Low dose of oleanolic acid protects against lithocholic acid-induced cholestasis in mice: potential involvement of Nrf2 mediated up-regulation of Mrps

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**Abbreviations:** OA, oleanolic acid; LCA, lithocholic acid; Mrp, multidrug resistance-associated protein; Nrf2, nuclear factor-E2-related factor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; CDCA, chenodeoxycholic acid; PCN, Pregnenolone 16α-carbonitrile; PXR, pregnane X receptor; FXR, farnesoid X receptor; CAR, constitutive androstane receptor; Nqo-1, NAD(P)H quinone oxidoreductase 1; Ho-1, heme oxygenase-1; Gclc and Gclm, glutamate–cysteine ligases; SFN, sulforaphane; RIF, rifampicin; Ntcp, Na+/taurocholate co-transporter; Oatp1b2, organic anion transporter 1b2; Bsep, bile salt export pump; Mdr3, multidrug resistance protein 3
Abstract:

Oleanolic acid (OA) is a natural triterpenoid and has been demonstrated to protect against varieties of hepatotoxicants. However recently, OA at high doses was reported to produce apparent cholestasis in mice. In this study, we characterized the protective effect of OA at low doses against lithocholic acid (LCA)-induced cholestasis in mice and explored further mechanisms. OA co-treatment (5, 10, 20 mg/kg, i.p.) significantly improved mice survival rate, attenuated liver necrosis and decreased serum ALT, AST and ALP, more importantly, serum total bile acids and bilirubin, as well as hepatic total bile acids were also remarkably reduced. Gene and protein expression analysis showed that hepatic expression of Mrp2, Mrp3 and Mrp4 were significantly increased by OA co-treatment, whereas other bile acid metabolism and transport related genes including Ntcp, Oatp1b2, Bsep, Mdr3, Cyp3a11, Cyp2b10, Sult2a1 and Ugt1a1 were only slightly changed. OA also caused increased Nrf2 mRNA expression and nuclear protein accumulation, whereas nuclear receptors FXR, PXR and CAR were not significantly influenced by OA. Luciferase assays performed in HepG2 cells illustrated that OA was a strong Nrf2 agonist with moderate PXR and weak FXR agonism. Finally, in mice primary cultured hepatocytes, OA dose- and time-dependently induced expression of Mrp2, Mrp3 and Mrp4, however, this up-regulation were abrogated when Nrf2 was silenced. In conclusion, OA produces protective effect against LCA-induced hepatotoxicity and cholestasis, possibly due to Nrf2-mediated up-regulation of Mrp2, Mrp3 and Mrp4.
Introduction

Cholestasis is a clinical syndrome with systemic and intrahepatic retention of excessive toxic bile acids that causes liver injury (Trauner et al., 1998). Among varieties of bile acids, lithocholic acid (LCA) is a toxic hydrophobic secondary bile acid formed in the large intestine by bacterial 7α-dehydroxylation of CDCA (Ridlon et al., 2006). The accumulation of LCA in circulating bile acids was thought to contribute to liver injury in patients (Carey et al., 1966). Mice feeding with LCA led to development of segmental bile duct obstruction and destructive cholangitis (Fickert et al., 2006). The hepatotoxicity of LCA or other bile acids are normally attenuated by enhancement of hepatic bile acid detoxification and output. Nuclear receptors, transcription factors and their targeting genes have been proved to play crucial roles in bile acid detoxification and output, such as PXR, FXR, CAR and Nrf2, as well as their targeting genes Cyp3a11, Bsep, Cyp2b10, Mrp (Zollner et al., 2010).

Oleanolic acid (OA) is a triterpenoid that exists naturally in food and medicinal plants (Liu, 1995). Many pharmacological studies have revealed its beneficial effects in the treatment of inflammatory diseases, type-II diabetes and cancer (Liu, 2005; Castellano et al., 2013). Importantly, OA has been shown to protect against varieties of hepatotoxicants such as acetaminophen, carbon tetrachloride and cadmium (Liu et al., 1993a; Liu et al., 1993b; Jeong, 1999), and has been used as an over-the-counter Chinese medicine for the treatment of liver diseases such as acute or chronic hepatitis. However, as a note of caution, it was recently reported that high doses (90 mg/kg and above) and longer-term use of OA produces cholestatic liver injury in mice (Liu et al., 2013; Lu et al., 2013), indicating the hepatotoxic potential of this hepatoprotective compound. Therefore, an
important and intriguing question arises whether OA has hepatoprotective effect on cholestatic liver injury such as LCA-induced cholestasis under normal low hepatoprotective doses. Another further question is that whether and how those bile acid homeostasis-related nuclear receptors, transcription factors and their targeting genes contribute to its hepatoprotection if OA possesses protective effect against cholestasis.

Therefore, the present study aimed to investigate the protective effect of OA against LCA-induced cholestasis in mice, and to explore the potential mechanisms \textit{in vivo} and \textit{in vitro}.
Materials and methods

Chemicals and reagents

LCA (purity >98%), OA (purity >98%) and corn oil were purchased from Aladdin Company (Shanghai, China). PCN was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SFN, RIF, CDCA and CITCO were all purchased from Sigma-Aldrich (St. Louis, MO). The plasmid pGL3-ARE-Luc was a kind gift from Dr. Athanassios Fragouli (University Hospital Aachen, RWTH Aachen University, Germany) (Fragouli et al., 2012), ARE refers to the antioxidant response element (ARE) consensus sequence of Nqo1 promoter region. The expression vector for dominant positive Nrf2 (pEF-Nrf2) was kindly provided by Dr. Shinya Ito (The Hospital for Sick Children, Toronto, Canada) (Tan et al., 2008). The pSG5-hPXR expression vector was generously provided by Dr. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX, USA) (Lehmann et al., 1998). The pGL3-CYP3A4-XREM luciferase reporter construct containing the basal promoter (−362/+53) with the proximal PXR response element (ER6) and the distal xenobiotic responsive enhancer module (XREM, −7836/−7208) of the CYP3A4 gene 5'-flanking region inserted to pGL3-Basic reporter vector was generously provided by Dr. Jeff Staudinger (University of Kansas, Lawrence, KS, USA) (Goodwin et al., 1999). FXR-responsive reporter tk-EcRE-Luc and expression vector for FXR were kindly provided by Dr. Wen Xie (University of Pittsburgh, PA, USA) (Forman et al., 1995; Xie et al., 2001a). pRL-TK Rotylenchulus reniformis control vector was obtained from Promega (Madison, WI, USA). All other chemicals and solvents were commercially available and of analytical grade.
Animals and treatments

Male C57BL/6 mice (8-9 weeks), weighting 21-25 g, were purchased from Guangdong Animal Experimental Centre (Guangzhou, China). Mice were kept in a room at 22-24°C with a light/dark cycle of 12/12 h and 55-60% relative humidity with free access to standard rodent food and water. All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of China (http://www.most.gov.cn). The animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Sun Yat-sen University, Guangzhou, China.

OA (5, 10, 20 mg/kg, i.p.) were prepared by suspending it in 2% Tween-80 in saline. PCN (50 mg/kg, i.p.) was dissolved in corn oil as described previously (Staudinger et al., 2001; Xie et al., 2001b). OA or PCN was administered to mice for 7 days and LCA dissolving in corn oil (125 mg/kg, i.p.) was initiated since the fourth day, as described previously (Staudinger et al., 2001). Mice in OA alone group were treated with highest dose of OA once daily for 7 days. Additionally, more mice were treated with LCA alone (n=12) than were used in the other groups to obtain enough survivors to perform the various analyses in an adequate number of animals. 24 h after the final treatment, serum and liver tissue samples were obtained and snap-frozen on liquid nitrogen, then stored at -80°C until use.

Histological and biochemical assessment

Liver tissues were immediately formalin-fixed, paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E) following a standard protocol. The
H&E-stained liver sections were examined using an Olympus BX41 microscope.

Liver injury or cholestasis was also evaluated by measuring serum ALT, AST and ALP activities, serum total bile acids and total bilirubin and hepatic total bile acids using commercially available kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer’s instructions.

**Isolation and culture of mice primary hepatocytes**

Hepatocytes from male C57BL/6 mice (8-9 weeks) were isolated by the two-step collagenase digestion method as described previously (Klaunig et al., 1981a; Klaunig et al., 1981b). Viability of the obtained hepatocytes was routinely more than 90% by trypan blue exclusion. Hepatocytes were cultured with the William's E Medium supplemented with 0.1 μM Dexamethasone (Sigma, St. Louis, MO), 10% heat-inactivated fetal bovine serum (FBS), 100 U penicillin/streptomycin, 1× glutamine and 1×Insulin-Transferrin-Selenium-Sodium Pyruvate Solution (ITS-A) (Gibco, NY) on plates coated with rat tail collagen (Shengyou Biotechnology, China) and incubated for 3 h, then replaced with the fresh William's E Medium and incubated for another 9 h before treatment. Only hepatocytes with viability greater than 90% were used for the study.

**Quantitative real time PCR (qRT-PCR) analysis**

Total RNA from mice liver tissues or mice primary cultured hepatocytes was isolated using Trizol reagent according to the manufacturer’s instruction (Invitrogen, Grand Island, NY). 1 μg RNA was purified and randomly reverse-transcribed to cDNA by using PrimeScript RT reagent kit with gDNA eraser (TaKaRa Biotech, Kyoto, Japan).
qRT-PCR analysis was performed using SYBR Premix Ex Taq II (Tli RnaseH Plus) kit (TaKaRa Biotech, Kyoto, Japan) in Applied Biosystems 7500 Real-Time PCR System. GAPDH was run for each sample to normalize expression. The gene-specific primers were obtained from a primerbank (Spandidos et al., 2010; Wang et al., 2012) and the sequences are listed in Supplemental Table 1.

**Western blot analysis**

Protein extracted from mice liver tissue or primary cultured mice hepatocytes were prepared using RIPA lysis buffer or Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Protein concentration was determined by BCA protein assay (Thermo Scientific, Rockford, IL). 40 μg of protein extracts was separated in 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrophoretically transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat dry milk in Tris-buffered saline, membranes were incubated overnight with primary antibodies, including Mrp2 (H-17), Mrp3 (C-18) and Nrf2 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA); Mrp4 (M41-10) (Abcam, Cambridge, MA). Subsequently, a secondary horseradish peroxidase-conjugated anti-rabbit, anti-rat or anti-goat IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was applied, and then specific bands were visualized using ECL detection kit (Engreen Biosystem, China).

**Drug or siRNA treatments in mice primary cultured hepatocytes**

Mice primary cultured hepatocytes were seeded onto 12-well plate at a density of 6×10^5 cells per well. 12 h later, the cells were treated with vehicle (0.1% DMSO) or OA
DMD#56549

1, 5, 25 μM for 48h or 25 μM for 1, 3, 6, 12, 24, 48 h; for RNA silencing experiment, 100 nM negative control siRNA or siRNA targeting at mouse *Nrf2* (Product ID: siG091224150819. Sense: 5'-GCAGGAGAGGUAAGAAUAA dTdT-3'; Anti-sense: 3'-dTdT CGUCCUCUCCAUUCUUAUU-5') (RiboBio, Guangzhou, China) were transiently transfected to mice primary cultured hepatocytes using X-tremeGENE siRNA Transfection Reagent (Roche diagnostics, Mannheim, Germany) and incubated for 60 h, 25 μM OA was added to the culture medium for the last 48 h. The culture medium at the volume of 1.5 mL per well were not changed during the incubation period according to the instruction of transfection reagent. After that, the cells were either harvested for qRT-PCR or western blot analysis.

**Transient transfection assays**

HepG2 cells (ATCC) were maintained in DMEM containing 10% FBS and 100 U penicillin/streptomycin. Cells were seeded to 96 well plate at a density of 1.5×10⁴ cells per well. For Nrf2 transactivation assay, each well contains 100 ng pGL3-ARE-Luc, 50 ng pEF-Nrf2 and 5 ng pRL-TK; For PXR transactivation assay, each well contains 100 ng pGL3-CYP3A4-XREM-Luc, 50 ng pSG5-hPXR and 5 ng pRL-TK; For FXR transactivation assay, each well contains 100 ng tk-EcRE-Luc, 50 ng FXR expression vector and 5 ng pRL-TK. The transfection procedure was followed by instruction of Lipofectamine 2000 (Invitrogen, Grand Island, NY). 6 h later, the transfection mixtures were removed and replaced with phenol red free DMEM containing 10% charcoal-stripped delipidated FBS. Transfected cells were then treated with OA (1, 5, 25 μM) or respective positive agonist SFN (10 μM), RIF (10 μM) or CDCA (100 μM) for 24
Luciferase activity was assayed in an Amersham Pharmacia Biotech luminometer using Dual Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s directions. Firefly luciferase activity was normalized to Renilla activity for each well.

**Statistical Analysis**

All values were expressed as mean ± S.E.M. One-way ANOVA followed by Dunnett’s multiple comparison post hoc test or unpaired Student’s *t* test was used for statistical analysis of data using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Only the comparisons indicated above the bars are being made and difference was considered as significant if the probability (*P* value) was less than 0.05 (*P* < 0.05).
Results

Protection against LCA-induced hepatotoxicity and cholestasis by OA

The morphological and histological assessments clearly indicated the severe hepatotoxicity and cholestasis caused by LCA (Figure 1A-1B). Mice co-treated with 20 mg/kg OA or 50 mg/kg PCN exhibited remarkable less subcapsular foci of white discolorations in the liver and shrunken gallbladder size, as compared to mice treated with LCA (Figure 1A). This morphological change was corresponding with histological assessment results (Figure 1B), H&E stained liver sections showed large-scaled necrotic foci in mice treated with LCA and this pattern was significantly attenuated by co-administration of OA or PCN. Furthermore, LCA treatment for 4 days caused 42% of the mice death, as shown in Table 1, more mice were treated with LCA alone (n=12) than were used in the other groups to obtain enough survivors for various analyses in an adequate number of animals. This lethal effect was greatly reversed by co-treatment of 5, 10, 20 mg/kg OA, especially the mice in high-dose OA group and PCN co-treatment group all survived.

Serum biochemistry results were in good agreement with liver histology. The severe cholestasis caused by LCA was also indicated by huge elevation of serum ALT, AST and ALP to 4147, 4910 and 437 U/L, respectively (Figure 1C-1E). In contrast, co-administration of OA dose-dependently reversed the LCA-induced increase of ALT to 39%, 22% and 14%, AST to 33%, 14% and 6%, ALP to 71%, 69% and 42%, respectively. Likewise, Figure 1F-IG illustrated that serum total bile acids and total bilirubin were dramatically increased by LCA to 590 and 290 μmol/L, which was 17 and 32 fold higher than that of vehicle group, whereas OA co-treatment dose-dependently reduced
LCA-induced increase of serum total bile acids to 47%, 47% and 35%, total bilirubin to 27%, 24% and 14%, respectively. Consistent with serum biochemistry findings, OA also dose-dependently reduced LCA-induced elevation of hepatic total bile acids (3 fold higher than that of vehicle group) to 69%, 57% and 55%, respectively (Figure 1H). In addition, 50 mg/kg of PCN co-treatment produced similar effects found in the high-dose OA group. Taken together, these data clearly demonstrate that OA at relatively low doses (20 mg/kg or below) can protect against LCA-induced hepatotoxicity and cholestasis.

**OA altered gene and protein expression profile involved in bile acid homeostasis**

First, the hepatic gene expression profile of bile acid transporters was analyzed by qRT-PCR. Bile acids are taken up by basolateral Ntcp and Oatp1b2 and are exported into bile by canalicular Bsep and Mrp2. Besides these, canalicular Mdr3 is also involved in bile acid transport by mediating excretion of Phosphatidylcholine. It is noteworthy that Mrp3 and Mrp4 stand for alternative basolateral bile acid export (Zollner et al., 2010). For the best hepatoprotective effect was found in the high-dose group, 20 mg/kg OA was chose for the following study. Figure 2A-2C illustrated that LCA markedly decreased the expression of Ntcp, Oatp1b2 and Mrp2, increased Mdr3 and Mrp4 mRNA, while Bsep and Mrp3 expression were slightly changed. In comparison, OA co-treatment reversed LCA-induced decrease of Mrp2 to normal level, meanwhile Oatp1b2 and Ntcp decrease were partially recovered and Mrp3 was significantly induced. Notably, OA co-treatment further enhanced the up-regulation of Mrp4 that observed in LCA group, whereas the elevation of Mdr3 by LCA was markedly weakened. Furthermore, OA alone significantly caused Mrp2, Mrp3, Mrp4 and Mdr3 up-regulation (1.8, 1.9, 3.5 and 1.9
fold higher than that in vehicle group, respectively), and resulted in the down-regulation of Ntcp and Oatp1b2. PCN co-treatment caused similar effect on gene expression of hepatic transporters altered by OA, except that LCA-induced Mdr3 up-regulation was not reversed by PCN. Together, the above data suggests that hepatoprotection of OA against LCA may due to up-regulation of Mrp2, Mrp3 and Mrp4 instead of other transporters.

Besides the above bile acid transporters, hepatic metabolism enzymes involving in bile acid homeostasis were also examined. Cyp7a1 is an enzyme mediating the rate-limiting step in the classical bile acid synthesis. Bile acid detoxification was mainly mediated via phase I enzymes (Cyp3a11 and Cyp2b10) and phase II enzymes (Sult2a1 and Ugt1a1) (Zollner et al., 2010). As demonstrated in Figure 2D-2G, LCA dramatically inhibited the mRNA levels of Cyp7a1 and Ugt1a1 to 2.8% and 43.7%, whereas the mRNA levels of Cyp3a11, Cyp2b10 and Sult2a1 were induced to 2.8, 4.6 and 40.0 fold higher, respectively. In comparison, co-administration of OA markedly attenuated the suppression of Cyp7a1 and the increase of Sult2a1 by LCA, whereas the decreased Ugt1a1 and increased Cyp3a11 and Cyp2b10 by LCA were only slightly altered. OA alone treatment had little impact on the mRNA levels of Cyp7a1, Cyp3a11, Cyp2b10 and Ugt1a1, but induced Sult2a1 expression. In addition, PCN co-treatment induced the above five enzyme gene expression when compared to that in LCA alone group. Thus, these data indicate that the slight impact of OA on bile acid metabolism enzymes may not contribute to its hepatoprotection against LCA.

To further investigate the mechanisms of hepatoprotection of OA against LCA-induced cholestasis, we measured gene expression of some nuclear receptors and transcription factors related to bile acid homeostasis. Figure 3 showed that the mRNA of FXR, PXR
and CAR were all slightly changed by OA alone, whereas Nrf2 mRNA was significantly induced. LCA itself also increased Nrf2 mRNA expression, and co-treatment with OA further enhanced this induction. LCA resulted in decrease of FXR and CAR mRNA expression but markedly increased PXR mRNA expression. OA co-treatment mitigated these alteration, especially reversed the LCA induced up-regulation of PXR and down-regulation of FXR and CAR to normal levels. Besides, in contrast to LCA alone treatment, PCN co-treatment reversed the Nrf2 mRNA induction and reduced the three nuclear receptors. These data indicate that induction of Nrf2 but not FXR, PXR and CAR, plays a crucial role in the hepatoprotection of OA against LCA-induced cholestasis.

To confirm the qRT-PCR results regarding induction of Nrf2 and Mrps by OA, we further detected their protein levels using western blot analysis. As shown in Figure 4, LCA treatment resulted in significant increase in Mrp3 and Mrp4 protein expression and decrease of Mrp2 protein expression. OA co-treatment caused a more robust Mrp3 and Mrp4 induction, meanwhile reversed the down-regulation of Mrp2. OA alone treatment resulted in a significant up-regulation of Mrp2, Mrp3 and Mrp4 at protein level. In addition, nuclear Nrf2 protein level was also increased by OA alone and the combined treatment enhanced Nrf2 accumulation in the nucleus, however LCA alone had no impact on nuclear Nrf2 level. Therefore, OA possesses the ability to induce mRNA and protein expression of Mrp2, Mrp3 and Mrp4, which is possibly mediated via Nrf2 activation.

Taken together, these data indicate that the hepatoprotective effect of OA against LCA-induced cholestasis may due to enhancement of bile acid export, probably via Nrf2-mediated up-regulation of Mrp2, Mrp3 and Mrp4, whereas the impact of OA on bile acid metabolism enzymes and nuclear receptors may contributes little to its...
DMD#56549

hepatoprotective effect.

**OA induced Mrps gene expression via Nrf2 in mice primary cultured hepatocytes**

To further demonstrate whether Nrf2 activation is required for OA-mediated up-regulation of Mrp2, Mrp3 and Mrp4, Nrf2 gene silencing experiment was performed using mice primary cultured hepatocytes. **Figure 5A-5B** illustrated that OA could time- and dose-dependently resulted in elevation of *Mrp2*, *Mrp3* and *Mrp4* expression, with maximum level reached at 48 h post-incubation with 25 μM OA, as compared to that in vehicle group. Nrf2 gene silencing experiment was further conducted. As illustrated in **Figure 5C**, western blot analysis ensured that Nrf2 expression decreased by approximately 80% after transfection of specific siRNA sequence targeting Nrf2 mRNA. qRT-PCR analysis was used to measure the subsequent mRNA alterations (**Figure 5D**), as expected, Nrf2 silence remarkably reduced the gene expression of *Mrp2*, *Mrp3* and *Mrp4* to 62%, 64% and 5% respectively, as compared to that in negative control. Notably, the significant induction of *Mrp2*, *Mrp3* and *Mrp4* by OA were all abrogated by Nrf2 silence. Thus, it is further demonstrated in mice primary cultured hepatocytes that OA induces *Mrp2*, *Mrp3* and *Mrp4* gene expression via Nrf2 activation.

**Effects of OA on Nrf2 activation and nuclear receptor agonism in HepG2 cells**

In *in vivo* experiments, the effects of OA on mRNA expression of Nrf2, FXR, PXR and CAR had been measured; however, the mRNA level may not be enough to represent the effects of OA on these gene functions. Thus, effects of OA on Nrf2 activation and nuclear receptor agonism were subsequently examined using luciferase reporter assay in
HepG2 cells transiently transfected with reporter plasmids. As demonstrated in Figure 6A, SFN, a characterized Nrf2 activator, significantly increased the luciferase activity of Nrf2 reporter gene by 3.5 fold higher than that in vehicle group, while OA dose-dependently increased the Nrf2 reporter gene activity, with a significant 2.5-fold elevation at 25 μM. We next detected the nuclear receptor agonism and found that OA is a moderate PXR agonist. As shown in Figure 6B-6C, compared to vehicle group, 5 μM and 25 μM of OA resulted in significant elevation of luciferase activity of PXR reporter gene (2.5 and 2.6 fold, respectively) and 25 μM OA caused weak FXR agonism (2.3 fold), while classical PXR agonist rifampicin and FXR agonist CDCA increase the luciferase activity by 5.6 and 14.5 fold, respectively. The above data suggest that OA is a strong Nrf2 activator with moderate PXR and weak FXR agonism.
Discussions

The present study clearly demonstrated that OA held the potential to protect against LCA-induced liver cholestasis in a dose-dependent manner, as evidenced by increased survival rate and ameliorative liver morphology and histology, as well as significant decrease in serum ALT/AST/ALP, serum total bile acids, total bilirubin, and hepatic total bile acids. Further in vivo and in vitro studies indicate that the hepatoprotective effect of OA against LCA-induced cholestasis was possibly due to Nrf2-mediated up-regulation of Mrp2, Mrp3 and Mrp4.

As a note of caution, it should be mentioned that although relative low dose (20 mg/kg, or below, i.p.) of OA used in our study is hepatoprotective against cholestasis, higher dose of OA produces cholestasis and liver injury, indicating the hepatotoxic potential of this hepatoprotective compound. In C57BL/6 mice, OA produced cholestasis when fed in the diet at a dose of 100 mg/kg/d for 7 days (Sato et al., 2007). Very recently, Liu reported that higher doses (90 mg/kg and above) of OA could produce cholestatic liver injury in mice by altering bile acid homeostasis (Liu et al., 2013; Lu et al., 2013). In the reported study, C57BL/6 mice were given OA at doses of 0, 22.5, 45, 90, and 135 mg/kg, s.c., once daily for 5 days, liver injury was apparent at doses of 90 mg/kg and above, however 22.5 mg/kg OA did not cause any liver injury and cholestasis in mice, suggesting a quite narrow margin of the safety of OA (the ratio of toxic dose at 90 mg/kg over effective dose at 22.5 mg/kg is about 4). In our study, mice were treated with 0, 5, 10 and 20 mg/kg OA once daily for 7 days, i.p., the highest dose (20 mg/kg) is a safe and effective dose, and no any liver injury or cholestasis was observed. In addition, we focused on the effect of OA in bile acid output and noted Nrf2-mediated induction of
Mrp2, Mrp3 and Mrp4 as the possible mechanism of hepatoprotection of OA, which was not presented in Liu’s study. Taken together, it suggests that for the therapy of cholestasis, OA is a double-edged sword, at doses 90 mg/kg and above, OA can cause apparent liver injury and cholestasis, whereas OA at doses below 22.5 mg/kg, especially at 20 mg/kg, can protect against LCA-induced cholestasis. Therefore, cautions should be taken when high dose use of OA and the beneficial effects of OA should be balanced between hepatoprotection and hepatotoxicity.

Varieties of metabolism enzymes and transporters play crucial roles in bile acid homeostasis (Trauner et al., 1998; Zollner et al., 2010). This study demonstrated that excessive LCA caused adaptive hepatic gene expression changes in favor of lessening hepatotoxicity, which was in line with previous reports (Beilke et al., 2008; Tan et al., 2010). In the current study, little impact of OA was found on the five key metabolism enzyme genes Cyp7a1, Cyp3a11, Cyp2b10, Sult2a1 and Ugt1a1. However, PCN co-treatment induced significant gene expression of Cyp3a11, Sult2a1 and Ugt1a1, since PCN is a classical PXR agonist that targets at these genes to facilitate bile acid detoxification (Staudinger et al., 2001; Xie et al., 2001b; Chen et al., 2003; Kitada et al., 2003). Ntcp and Oatp1b2 were greatly inhibited by LCA to defense against excessive bile acids entering hepatocytes. OA also caused decrease in the gene expression of these two uptake transporters in normal mice, whereas co-treatment of OA with LCA leaded to partial recovery of Ntcp and Oatp1b2, which may be explained by the possibility that OA treatment resulted in a lower abundance of LCA in hepatocytes, which in turn weakened the adaptive response of mouse liver, instead of a direct effect of OA on Ntcp and Oatp1b2 expression. Bsep, as an essential transporter mediating canalicular bile acid
output (Strautnieks et al., 1998), was slightly changed by OA, LCA and PCN, suggesting that Bsep may play little role in this cholestasis model, although its induction in other cholestasis models such as ANIT and bile duct ligation (BDL) was proved beneficial (Liu et al., 2003). The Mdr3 transporter, which can excrete phosphatidylcholine into bile to prevent bile duct injury (Klaassen and Aleksunes, 2010), was highly up-regulated by LCA in an adaptive responsive way, however, OA co-treatment alleviated this up-regulation, possibly due to the same reason found in Ntcp and Oatp1b2. During the process of cholestasis, Mrp transporters including Mrp2, Mrp3 and Mrp4 exert their effects in favoring output of bile acid or bilirubin conjugated with glucuronide or sulfate (Keppler and König, 2000). In this study, OA remarkably induced these three Mrps expression at both mRNA and protein levels with or without LCA treatment, indicating that this induction may contribute to the hepatoprotection of OA by enhancing bile acid output.

Mrp2, Mrp3 and Mrp4 are regulated by many nuclear transporters and transcription factors (Zollner et al., 2010), among them, Nrf2 has been proved to be one of the most important upstream transcription factors and recent studies showed that Mrp2, Mrp3 and Mrp4 were direct target genes of Nrf2 and could be induced by Nrf2 activators in rodent livers (Maher et al., 2007; Okada et al., 2008; Anwar-Mohamed et al., 2011). The binding of Nrf2 to antioxidant response elements in the promoter regions of mouse Mrp2 (185 bp), Mrp3 (9919 bp) and Mrp4 (3767 bp) was demonstrated using CHIP assays (Maher et al., 2007). Interestingly, administration of LCA was demonstrated to orchestrate adaptive responses by activating Nrf2 (Tan et al., 2010), indicating that activation of Nrf2 might hold promise for the treatment of LCA-induced cholestasis. Nrf2 activation was
DMD#56549

demonstrated to be a mechanism of OA protection against acetaminophen-induced liver injury (Liu et al., 2008; Reisman et al., 2009), however these studies only focused on the anti-oxidative property of OA involving Nrf2 mediated up-regulation of Gclc, Nqo-1 and Ho-1. There were no reports on the effect of OA on the bile acid output transporters Mrp2, Mrp3 and Mrp4, which are also downstream genes of Nrf2. In this study, we proved that Nrf2 was activated by OA, as evidenced by increased gene transcription of Nrf2 and nuclear accumulation of Nrf2 protein in mice liver, as well as Nrf2 activation using luciferase reporter assay in HepG2 cells. Furthermore, Nrf2 silencing experiment conducted in mice primary cultured hepatocytes directly proved that up-regulation of Mrp2, Mrp3 and Mrp4 by OA was mediated via Nrf2, which was consistent with our in vivo results. In addition, we also demonstrated that the effects of OA on the gene expression of Oatp1b2 and Ntcp in mice primary cultured hepatocytes were not affected when Nrf2 was silenced, as shown in Supplemental Figure 1.

Besides Nrf2, three nuclear receptors FXR, PXR and CAR also play important roles in protecting against bile acid toxicity (Guo et al., 2003; Zhang et al., 2004). However, no significant change of FXR gene expression was observed in the LCA model and the luciferase reporter assay also indicated that OA was a weak FXR agonist, which is in line with previous study (Sato et al., 2007). Furthermore, it should be noted that OA is a FXR antagonist as manifested in HepG2 cells (Liu and Wong, 2010), in our study, OA at 20 mg/kg caused no statistical change of FXR target genes such as Bsep, however, OA at doses of 90 mg/kg or above produced significant Bsep decrease (Liu et al., 2013; Lu et al., 2013), suggesting FXR antagonism may be one of the mechanisms that OA produces cholestasis at high doses. In addition, the data from human PXR reporter assay indicated
that OA could cause moderate PXR agonism however, *in vivo* experiment showed no significant induction of PXR target genes *Cyp3a11*, *Sult2a1* and *Ugt1a1* by OA, which may be explained that species difference exists in PXR agonism by OA, or OA at relatively low doses used in our study may not be sufficient to activate PXR and induce significant target gene expression. CAR was not examined in current study because the HepG2 model is not effective for evaluating treatment effects on CAR activity (Honkakoski et al., 1998).

Sensitivity to cholestasis can be sex specific and the female are more susceptible to cholestasis (Kaplan and Gershwin, 2005), thus we used male mice only in the current study, in order to avoid confounding by changes in estrogen levels associated with the estrous cycle, and it remains to be determined whether the same phenotype will be conserved in female mice. Besides, without the use of *Mrp* knockout mice, we can’t directly demonstrate that Mrp transporters are effectors of OA hepatoprotection, and this issue also requires to be further studied.

In summary, the current study clearly demonstrated that OA at relative low doses (20 mg/kg or below) can dose-dependently protect against LCA-induced hepatotoxicity and cholestasis, and this hepatoprotective effect is possibly due to Nrf2-mediated up-regulation of Mrp2, Mrp3 and Mrp4, suggesting that Nrf2 may be a promising cholestasis therapeutic target and discovering more effective and safe Nrf2 activators would be necessary.
Authorship Contributions

Participated in research design: Bi, Huang, Chen.


Contributed to new reagents and analytic tools: Chen, Zhou, Deng.

Performed data analysis: Chen, Bi, Xu.

Wrote or contributed to the writing of the manuscript: Chen, Bi.
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DMD#56549


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126:228-243.
Footnotes:

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Legends for Figure:

Figure 1. Hepatoprotection of OA against LCA-induced cholestasis. (A) Photographs of representative livers. Gall bladders were marked by arrows and the apparent subcapsular necrotic foci in the liver could be seen in LCA group. (B) Representative H&E stained liver sections (10 × magnification). Areas of severe liver necrosis were marked by arrows. (C-H) Serum ALT, AST and ALP activity, as well as serum total bile acids, total bilirubin and hepatic total bile acids levels elevated by LCA were significantly reduced by co-treatment with different doses of OA or 50 mg/kg PCN. Data are the mean ± S.E.M (n = 6-10). *P <0.05 versus vehicle; #P<0.05 versus LCA alone.

Figure 2. OA altered hepatic gene expression profile involved in bile acid metabolism and transport in LCA-induced mice model of cholestasis. qRT-PCR analysis was performed to measure the gene expression of (A) Ntcp and Oatp1b2, (B) Bsep, Mrp2 and Mdr3, (C) Mrp3 and Mrp4, (D) Cyp7a1, (E) Cyp3a11 and Cyp2b10, (F) Sult2a1, (G) Ugt1a1. Data are the mean ± S.E.M. (n=5-6). *P<0.05 versus vehicle, #P<0.05 versus LCA alone.

Figure 3. Effects of OA on hepatic gene expression of Nrf2 and nuclear receptors in LCA-induced mice model of cholestasis. qRT-PCR analysis was performed to measure the gene expression of (A) Nrf2, (B) FXR, PXR and CAR. Data are the mean ± S.E. (n=5-6). *P<0.05 versus vehicle, #P<0.05 versus LCA alone.
Figure 4. OA increased hepatic protein expression of Mrps and nuclear Nrf2 accumulation in LCA-induced cholestasis model. (A) Western blot was used to measure Mrp2, Mrp3, Mrp4 and nuclear Nrf2 protein expression. (B) Specific band intensity was quantified, normalized to GAPDH, and expressed as mean ± S.E.M. (n=3). *P<0.05 versus vehicle, #P<0.05 versus LCA alone.

Figure 5. In vitro evidences on the up-regulation of Nrf2-Mrps pathway by OA. (A-B) OA dose-dependently and time-dependently induced Mrp2, Mrp3 and Mrp4 in mice primary cultured hepatocytes. qRT-PCR analysis was performed to measure the gene expression and data are expressed as mean ± S.E.M (n=3). *P<0.05 versus vehicle. (C) Nrf2 silencing efficiency was measured by Western blot, band intensity was quantified and normalized to GAPDH and expressed as mean ± S.E.M. (n=3). *P<0.05 versus control. (D) Nrf2 knockdown abrogated the induction of Mrp2, Mrp3 and Mrp4 by OA in mice primary hepatocytes. qRT-PCR analysis was performed to measure the gene expression and data are the mean ± S.E.M (n=3). *P<0.05 versus vehicle, #P<0.05 versus OA alone.

Figure 6. Effects of OA on Nrf2 activation and nuclear receptor agonism. (A-C) HepG2 cells were transiently transfected with respective plasmids as described in materials and methods, then the cells were treated with different concentrations of OA (1, 5, 25 μM) or respective positive agonist SFN (10 μM), RIF (10 μM) or CDCA (100 μM) for 24 h. Data are the mean ± S.E.M. (n=4). *P<0.05 versus vehicle.
Table 1. OA increases mice survival rate against LCA-induced death.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival/Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>OA 20mg/kg</td>
<td>6/6 (100%)</td>
</tr>
<tr>
<td>LCA</td>
<td>7/12 (58%)</td>
</tr>
<tr>
<td>OA 5 mg/kg + LCA</td>
<td>9/10 (100%)</td>
</tr>
<tr>
<td>OA 10 mg/kg + LCA</td>
<td>8/10 (100%)</td>
</tr>
<tr>
<td>OA 20 mg/kg + LCA</td>
<td>10/10 (80%)</td>
</tr>
<tr>
<td>PCN 50 mg/kg + LCA</td>
<td>6/6 (100%)</td>
</tr>
</tbody>
</table>
Figure 5

A

Mrp transporters

Relative mRNA expression

Vehicle 1 μM 5 μM 25 μM 50 μM Vehicle 1 μM 5 μM 25 μM 50 μM Vehicle 1 μM 5 μM 25 μM 50 μM

OA 48h OA 48h OA 48h

B

Mrp transporters

Relative mRNA expression

OA 25 μM OA 25 μM OA 25 μM

C

Nrf2

GAPDH

Control siNrf2

Nrf2

Relative protein expression

con siNrf2

D

Mrp transporters

Relative mRNA expression

Vehicle OA Vehicle OA Vehicle OA Vehicle OA

siNrf2 siNrf2 siNrf2 siNrf2