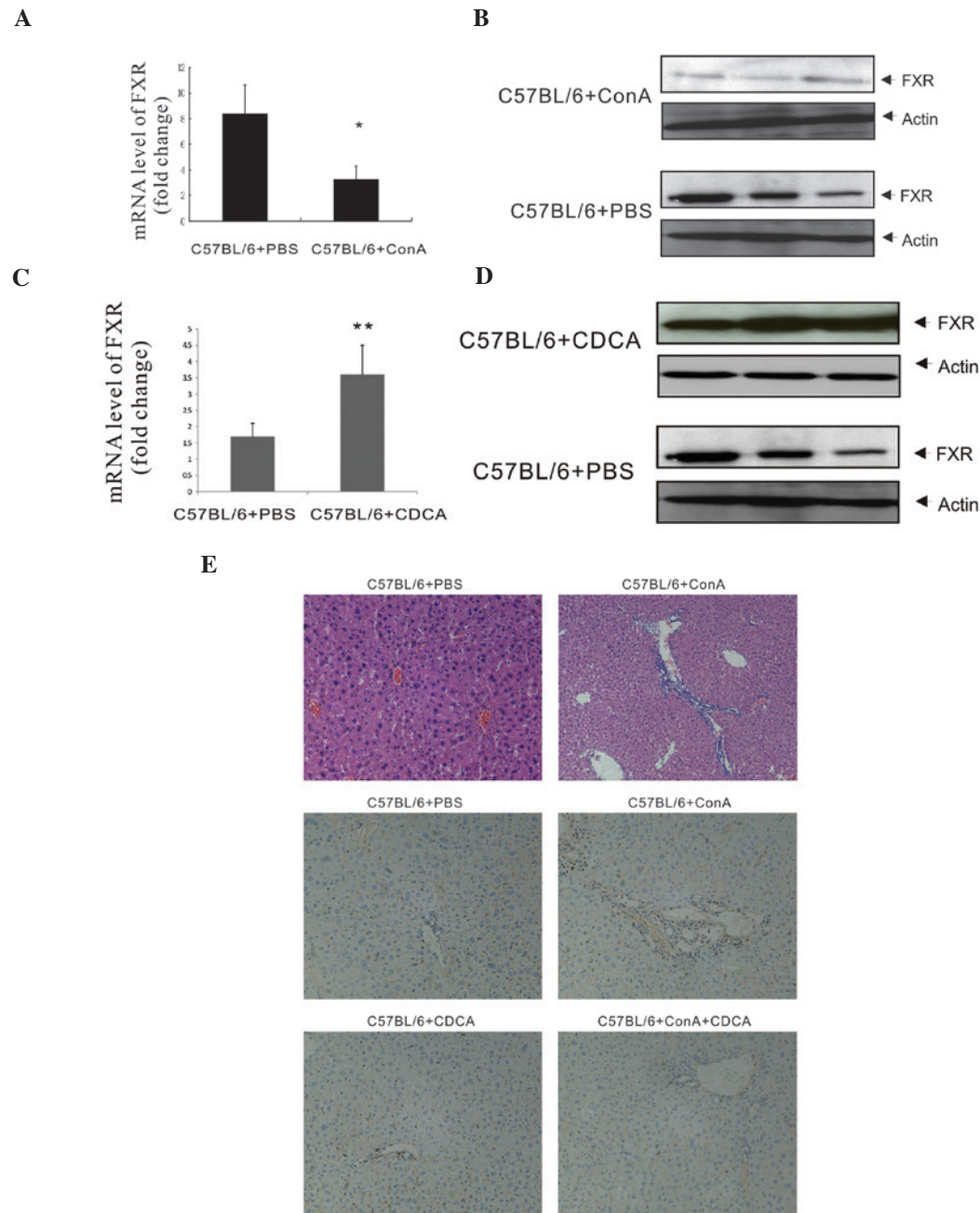


Figure 1. The expression of FXR in ConA-induced hepatitis. (A) The mRNA expression of FXR in ConA-induced hepatitis and normal controls was detected by reverse transcription-quantitative polymerase chain reaction. (B) The protein expression of FXR in ConA-induced hepatitis and normal controls was detected by western blotting. (C) The mRNA expression of FXR in CDCA-treated hepatitis and normal controls was detected by reverse transcription-quantitative polymerase chain reaction. (D) The protein expression of FXR in CDCA-treated hepatitis and normal controls was detected by western blotting. (E) Hematoxylin-eosin staining (original magnification x100) of paraffin-embedded liver sections of ConA-induced hepatitis and normal controls. Immunohistochemical staining (original magnification x200) for the detection of FXR. * $P < 0.05$, ConA-induced hepatitis vs. normal controls, and ** $P < 0.05$, C57BL/6+CDCA mice vs. C57BL/6+PBS. CDCA, chenodeoxycholic acid; ConA, concanavalin A; FXR, farnesoid receptor X; PBS, phosphate-buffered saline.

induced by ConA injection was characterized by hepatocellular necrosis, portal inflammation, mononuclear cell infiltration into the parenchyma and sinusoidal hyperemia. The liver structures of the untreated mice were normal. Mice, which were administered CDCA alone, developed no liver injury. As shown in Fig. 1, the expression of FXR was detected in the liver of all mice. CDCA treatment increased the expression of FXR and FXR was demonstrated to be present in smaller quantities in mice inflicted with ConA-induced hepatitis (Fig. 1).

Activation of FXR attenuates ConA-induced liver injury. A total of 15 C57BL/6 mice were fed a chow diet enriched with 1% (w/w) CDCA to stimulate the expression of FXR 1 week prior to ConA injection. Another 15 C57BL/6 mice were fed a normal diet. All the mice were subsequently injected with ConA (20 mg/kg) through their tail veins. The two control groups comprised mice on a CDCA diet without ConA injection, or mice on normal food injected with PBS. Two of the mice fed a normal diet died within 24 h of the ConA injection,

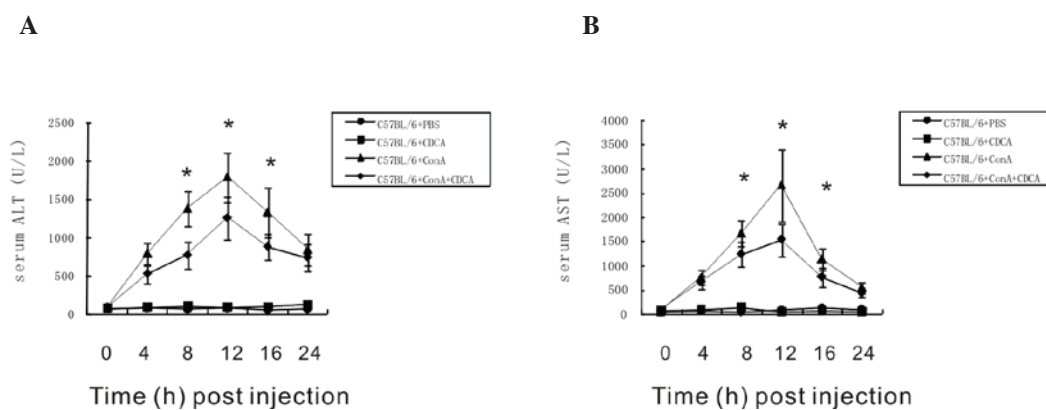


Figure 2. Activation of FXR decreased the serum levels of ALT and AST. (A) The serum levels of ALT and (B) AST were measured in the ConA-treated mice and normal controls in the presence or absence of CDCA at various time points. * $P<0.05$, ConA-induced hepatitis in the presence of CDCA, vs. ConA-induced hepatitis without CDCA. ALT, alanine transaminase; AST, aspartate aminotransferase; CDCA, chenodeoxycholic acid; ConA, concanavalin A; FXR, farnesoid receptor X; PBS, phosphate-buffered saline.

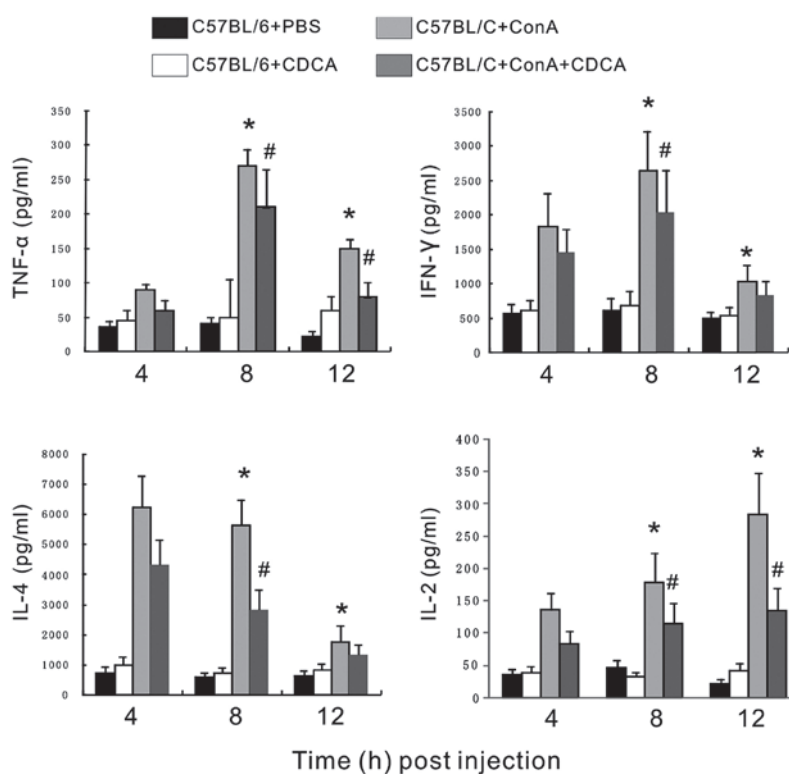


Figure 3. Activation of FXR suppressed inflammatory cytokines in ConA-induced hepatitis. Serum levels of IFN- γ , TNF- α , IL-4 and IL-2 were measured in ConA-treated mice and normal controls in the presence or absence of CDCA at different time points. * $P<0.05$, ConA-induced hepatitis in the presence of CDCA, vs. ConA-induced hepatitis without CDCA; # $P<0.05$, ConA induced hepatitis without CDCA, vs. control. CDCA, chenodeoxycholic acid; ConA, concanavalin A; FXR, farnesoid receptor X; IFN- γ , interferon- γ ; IL, interleukin; TNF- α , tumor necrosis factor- α ; PBS, phosphate-buffered saline.

and all mice on the CDCA diet survived. No controls died within the experimental period.

To assess ConA-induced liver injury between the two groups with or without FXR activation, the level of aminotransferase enzymes was measured. No appreciable level of liver enzyme was identified at the baseline among any of the experimental or control groups; however, increased ALT and AST activities were detected in the ConA-treated mice. The increase in aminotransferase activity peaked at ~12 h following ConA injection, prior to subsiding. The liver enzyme levels were increased in each group treated with ConA, although

they were more significantly increased in the group without CDCA treatment (Fig. 2; $P<0.05$).

Activation of FXR suppresses the inflammatory cytokines induced by ConA. To determine the effect of FXR on inflammatory responses, the serum cytokine levels of IFN- γ , TNF- α , IL-4 and IL-2 were measured. A significant increase ($P<0.05$) in the level of the inflammatory cytokines was identified following ConA injection, particularly in the mice fed a normal diet. The levels of these cytokines were reduced by CDCA treatment (Fig. 3).

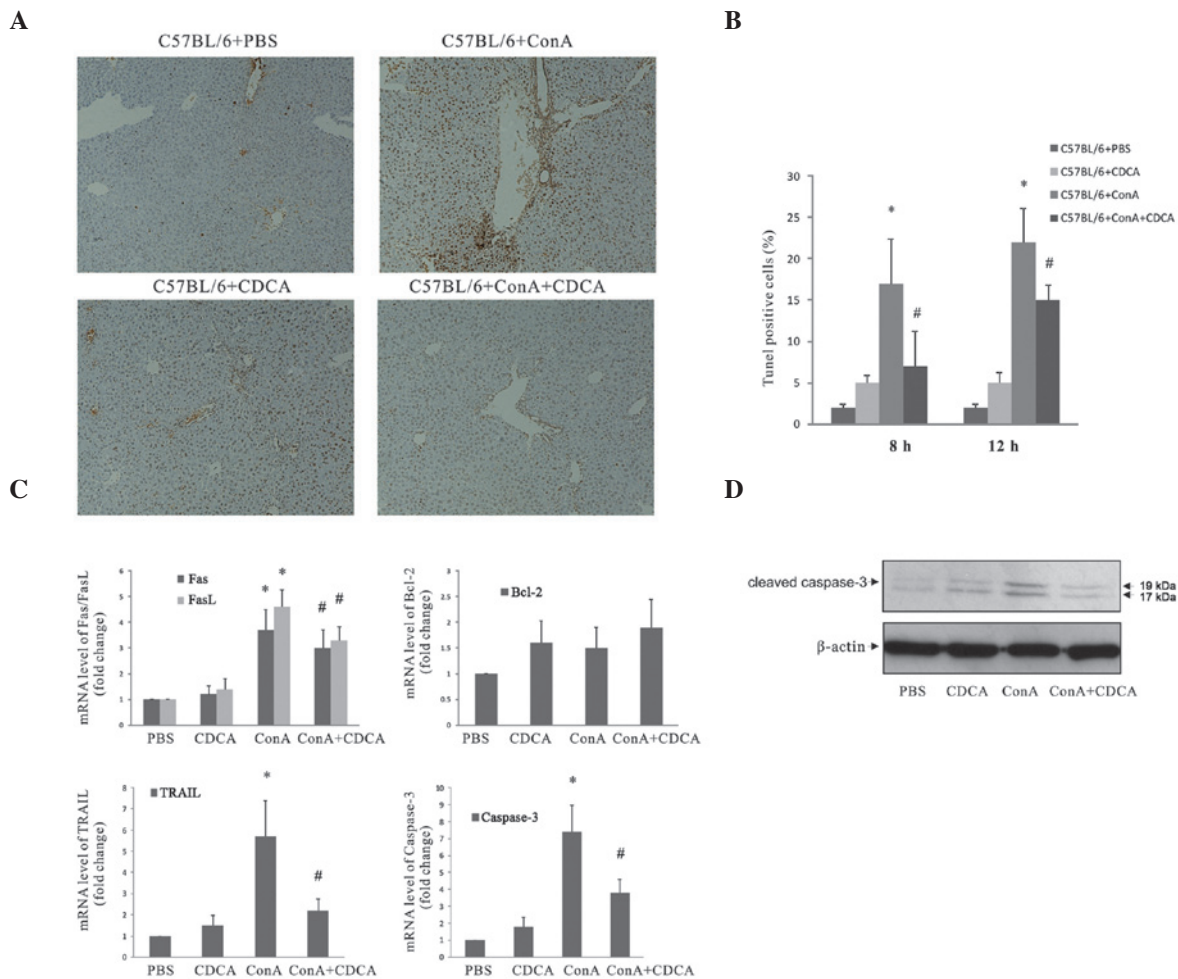


Figure 4. Activation of FXR reduced hepatic apoptosis. (A) Representative images of the TUNEL assay (original magnification x100). The cells with dark brown nuclei were considered TUNEL-positive. (B) The ratio of TUNEL-positive cells was calculated and the percentage of TUNEL-positive cells significantly decreased when FXR was activated upon treatment with CDCA. (C) The mRNA expression level of the apoptosis markers, Fas/FasL, Bcl-2, TRAIL and caspase-3 were determined by RT-qPCR. The data are expressed as the mean \pm standard deviation. (D) The protein expression of caspase-3 in ConA-induced hepatitis and normal controls was detected by western blotting. * $P < 0.05$, ConA-induced hepatitis in the presence of CDCA, vs. ConA-induced hepatitis without CDCA; # $P < 0.05$, ConA-induced hepatitis without CDCA, vs. control. CDCA, chenodeoxycholic acid; ConA, concanavalin A; FasL, Fas ligand; FXR, farnesoid receptor X; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; PBS, phosphate-buffered saline.

Activation of FXR suppresses ConA-induced apoptosis in the liver. Following ConA injection, hepatic apoptosis was less evident in the C57BL/6 mice pretreated with CDCA compared with the mice without CDCA. TUNEL assays were used to analyze liver sections (Fig. 4A). As shown in Fig. 4B, the TUNEL index was decreased in the livers pretreated with CDCA.

The mRNA expression levels of Fas/FasL, Bcl-2, TRAIL, Bcl-2 and caspase-3 were detected by RT-qPCR. The mRNA expression levels of Fas/FasL, TRAIL and caspase-3 were low in the control groups, although their expression levels were significantly increased following ConA injection ($P < 0.05$). Treatment with CDCA significantly downregulated the mRNA expression levels of Fas/FasL, TRAIL and caspase-3. No significant differences in the level of Bcl-2 protein were identified among the four groups (Fig. 4C). The level of caspase-3 protein detected by western blotting exhibited a similar trend, suggesting that FXR exerts an antiapoptotic role in ConA-induced hepatitis.

Discussion

AIH is a chronic inflammatory liver disease, the cause of which remains to be elucidated. AIH is characterized by increased levels of autoantibodies and immunoglobulins, hepatic inflammation and liver dysfunction (6,23,24). A subset of patients with AIH may gradually progress to liver failure.

FXR is a member of the ligand-activated nuclear receptor superfamily and bile acid sensor, highly expressed in the liver. Previous studies have associated FXR with inflammatory and immune-mediated diseases (25,26). In the present study, to detect any putative protective role of FXR *in vivo*, an experimental mouse model of AIH was established by treatment with ConA. The ConA-induced hepatitis, T cell-activated murine model system, which models several aspects of pathological lesions and mechanisms of pathogenesis in human AIH, has been used as an experimental model for AIH research (16,27). ConA-induced hepatitis typically demonstrates T-cell infiltration and portal inflammation, which results in cytokine

overproduction, hepatocyte apoptosis and necrosis (28-31). On establishing this model, it was identified that the mRNA and protein expression level of FXR was downregulated in ConA-induced hepatitis.

To gain an insight into the biological roles of FXR in the development of AIH, the CDCA FXR agonist, which increases the expression of FXR was used. FXR activation alleviated symptoms of portal inflammation, lipid deposition and focal necrosis. The activity of ALT and AST is widely used for the assessment of liver injury (32). In the present study, the levels of aminotransferases were subsequently measured, and it was demonstrated that FXR protected mice against the development of ConA-induced liver injury by significantly lowering the levels of ALT and AST, suggesting that administration of the FXR agonist may attenuate AIH.

ConA-induced hepatitis is, to a certain extent, regulated by the balance of protective and stimulatory cytokines (33). It is possible that the effects of FXR in counteracting ConA-induced hepatitis are mediated by modulating inflammatory cytokines and it is considered that proinflammatory cytokines are involved in ConA-induced liver damage. Inflammatory mediators, including TNF- α , IFN- γ , IL-4 and IL-2, are essential for the progression of hepatitis, and liver injury was attenuated by IL-6, IL-10 and IL-22. The present data suggested that FXR inhibited the production of inflammatory cytokines and was involved in the inflammatory responses as a negative regulator. TNF- α and IFN- γ are key to the inflammatory hepatocellular apoptosis induced by ConA (34). Mice deficient in tumor necrosis factor receptor 1 (TNFR) 1 and TNFR2 or TNF- α inhibitor are less likely to develop ConA-induced hepatitis (35). The present study demonstrated that the upregulation of TNF- α and IFN- γ occurred following ConA injection, which is compatible with previous reports (36). These changes were reversed in the experimental models by pretreatment with an FXR agonist. The present study suggested that FXR exerts a protective role at the early stage and continues to subsequently have an effect, and therefore may partly prevent hepatocyte necrosis at a late stage. IL-2 and IL-4 are also major cytokines involved in ConA-induced hepatitis (37,38). Similar to previous reports, the serum levels of IL-2 and IL-4 were markedly increased following ConA injection in the present study. These changes were less pronounced in the mice pretreated with CDCA and the results suggested a protective role of FXR against inflammatory liver injury.

Hepatocyte apoptosis is a critical event in the pathogenesis of ConA-induced liver damage. Several apoptosis-associated genes and enzymes are considered to exert important roles in the development of hepatitis (39). Previous reports have linked FXR to the regulation of ConA-induced hepatitis by postulating a mechanism, which focuses on the inhibition of natural killer T (NKT) cell activation (13). ConA-induced activation of NKT cells triggers the upregulation of FasL, which interacts with its death receptor, Fas, on the surface of hepatocytes, leading to apoptosis. It was observed that mice with mutations in the Fas-FasL apoptotic pathway were resistant to hepatitis (40-42). In addition, activated NKT cells are responsible for the increased level of IFN- γ , which is required by NKT cells for the expression of TRAIL, another effector for hepatocyte apoptosis (43). TRAIL is associated with the inflammatory status of the liver (44). Similarly, caspase

activation is also involved. Caspase-3, a member of the IL-1 β -converting enzyme family and downstream effector, is the key element promoting apoptosis (45). Based on the results of the present study, mice pretreated with the FXR agonist were less sensitive to ConA-induced apoptosis. The TUNEL assay confirmed the protective effect of FXR on apoptosis. FXR activation markedly downregulated Fas/FasL, TRAIL and caspase-3; however, no significant difference in the level of Bcl-2 was observed. These data suggested that the underlying antiapoptotic mechanisms of FXR may be associated with several signaling pathways, including Fas/FasL, TRAIL and caspase-3, and that Bcl-2 was not involved.

Taken together, the present study suggested that FXR was downregulated in ConA-induced hepatitis. FXR activation dramatically attenuated liver injury and suppressed inflammatory cytokines. The protective effect of FXR may depend on suppressing apoptosis through several signaling pathways, including Fas/FasL, TRAIL and caspase-3. The present data corroborate the essential role of FXR in the ConA model of liver injury, and will pave the way for further studies in AIH to target FXR for potential therapeutic alternatives.

Acknowledgements

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