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Research paper

Excessive bile acid activated NF-kappa B and promoted the development of alcoholic steatohepatitis in farnesoid X receptor deficient mice

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ABSTRACT

Chronic and excessive alcohol consumption can lead to alcoholic liver disease (ALD), which is characterized by a spectrum of liver disorders, including fatty liver, alcoholic steatohepatitis (ASH), fibrosis/cirrhosis and hepatocellular carcinoma (HCC). The mechanism of the progression from alcoholic steatosis to steatohepatitis and fibrosis is still not fully understood. As a nuclear receptor, farnesoid X receptor (FXR) plays a critical role in maintaining hepatic lipid and bile acid homeostasis. To clarify the role of FXR in the progression of steatohepatitis, we studied the effect of ethanol feeding on FXR-deficient mice. Wild-type and FXR-deficient mice were fed with *Lieber-DeCarli* ethanol liquid diet or an isocaloric control diet. We found that FXR-deficient mice fed with ethanol diet developed more severe liver injury and steatosis, even progressed to steatohepatitis and moderate fibrosis. Whereas, wild-type (WT) mice only developed mild level of steatosis, with rarely observed inflammatory foci and collagen accumulation. We also found that ethanol induced hepatic bile acid accumulation and NF-κB activation in FXR-deficient mice, which could be attenuated by ursodeoxycholic acid (UDCA). Thus, FXR deficient mice were more prone to develop alcoholic steatohepatitis and fibrosis upon ethanol diet feeding. Our results highlight the role of FXR in hepatoprotection during ALD development. Moreover, attenuating alcoholic liver cholestasis would be beneficial in preventing the progression of hepatic hepatitis in patients with ALD.

1. Introduction

Chronic consumption of excessive alcohol would lead to alcoholic liver disease (ALD), which is a major health problem worldwide [1,2]. ALD can be developed from simple fatty liver (steatosis) to alcoholic steatohepatitis (ASH), fibrosis/cirrhosis, even to end-stage hepatocellular carcinoma (HCC) [3].

In histological examination, one hallmark in patients with ALD is the elevated hepatic inflammation accompanied with steatosis. The association between heavy alcohol consumption and alcoholic steatohepatitis was supported by some clinical studies [4]. However, the mechanism of ALD progression is complex and not fully elucidated. To better understand the underlying molecular mechanisms in the development of hepatitis and liver fibrosis in ALD is of significance. In addition, to duplicate the pathogenic process of ALD in animal model is a good way to study the disease.

Abbreviations: FXR, farnesoid X receptor; *ALD*, alcoholic liver disease; *ASH*, alcoholic steatohepatitis; *ADH*, alcohol dehydrogenase; *ALDH*, aldehyde dehydrogenase; *CYP2E1*, cytochrome P450 2E1 enzyme; *FXR*$^{-/-}$, FXR deficient; *ALT*, alanine aminotransferase; TBARS, thiobarbituric acid reactive substances; *Tnf-α*, tumor necrosis factor alpha; *Mcp-1*, monocyte chemotactic protein-1; *Timp-1*, tissue inhibitor of metalloproteinase 1; *Col1a1*, collagen; type I, alpha 1; *OCT*, Optimal Cutting Temperature; *SREBP-1c*, sterol regulatory element-binding protein 1c; NF-κB, nuclear factor kappa B; UDCA, ursodeoxycholic Acid.

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The Farnesoid X Receptor (FXR; NR1H4), which is a member of the nuclear receptor superfamily and primarily expressed in the gastroenterological system, plays an essential role in maintaining bile acid and lipid homeostasis [5,6]. FXR regulates a series of genes involved in the processes of bile acid and lipid biosynthesis and transportation, including small heterodimer partner (SHP), cholesterol 7α-hydroxylase (CYP7A1), bile salt export pump (BSEP) and sterol regulatory element-binding protein 1c (SREBP-1c) [7–9]. In addition, FXR was reported to modulate inflammatory response in liver and intestine [10–12]. It was also demonstrated that FXR activation inhibited NF-κB activation in hepatocyte, whereas inflammation-induced NF-κB activation could reciprocally antagonize FXR activity [11].

In the previous study, we demonstrated that activation of FXR by a specific agonist WAY-362450 attenuates ethanol-induced hepatic liver injury and steatosis [13]. Meanwhile, we observed that $FXR^{-/-}$ mice developed severe liver injury and steatohepatitis, while wild-type mice developed steatosis but rare inflammation upon *Lieber-DeCarli* ethanol diet feeding. These results suggested that FXR deficiency might promote the progression of severe ALD. In the present work, we further studied the role and the underlying mechanism of FXR in the development of alcoholic steatohepatitis with $FXR^{-/-}$ mice.

2. Materials and methods

2.1. Materials

Protease inhibitors (including leupeptin, aprotinin and phenylmethylsulfonyl fluoride) were purchased from Sigma–Aldrich Inc (St. Louis, MO). Antibodies against CYP2E1, ADH1 (an isoform of alcohol dehydrogenase), ALDH2, NF-κB p65 and lamin B1 were from Abcam (Cambridge, MA). Anti-FXR antibody was from R&D system (Minneapolis, MN) and anti β-actin antibody was from Abmart Inc. (Shanghai, China). HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies were products of Jackson ImmunoResearch (West Grove, PA). Other chemical reagents were commercially available in China.

2.2. Animal experiments

FXR-knockout ($FXR^{-/-}$) mice on C57BL/6J background were obtained from the Jackson Laboratories (BarHarbor, ME). Wild-type C57BL/6J mice were from Shanghai Laboratory Animal Center (Chinese Academy of Sciences, Shanghai, China). All animal procedures were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals and with approval of the Animal Care and Use Committee of Fudan University. Eight to 10-week-old male mice were fed either a *Lieber-DeCarli* liquid diet (contain 5% ethanol, *v/v*) or an isocaloric control diet (Bio-Serv, Frenchtown, NJ) for 4 weeks as previously described [14,15]. There was no significant difference in food intake and weight gain among the different groups of mice (data not shown). At sacrifice, animals were anesthetized with sodium pentobarbital (75 mg/kg, IP). Blood was collected from the *vena cava* just prior to sacrifice by exsanguination and plasma was stored at −80 °C until further analysis. Liver tissues were collected. Portions were either frozen immediately in liquid nitrogen and stored at −80 °C, fixed in 10% neutral buffered formalin, or frozen-fixed in OCT mounting media (Tissue Tek, Hatfield, PA).

2.3. Serum and tissue biochemical assays

Serum alanine aminotransferase (ALT) was determined using a kinetic method (DiaSys Diagnostic Systems, Holzheim, Germany). Hepatic triglyceride and bile acid was extracted as previously described [13]. Triglyceride was measured according to manufacture's instruction (Biovision Inc., Mountain View, CA). Bile acid was determined using a kit from DiaSys Diagnostic Systems. Liver thiobarbituric acid reactive substances (TBARS) determination was measured as described previously [15].

2.4. Histopathological examination

In all experimental groups, Formalin-fixed and paraffin embedded liver tissues were cut at 5 μm thick, followed by staining wih hematoxylin and eosin (H&E), *Picro* Sirius Red, and *Masson* Trichrome according to previous report [10,16]. Frozen OCT-embedded liver tissues were cut at 10 μm thick and stained with Oil red O (Sigma, St. Louis, MO) to estimate the degree of hepatic steatosis [13].

2.5. Total RNA isolation and quantitative realtime-PCR assay

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. First strand cDNA was synthesized using CMV reverse transcriptase (Takara Biotech, Dalian, China) from DNase I treated RNA. Quantitative PCR was performed to determine target mRNA level using SYBR Green I premix (Takara biotech) on a StepOnePlus Realtime PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction. Primers for realtime PCR assays were provided in Supplementary Table 1. Relative mRNA levels of target genes were expressed using the $2^{-\Delta\Delta Ct}$ method with normalization to β-Actin mRNA.

2.6. Tissue lysate preparation and western blotting assay

Frozen liver tissues (50–100 mg) was homogenized in 1 ml RIPA lysis buffer (150 mM NaCl, 1% NP- 40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris–HCl, 1 mM aprotinin, 1 mM PMSF and 10 μg/ml leupeptin). Western blotting assays were performed as described previously [13]. Briefly, equivalent amount of lysates were separated in an 8% SDS polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) membranes, which were then probed with primary antibodies and HRP-conjugated secondary antibodies. Protein band was visualized using the enhanced chemiluminescent (ECL) assay kit (Tiangen Biotech, Beijing, China). Beta-actin was used as the endogenous loading control. The nuclear and cytoplasmic extracts of mouse liver were prepared as reported previously [17]. The NF-κB activity was measured by detecting nuclear NF-κB p65 level in nuclear extracts as described [11,18].

2.7. Statistics

Data are shown as means ± SEM. Statistical analysis was determined by two-tailed Student's t test using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. FXR deficient mice fed Lieber-DeCarli diet developed more severe liver injury and steatosis

We fed FXR-deficient mice with *Lieber-DeCarli* ethanol diet to determine the role of FXR in the development of ALD. As shown in Fig. 1A–B, $FXR^{-/-}$ mice exhibited higher levels of serum ALT and hepatic triglyceride upon ethanol feeding, when compared the condition with wild-type mice. Oil red O staining also confirmed