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Alpha-naphthylisothiocyanate impairs bile acid homeostasis through AMPK-FXR pathways in rat primary hepatocytes

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ABSTRACT

Alpha-naphthylisothiocyanate (ANIT) is widely used to induce cholestasis in basic researches. Although direct damage induced by ANIT to bile duct epithelial cells has been documented in previous studies, few works investigated ANIT-induced effects on hepatocytes. Our previous study indicated that activated AMP-activated protein kinase (AMPK) inhibited farnesoid X receptor (FXR) expression and further participated in the pathogenesis of estrogen-induced cholestasis. However, whether ANIT has effects on bile acid homeostasis in hepatocytes, and the role of AMPK-FXR pathway played in these effects remain unclear. In this study, our results showed that ANIT induced intracellular bile acid accumulation without obvious cellular toxicity in sandwich cultured rat primary hepatocytes (SCRHs), accompanied with significant decreased expression of FXR and bile acid transporters. AMPK activation *via* ERK1/2-LKB1 pathway was critical for ANIT-induced effects on hepatocytes. Compound C, specific AMPK inhibitor, blocked ANIT-regulated gene expression, decreased bile acid accumulation and recovered bile canalicular structure both *in vitro* and *in vivo*. Furthermore, the expression of A_1 adenosine receptor (A_1AR), a potential cholestatic target, was relatively low in hepatocytes compared with expression in rat whole livers. Consistent with these findings, DPCPX, a classic antagonist of A_1AR, had no effect on ANIT-induced hepatocytes injury. In summary, our results indicate that AMPK-FXR signaling is critical for ANIT-induced toxic effects on hepatocytes, provide new insights into the pathogenesis of ANIT-induced cholestasis, and suggest AMPK-FXR pathway as a potential therapeutic target for cholestasis.

1. Introduction

Cholestasis, induced by drugs, hormones, cytokines, stones or progressive bile duct destruction, increase the risk of liver cirrhosis, hepatitis or other hepatic and gall-bladder diseases (Kullak-Ublick and Meier, 2000; Liu et al., 2015, 2014). Alpha-naphthylisothiocyanate (ANIT) is widely used to cause cholestasis *in vitro* and *in vivo* for toxicological researches. Emerging evidence suggested that bile duct epithelial cells (BECs) are primary targets of ANIT during cholestatic conditions. Unstable glutathione-conjugated ANIT was transported by multidrug resistance-associated protein 2 (Mrp2) into biliary canalicular. Free ANIT was then dissociated and impaired bile duct acutely or chronically (Kong et al., 2012; Orsler et al., 1999). In addition, ANIT was also demonstrated to decrease bile acid transporters expression in rat hepatocytes and subsequently induce intrahepatic cholestasis (Guo et al., 2014; Luyendyk et al., 2011). Nuclear factor erythroid 2–related factor 2 (Nrf2) was proved to regulate bile acid transporters and acted as an essential factor involved in hepatotoxicity. Unexpectedly, after ANIT administration, bile acid transporters in Nrf2-null mice exhibited similar changes compared with wild-type group (Tanaka et al., 2009). Recent study indicated

Abbreviations: ANIT, alpha-naphthylisothiocyanate; A_1AR, A1 adenosine receptor; FXR, farnesoid X receptor; SHP, small heterodimer partner; Bsep, bile salt export pump; Mrp2, multidrug resistance-associated protein 2; Ntcp, Na^+-dependent taurocholate cotransporter; Oatp2, organic anion transporters 2; Mrp3, multidrug resistance-associated protein 3; AMPK, AMP-activated protein kinase; LKB1, liver kinase B1; ERK1/2, extracellular regulated protein kinases 1/2; PKA, cyclic AMP dependent protein kinase.

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that some cell-surface receptors were also involved in ANIT toxicity, and found that A_1 adenosine receptor (A_1AR) deficiency protected mice against ANIT-induced hepatotoxicity (Yang et al., 2013). Taken together, specific mechanism and signaling pathways involved in ANIT-induced effects on hepatocytes, including regulation of bile acids transporters and intra-cellular accumulation of bile acid are still in vague.

Hepatocytes are epithelial cells with polarity structure, which are distinguished as bile canalicular domains and basolateral domains, localized with specific bile acid transporters and tight junction proteins. The transporters on bile canalicular domains regulate transportation of bile acid from hepatocyte to bile canalicular, including bile salt export pump (Bsep) and Mrp2. While, Na^+-dependent taurocholate cotransporter (Ntcp) and organic anion transporters 2 (Oatp2) locate on basolateral domains, responsible for reabsorption of bile acid from blood to hepatocyte (Dawson et al., 2009; Halilbasic et al., 2013). These bile acid transporters were mediated by specific nuclear receptor (NR), including FXR. FXR heterodimerizes with RXR and regulates bile acid homeostasis *via* activating small heterodimer partner (SHP), which binds to promoters of bile acid transporters and bile acid synthases (Inagaki et al., 2006; Kakiyama et al., 2014; Li and Chiang, 2013; Plass et al., 2002). Previous study demonstrated that ANIT induced higher alkaline phosphatase levels and lower expression of bile acid transporters in FXR-null mice, suggesting that FXR deficiency aggravated the ANIT-induced cholestasis injury in liver (Cui et al., 2009).

Given the critical regulatory role of FXR in cholestasis, previous researches regarded the changes of downstream bile acid transporters as the consequence rather than the pathogenesis of cholestasis, and further focused on exploring the upstream regulatory signals responsible for hepatic cholestasis. Recently, in addition to the central role of AMP-activated protein kinase (AMPK) played in energy balance (Hardie and Ashford 2014; Hardie et al., 2012), several studies revealed that AMPK activation directly interacts with FXR, phosphorylates FXR at Ser250 site and inhibits its transcriptional activity, or influenced the formation of polarity and tight junction assembly in specific cell lines, such as epithelial cells and hepatocytes (Hardie et al., 2012; Lien et al., 2014; Zhang et al., 2009, 2006). Interestingly, our previous study demonstrated that AMPK was activated by liver kinase B1 (LKB1) in estrogen-induced cholestasis and dysregulated bile acid homeostasis by down-regulation of FXR, which revealed a novel pathogenesis mechanism of cholestasis (Li et al., 2016). However, whether ANIT contributes to cholestasis *via* activation of AMPK is still unknown.

In the current study, SD rats and sandwich-cultured rat primary hepatocytes were used to investigate the ANIT-induced effects on hepatocytes and bile canalicular networks *in vitro* and *in vivo*. Here, we demonstrated that ANIT activated AMPK phosphorylation, repressed FXR expression, and down-regulated bile acid transporters. Collectively, our findings strongly supported a critical role of AMPK-FXR pathway played in ANIT-induced dysregulation of bile acid homeostasis

2. Methods and materials

2.1. Materials and reagents

ANIT (more than 98% purity) was obtained from Acros Organics (Geel, Belgium). Compound C and DPCPX were purchased from Sigma (MO, USA). U1026 was purchased from Selleck Chemicals (TX, USA). Anti-Bsep antibody, anti-Ntcp antibody, anti-p-ERK1/2 antibody, anti-ERK1/2 antibody, anti-FXR antibody, anti-β-actin antibody, HRP-conjugated goat anti-mouse and goat anti-rabbit IgGs were from Santa Cruz Biotechnology (CA, USA). Anti-LKB1, anti-p-LKB1, anti-AMPK, anti-p-AMPK, anti-CAMKII, anti-p-CAMKII antibodies were from Cell Signaling Technology (MA, USA). Alexa Fluor Donkey anti-rabbit lgG Antibody and Donkey anti-goat lgG Antibody for immunofluorescence were provided by Invitrogen Life Science (CA, USA).

2.2. The isolation and sandwich culture of rat primary hepatocyte

After anesthetized, livers of Sprague-Dawley (SD) rats were exposed and digested according to the method of two-steps perfusion (Studer et al., 2012). Hepatocytes were collected and re-suspended in William's E medium (Invitrogen, CA, USA), containing dexamethasone (0.1 μM), penicillin (100 U/mL), streptomycin (100 U/mL), and thyroxine (1 μM). Hepatocytes were then seeded into collagen type I (Sigma, MO, USA) pre-coated cell culture dishes as described previously. After 24 h incubation, hepatocytes were covered by collagen I solution for 40 min and then cultured in complete medium to achieve sandwich culture model (Nagahashi et al., 2015).

2.3. Cell viability assay

Rat hepatocytes were seeded into 24-well plates with density of 2.5×10^4 per cell in William's E medium and then were exposed to 1–200 μM of ANIT for 24 h in 5% CO_2 incubator at 37 °C for 24 h. At the end of treatment, the Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) was added into the medium and incubated for another 2 h to assess the cell viability and the absorbance at A450 nm was measured by a microplate reader from Tecan (Austria GmbH, Austria).

2.4. Animal studies

Male Sprague-Dawley (SD) rats, 8 weeks old, were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All of the rats were housed at the humidity of 40 ± 10% and the temperature of 24 ± 2 °C under a standard 12:12-h light/dark cycle, which were provided with standard diet and free water. All animal studies and procedures were approved by the Animal Ethics Committee of China Pharmaceutical University and were carried out in accordance with the Declaration of Helsinki. After a standard rodent chow for 7 days, 18 rats were divided into three groups randomly. (n = 6): (1) control, (2) ANIT, (3) ANIT + compound C. After pretreated with saline or compound C (10 mg/kg) intraperitoneally for 1 h, rats were received gavage in a single dosage of ANIT (50 mg/kg) (Pinli, Shanghai, China). After 24 h treatment, rats were sacrificed to collect blood and livers. Tissues were weighted, fixed in formalin, prepared for frozen section by embedding with O.C.T gel (Sakura Finetek, CA, USA) or frozen in liquid nitrogen for total RNA and protein isolation.

2.5. Biochemistry analysis

Hepatocytes were treated with ANIT for 24 h and then washed by ice-cold PBS. RIPA buffer was used to harvested cells for further detection. After sacrificed, blood of rats was centrifuged for serum and 50 mg of livers were collected to homogenize in RIPA buffer. The contents of Total Bile Acid (TBA), the enzyme activities of Alkaline Phosphatase (ALP), γ-Glutamyl Transpeptadase (γ-GGT), Lactate Dehydrogenase (LDH), Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) in hepatocytes lysate, rat serum and liver samples were then measured by corresponding detection kit (Weiteman, China). For hepatocytes lysate and rat liver samples, results were normalized with total protein concentration, which was determined by BCA Protein Assay Kit (Beyotime, China).