Research Article

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Insufficient bile acid signaling impairs liver repair in *CYP27*$^{-/-}$ mice

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Background & Aims: Previous studies indicate that bile acids (BAs) promote normal liver regeneration and repair after injury. However, the impact of insufficient BA signaling, which is observed in patients with BA sequestrant medication or cerebrotendinous xanthomatosis (CTX) disease, on liver injury is still unknown. Our aim is to determine the outcomes of reduced BA levels upon liver injury.

Methods: Seventy percent partial hepatectomy (PH) and carbon tetrachloride (CCl_4) treatment were performed using *CYP27*$^{-/-}$ mice, a genetic animal model with low BA levels. The liver repair of *CYP27*$^{-/-}$ mice after the treatments was characterized by histological staining, chemical analysis, and quantitative real-time PCR.

Results: *CYP27*$^{-/-}$ mice exhibited enhanced CCl_4-induce liver injury, and defective liver regeneration and prolonged steatosis after 70% PH. Due to the insufficient BA signaling, farnesoid X receptor (FXR) activities were significantly reduced in *CYP27*$^{-/-}$ livers after 70% PH. Activation of FXR by either 0.2% cholic acid feeding or oral infusion of an FXR agonist greatly promoted liver regeneration in *CYP27*$^{-/-}$ mice.

Conclusions: Normal physiological levels of BAs are required for liver repair. Patients with BA sequestrant medications or CTX disease due to *CYP27* gene mutations may have an increased risk of liver failure, and treatment with FXR ligands can promote liver regeneration of patients with low BA levels.

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Abbreviations: BA, bile acid; CTX, cerebrotendinous xanthomatosis; PH, partial hepatectomy; CCl_4, carbon tetrachloride; FXR, farnesoid X receptor; CYP27, sterol 27-hydroxylase; CYP7A1, cholesterol-7α-hydroxylase; ALT, alanine aminotransferase; CA, cholic acid; FoxM1B, Forkhead Box M1B; CAR, constitutive androstane receptor; PXR, pregnane X receptor.

Introduction

It is well known that abnormally high bile acid (BA) levels result in cholestasis and liver toxicity [1,2]. However, the potential impact of low BA levels on liver injury is unknown. We previously showed that BAs promoted liver regeneration by activating the farnesoid X receptor (FXR) [3], and loss of FXR leads to deficient liver regeneration and increases the susceptibility to toxin-induced liver injury [3,4]. Nevertheless, it is unclear whether a reduced BA flow will impair liver regeneration and increase the risk of liver injury.

The low BA flow is observed in patients with cerebrotendinous xanthomatosis (CTX), an autosomal recessive disease [5]. CTX patients exhibit tendon xanthomas, juvenile cataracts, progressive neurological dysfunction, and elevated plasma cholestanol levels. These pathological hallmarks were attributed to the heterogeneous mutations in the gene encoding the sterol 27-hydroxylase (*CYP27*) [6,7]. BAs are converted from cholesterol through two pathways in the liver. The classical pathway is initiated by cholesterol-7α-hydroxylase (CYP7A1) [8,9], while the alternative pathway is initiated by CYP27 [10,11]. The complete synthesis of BAs requires the alternative pathway mediated by CYP27, which catalyzes degradation of the steroid side chain. In CTX patients, dysfunctional CYP27 leads to cholestanol accumulation, and thus causes the neurodegenerative pathogenesis. CTX has been identified in a number of populations, particularly in Japanese, Sephardic Jewish, and Italian populations [12]. In the United States, the incidence was regarded to be rare. However, recent studies suggested that the prevalence of this disease might be greater than previously recognized [13]. Only a few studies were conducted to demonstrate that CTX patients were more susceptible to hepatitis in infancy [14,15], but the potential impact of low BA flow on liver injury is not known.

An animal model with deletion of CYP27 has been generated [11]. *CYP27*$^{-/-}$ mice displayed low BA levels but normal cholesterol homeostasis and vitamin D metabolism. Due to compensatory activation of CYP3A4, *CYP27*$^{-/-}$ mice do not have neurological dysfunction or tendon xanthomas [16]. *CYP27*$^{-/-}$ mice thus provided a unique animal model to determine the impacts of reduced BA levels on liver injury in CTX patients. Additionally, the animal model is useful to evaluate whether the intake of BA sequestrants that are used to lower cholesterol levels would increase the risk of liver injury.

In this study, we performed CCl_4 treatment and 70% PH in *CYP27*$^{-/-}$ mice to determine the impact of deficient BA signaling after liver injury. Our results demonstrated an essential role of physiological BA levels in liver regeneration, and thus provide insights into the risk of liver injury for CTX patients and those who take BA sequestrants.

Materials and methods

Animals

CYP27$^{+/+}$, *CYP27*$^{+/-}$, and *CYP27*$^{-/-}$ mice with C57BL/6 background were maintained in a pathogen-free animal facility under a standard 12:12 h light:dark cycle. Mice were fed with the standard rodent chow and water *ad libitum*. 70% PH was performed as previously described [3,17]. Px20350 (20 mg/kg) or vehicle (10% HPBCD (Sigma) in 500 mM phosphate pH 7.0) alone was treated to mice by oral garage twice a day for 2 days. Liver remnants were weighed after removal of necrotic stumps and sutures. All procedures followed the National Institute of Health guidelines for the care and use of laboratory animals.

Liver histology

Livers were fixed in 4% PBS-buffered formalin, dehydrated and embedded in paraffin, sectioned at 5 μm, and processed for H&E, BrdU and TUNEL staining, respectively. For BrdU staining, mice were injected intraperitoneally with the BrdU solution (100 mg/kg body weight) 2 h before being sacrificed. Liver sections were prepared and stained using a BrdU staining kit or a TUNEL staining kit (Roche Applied Science) according to the manufacturer's instructions. Hepatocyte mitotic figures were presented as fractions of the total number of hepatocytes examined. Steatosis in the livers was graded as previously described [18]. Oil-Red O staining was performed on cryosections of snap-frozen livers.

RNA preparation and reverse transcription

First-strand cDNA was synthesized from total RNAs using MMLV reverse transcriptase (Invitrogen). mRNAs were quantified by Quantitative Real-time PCR using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems). The primers were provided in supporting documents. The quantity of mRNA was normalized with an internal standard mouse acidic ribosomal phosphoprotein P0 m36b4.

Electromobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP)

EMSA was performed according to a previous publication [4]. Briefly, snap-frozen livers were homogenized in a hypotonic buffer and then the insoluble pellets were placed in a hypertonic buffer, lysed and centrifuged. The supernatant was collected as nuclear lysates. Liver nuclear extracts (6 μg), poly-(dI–dC) (3 μg), and a ^{32}P-radiolabeled probe containing IR-0 element, which was previously described by Chen *et al.* [19], were co-incubated for 30 min at room temperature and then subjected to 6% non-denaturing polyacrylamide gel electrophoresis. The gel was dried on chromatography paper (Whatman) and exposed overnight at −80 °C using BioMax MS film (Kodak) and an intensify screen. The ChIP assay on the FoxM1B IR-0 element was performed as described previously [19] with an anti-FXR antibody and normal rabbit IgG control from Santa Cruz Biotechnology using a kit from Upstate according to the manufacturer's protocol. The genomic DNA fragments precipitated by the antibodies were analyzed with an ABI-7300 Real-Time PCR System and Sybgreen PCR Mastermix, and further confirmed by a semi-quantitative regular PCR.

Analysis of BAs, alanine aminotransferase (ALT), triglyceride (TRIG) and cholesterol (CHOL)

Serum and liver BAs were measured according to the previous descriptions [3]. Serum ALT was measured at the City of Hope Helford Research Hospital. For hepatic TRIG measurement, frozen livers were homogenized in a buffer containing 50 mM Tris–HCl, 0.05% SDS, and 1 mM EDTA. The liver lysates were diluted with TBS and further analyzed by the City of Hope Helford Research Hospital. The hepatic CHOL levels in the same lysates were assayed with a cholesterol assay kit from Cayman Chemical Company.

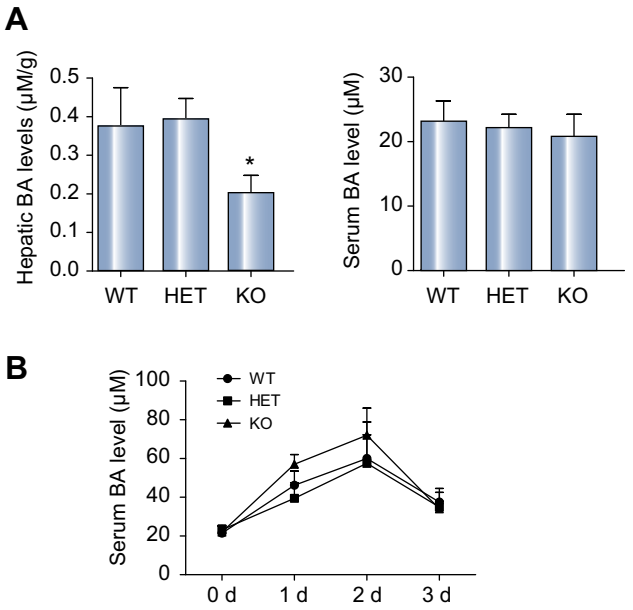

Fig. 1. Bile acid levels in *CYP27*$^{-/-}$ mice. (A) Hepatic and serum BA levels in *CYP27*$^{+/+}$ (WT), *CYP27*$^{+/-}$ (HET), and *CYP27*$^{-/-}$ (KO) mice. (B) Serum BA levels on different days after CCl_4 treatment. *KO *vs.* WT, $p < 0.05$.

Statistical analysis

All the data were reported as mean ± SD (standard deviation). Non-paired two-tailed Student's *t* test was used to determine the significance of differences. The *p* value and the statistical method were indicated individually for figures.

Results

CYP27$^{-/-}$ *mice displayed enhanced* CCl_4*-induced liver injury*

Though *CYP27*$^{-/-}$ mice were reported to have reduced BA pools, hepatic and serum BA levels of these mice were not evaluated in the previous studies [11]. We measured hepatic BA levels in *CYP27*$^{-/-}$ males and found that it was 50% of those in *CYP27*$^{+/+}$ and *CYP27*$^{+/-}$ males (Fig. 1A). However, serum BA levels were not different among the 3 genotypes (Fig. 1A), indicating that the reduction of BAs was mainly in gastrointestinal tract.

Rodents treated with i.p. injection of CCl_4 are widely used to study the mechanisms of hepatic injury. CCl_4 causes liver damages characterized by centrilobular necrosis and subsequent hepatic fibrosis [20,21]. Administration of CCl_4 to mice also stimulates cholestasis, but *CYP27*$^{-/-}$ mice exhibited no difference with *CYP27*$^{+/-}$ and *CYP27*$^{+/+}$ mice in serum BA levels (Fig. 1B). However, *CYP27*$^{-/-}$ mice showed augmented weight loss and elevated ALT levels compared with *CYP27*$^{+/+}$ controls (Fig. 2A and B). Unexpectedly, *CYP27*$^{+/-}$ mice also showed slightly augmented weight loss and elevated ALT levels compared with *CYP27*$^{+/+}$ mice. We observed less hepatocyte proliferation and exacerbated apoptosis in *CYP27*$^{-/-}$ mice (Fig. 2C and D, and Supplementary Fig. 1). Moreover, compared with *CYP27*$^{+/-}$ mice and *CYP27*$^{+/+}$ mice, *CYP27*$^{-/-}$ mice showed higher grades of necrosis and leukocyte infiltration on day 3 after CCl_4 treatment (Fig. 2E). These results demonstrated the enhanced toxin-induced liver injury in *CYP27*$^{-/-}$ mice.

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