Low Dose of Oleanolic Acid Protects against Lithocholic Acid–Induced Cholestasis in Mice: Potential Involvement of Nuclear Factor-E2-Related Factor 2-Mediated Upregulation of Multidrug Resistance-Associated Proteins

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ABSTRACT

Oleanolic acid (OA) is a natural triterpenoid and has been demonstrated to protect against varieties of hepatotoxins. Recently, however, 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 pretreatment (5, 10, and 20 mg/kg, i.p.) significantly improved mouse survival rate, attenuated liver necrosis, and decreased serum alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase; 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 multidrug resistance-associated protein 2 (Mrp2), Mrp3, and Mrp4 was significantly increased by OA cotreatment, whereas other bile acid metabolism- and transport-related genes, including Na+-taurocholate cotransporter, organic anion transporter 1b2, bile salt export pump, multidrug resistance protein 3, Cyp3a11, Cyp2b10, Sulfortransferase 2a1 (Sult2a1), and UDP-glucuronosyltransferase 1a1 (Ugt1a1), were only slightly changed. OA also caused increased nuclear factor-E2-related factor (Nrf2) mRNA expression and nuclear protein accumulation, whereas nuclear receptors farnesoid X receptor (FXR), pregnane X receptor (PXR), and constitutive androstane receptor were not significantly influenced by OA. Luciferase (Luc) assays performed in HepG2 cells illustrated that OA was a strong Nrf2 agonist with moderate PXR and weak FXR agonism. Finally, in mouse primary cultured hepatocytes, OA dose- and time-dependently induced expression of Mrp2, Mrp3, and Mrp4; however, this upregulation was abrogated when Nrf2 was silenced. In conclusion, OA produces a protective effect against LCA-induced hepatotoxicity and cholestasis, possibly due to Nrf2-mediated upregulation 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 chenodeoxycholic acid (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 fed 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 is 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 pregnane X receptor (PXR), farnesoid X receptor (FXR), constitutive androstane receptor (CAR), and nuclear factor-E2-related factor (Nrf2), as well as their targeting genes Cyp3a11, bile salt export pump (Bsep), Cyp2b10, and multidrug resistance-associated protein (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 hepatotoxins such as acetaminophen, carbon tetrachloride, and cadmium (Liu et al., 1993a,b; Jeong, 1999), and has been used as an

ABBREVIATIONS: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ARE, antioxidant response element; AST, aspartate aminotransferase; Bsep, bile salt export pump; CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; FBS, fetal bovine serum; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LCA, lithocholic acid; Luc, Luciferase; Mdr3, multidrug resistance protein 3; Mrp, multidrug resistance-associated protein; Nrf2, nuclear factor-E2-related factor; Ntcp, Na+/taurocholate cotransporter; OA, oleanolic acid; Oatp1b2, organic anion transporter 1b2; PCN, pregnenolone 16α-carbonitrile; PXR, pregnane X receptor; qRT-PCR, quantitative real-time polymerase chain reaction; RIF, rifampicin; SFN, sulforaphane; siRNA, small interfering RNA; XREM, xenobiotic responsive enhancer module.
Oleanolic acid protects against LCA-induced cholestasis

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

Isolation and Culture of Mouse 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,b). Viability of the obtained hepatocytes was routinely more than 90% by trypan blue exclusion. Hepatocytes were cultured with Williams’ E medium supplemented with 0.1 μM dexamethasone (Sigma), 10% heat-inactivated fetal bovine serum (FBS), 100 U penicillin/streptomycin, 1 μg/mL of insulin, and transferrin-selenium-sodium pyruvate solution (Gibco, Grand Island, NY) on plates coated with rat tail collagen (Shengyou Biotechnology, Hangzhou, China) and incubated for 3 hours, then replaced with the Williams’ E medium and incubated for another 9 hours before treatment. Only hepatocytes with viability greater than 90% were used for the study.

Quantitative Real-Time Polymerase Chain Reaction Analysis. Total RNA from mouse liver tissues or mouse primary cultured hepatocytes was isolated using Trizol reagent according to the manufacturer’s instructions (Invitrogen, Grand Island, NY). One microgram of RNA was purified and randomly reverse transcribed to cDNA using a PrimeScript RT reagent kit with dDNA eraser (TaKaRa Biotech, Kyoto, Japan). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using a SYBR Premix Ex Taq II (Tli RNaseH Plus) kit (TaKaRa Biotech) in an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was run for each sample to normalize expression. The gene-specific primers were obtained from a primerbank (Spandios et al., 2010; Wang et al., 2012), and the sequences are listed in Supplemental Table 1.

Western Blot Analysis. Protein extracted from mouse liver tissue or primary cultured mouse hepatocytes was prepared using Radiolabeled protein precipitation assay (RIPA) lysis buffer or Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. Protein concentration was determined by bicinchoninic acid assay (BCA) protein assay (Thermo Scientific, Rockford, IL). Forty micrograms 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), and Mrp4 (M4H-10) (Abcam, Cambridge, MA). Subsequently, a secondary horseradish peroxidase–conjugated antirabbit, antirat, or antigoat IgG antibody (Santa Cruz Biotechnology) was applied, and then specific bands were visualized using an enhanced chemiluminescence (ECL) detection kit (Enorr Bio system, Beijing, China).

Drug or Small Interfering RNA Treatments in Mouse Primary Cultured Hepatocytes. Mouse primary cultured hepatocytes were seeded on a 12-well plate at a density of 6 × 10^4 cells per well. Twelve hours later, the cells were treated with vehicle (0.1% dimethylsulfoxide) or 1, 5, or 25 μM OA for 48 hours or 25 μM for 1, 3, 6, 12, 24, and 48 hours. For the RNA silencing experiment, 100 nM negative control small interfering RNA (siRNA) or siRNA targeting mouse Nrf2 (product ID: s10901224150819; sense: 5’-GCCAGGA-GAGGUUACAGAAUAAGTdTdT3’; antisense: 3’-dTdT CGUCCUCUCCAUUCUAAU-5’) (RiboBio, Guangzhou, China) was transiently transfected to mouse primary cultured hepatocytes using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics, Mannheim, Germany) and incubated for 60 hours; 25 μM OA was added to the culture medium for the last 48 hours. The culture medium at a volume of 1.5 ml per well was 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 (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS and 100 U penicillin/streptomycin. Cells were seeded in a 96-well plate at a density of 1.5 × 10^4 cells per well. For Nrf2 transactivation assay, each well contains 100 ng of pGL3-ARE-Luc, 50 ng of pEF-Nrf2, and 5 ng of pRL-TK. For PXR transactivation assay, each well contains 100 ng of pGL3-CYP3A4-XREM-Luc, 50 ng of pSG5-hPXR, and 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 hours 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 was approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University (Guangzhou, China). OA (5, 10, and 20 mg/kg, i.p.) was prepared by suspending it in 2% Tween-80 saline. PCN (50 mg/kg, i.p.) was dissolved in corn oil as described previously (Staudinger et al., 2001; Xie et al., 2001). OA or PCN was administered to mice for 7 days, and LCA dissolved in corn oil (125 mg/kg, i.p.) was initiated from the fourth day, as described previously (Staudinger et al., 2001). Mice in the OA alone group were treated with the 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. Twenty-four hours after the final treatment, serum and liver tissue samples were obtained and snap-frozen on liquid nitrogen, then stored at −80°C until use.

Histologic and Biochemical Assessment. Liver tissues were immediately formalin fixed, paraffin embedded, sectioned, and stained with H&E following a standard protocol. The H&E-stained liver sections were examined using an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan). Liver injury or cholestasis was also evaluated by measuring serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activities; serum total bile acids; and bilirubin and hepatic total bile acids using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer’s instructions.

Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan).
5 ng of pRL-TK. For FXR transactivation assay, each well contains 100 ng of tk-EcRE-Luc, 50 ng of FXR expression vector, and 5 ng of pRL-TK. The transfection procedure was followed by instruction of Lipofectamine 2000 (Invitrogen). Six hours later, the transfection mixtures were removed and replaced with phenol red free Dulbecco’s modified Eagle’s medium containing 10% charcoal-stripped delipidated FBS. Transfected cells were then treated with OA (1, 5, and 25 μM) or respective positive agonist SFN (10 μM), RIF (10 μM), or CDCA (100 μM) for 24 hours. Luciferase activity was assayed in

Fig. 1. Hepatoprotection of OA against LCA-induced cholestasis. (A) Photographs of representative livers. Gall bladders are marked by arrows, and the apparent subcapsular necrotic foci in the liver could be seen in the LCA group. (B) Representative H&E-stained liver sections (10 × magnification). Areas of severe liver necrosis are marked by arrows. (C–H) Serum ALT, AST, and ALP activity, as well as serum total bile acid, total bilirubin, and hepatic total bile acid levels elevated by LCA were significantly reduced by cotreatment 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.
an Amersham Pharmacia Biotech luminometer using a Dual Reporter Assay System (Promega) according to the manufacturer’s directions. Firefly luciferase activity was normalized to Renilla activity for each well.

Statistical Analysis. All values were expressed as the mean ± S.E.M. One-way analysis of variance 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). For all figures, only the comparisons indicated above the bars are 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 morphologic and histologic assessments clearly indicated the severe hepatotoxicity and cholestasis caused by LCA (Fig. 1, A and B). Mice cotreated with 20 mg/kg OA or 50 mg/kg PCN exhibited remarkably fewer subcapsular foci of white discolorations in the liver and shrunk gallbladder size, as compared with mice treated with LCA (Fig. 1A). This morphologic change corresponded with histologic assessment results (Fig. 1B); H&E-stained liver sections showed large-scale necrotic foci in mice treated with LCA, and this pattern was significantly attenuated by coadministration of OA or PCN. Furthermore, 4-day LCA treatment caused 42% of mouse deaths; 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 cotreatment of 5, 10, and 20 mg/kg OA; in particular, the mice in the high-dose OA group and PCN cotreatment group all survived.

Serum biochemistry results were in good agreement with liver histology. The severe cholestasis caused by LCA was also indicated by a huge elevation of serum ALT, AST, and ALP to 4147, 4910, and 437 U/l, respectively (Fig. 1, C–E). In contrast, coadministration of OA dose dependently reversed the LCA-induced increase of ALT to 39%, 22%, and 14%; AST to 33%, 14%, and 6%; and ALP to 71%, 69%, and 42%, respectively. Likewise, Fig. 1 (F and G) illustrates 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 the vehicle group, whereas OA cotreatment dose dependently reduced LCA-induced increase of serum total bile acids to 47%, 47%, and 35% and 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 the vehicle group) to 69%, 57%, and 55%, respectively (Fig. 1H). In addition, 50 mg/kg of PCN cotreatment 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.

### TABLE 1
OA increases mouse survival rate against LCA-induced death

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival/Total (Percentage)</th>
</tr>
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<tbody>
<tr>
<td>Vehicle</td>
<td>6/6 (100)</td>
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<tr>
<td>OA 20 mg/kg</td>
<td>6/6 (100)</td>
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<tr>
<td>LCA</td>
<td>7/12 (58)</td>
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<tr>
<td>OA 5 mg/kg + LCA</td>
<td>9/10 (90)</td>
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<tr>
<td>OA 10 mg/kg + LCA</td>
<td>8/10 (80)</td>
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<tr>
<td>OA 20 mg/kg + LCA</td>
<td>10/10 (100)</td>
</tr>
<tr>
<td>PCN 50 mg/kg + LCA</td>
<td>6/6 (100)</td>
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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 Na+/taurocholate cotransporter (Ntcp) and organic anion transporter 1b2 (Oatp1b2) and are exported into bile by canalicular Bsep and Mrp2. In addition, canalicular multidrug resistance protein 3 (Mrp3) 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). As the best hepatoprotective effect was found in the high-dose group, 20 mg/kg OA was chosen for the following study. Figure 2 (A–C) illustrates that LCA markedly decreased the expression of Ntcp, Oatp1b2, and Mrp2 and increased Mrd3 and Mrp4 mRNA, whereas Bsep and Mrp3 expression were slightly changed. In comparison, OA cotreatment reversed LCA-induced decrease of Mrp2 to a normal level; meanwhile, Oatp1b2 and Ntcp decreases were partially recovered and Mrp3 was significantly induced. Notably, OA cotreatment further enhanced the upregulation of Mrp4 that was observed in the LCA group, whereas the elevation of Mrd3 by LCA was markedly weakened. Furthermore, OA alone significantly caused Mrp2, Mrp3, Mrp4, and Mrd3 upregulation (1.8-, 1.9-, 3.5-, and 1.9-fold higher than that in the vehicle group, respectively) and resulted in the downregulation of Ntcp and Oatp1b2. PCN cotreatment caused a similar effect on gene expression of hepatic transporters altered by OA, except that LCA-induced Mrd3 upregulation was not reversed by PCN. Together, the aforementioned data suggest that hepatoprotection of OA against LCA may be due to upregulation of Mrp2, Mrp3, and Mrp4 instead of other transporters.

In addition to the previous bile acid transporters, hepatic metabolism enzymes involved in bile acid homeostasis were also examined. Cyp7a1 is an enzyme mediating the rate-limiting step in the classic 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 Fig. 2 (D–G), 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-, 4.5-, and 4.0-fold higher, respectively. In comparison, coadministration 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 treatment alone had little impact on the mRNA levels of Cyp7a1, Cyp3a11, Cyp2b10, and Ugt1a1, but induced Sult2a1 expression. In addition, PCN cotreatment induced the previous five enzyme gene expressions when compared with that in the 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 shows that the mRNA of Fxr, Pxr, and Car was slightly changed by OA alone, whereas Nrf2 mRNA was significantly induced. LCA itself also increased Nrf2 mRNA expression, and cotreatment with OA further enhanced this induction. LCA resulted in a decrease of Fxr and Car mRNA expression but markedly increased Pxr mRNA expression. OA cotreatment mitigated these alterations, and especially reversed the LCA induced upregulation of Pxr and downregulation of Fxr and Car to normal levels. In contrast to LCA treatment alone, PCN cotreatment 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 Fig. 4, LCA treatment resulted in a significant increase in Mrp3 and Mrp4 protein expression and a decrease in Mrp2 protein expression. OA cotreatment caused a more robust Mrp3 and Mrp4 induction, whereas it reversed the down-regulation of Mrp2. OA treatment alone resulted in a significant upregulation of Mrp2, Mrp3, and Mrp4 at the 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.

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

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

**OA Induced Mrp Gene Expression via Nrf2 in Mouse Primary Cultured Hepatocytes.** To further demonstrate whether Nrf2 activation is required for OA-mediated upregulation of Mrp2, Mrp3, and Mrp4, a Nrf2 gene silencing experiment was performed using mouse primary cultured hepatocytes. Figure 5 (A and B) illustrates that OA time and dose dependently resulted in elevation of Mrp2, Mrp3, and Mrp4 expression, with maximum levels reached at 48 hours post-incubation with 25 μM OA, as compared with that in the vehicle group. A Nrf2 gene silencing experiment was further conducted. As illustrated in Fig. 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 (Fig. 5D); as expected, Nrf2 silence remarkably reduced the gene expression of Mrp2, Mrp3, and Mrp4 to 62%, 64%, and 5%, respectively, as compared with that in the negative control. Notably, the significant induction of Mrp2, Mrp3, and Mrp4 by OA was abrogated by Nrf2 silence. Thus, it is further demonstrated in mouse 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 Fig. 6A, SFN, a characterized Nrf2 activator, significantly increased the luciferase activity of the Nrf2 reporter gene to a level 3.5-fold higher than that in the vehicle group, whereas 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 Fig. 6 (B and C), compared with the vehicle group, 5 and 25 μM 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), whereas classic PXR agonist rifampicin and FXR agonist CDCA increased the luciferase activity by 5.6- and 14.5-fold, respectively. The previous data suggest that OA is a strong Nrf2 activator with moderate PXR and weak FXR agonism.

**Discussion**

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 a 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 upregulation of Mrp2, Mrp3, and Mrp4.

As a note of caution, it should be mentioned that, although the relative low dose (20 mg/kg or below, i.p.) of OA used in our study is hepatoprotective against cholestasis, higher doses of OA produce 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/day for 7 days (Sato et al., 2007). Very recently, it was 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, and liver injury was apparent at doses of 90 mg/kg and above; however, 22.5 mg/kg OA did not cause any liver injury or cholestasis in mice, suggesting quite a narrow margin of safety of OA (the ratio of toxic dose at 90 mg/kg and above of OA could produce cholestatic liver injury increases the ratio of non-toxic 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 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 the studies by Liu et al. (2013) and Lu et al. (2013). Taken together, this suggests that, for the therapy of cholestasis, OA is a double-edged sword; at doses of 90 mg/kg and above, OA can cause apparent liver injury and cholestasis, whereas at doses below 22.5 mg/kg, especially at 20 mg/kg, OA can protect against LCA-induced cholestasis. Therefore, caution should be taken with high doses 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
in the current study, OA had little impact on the five key metabolism enzyme genes: Cyp7a1, Cyp3a11, Cyp2b10, Sult2a1, and Ugt1a1. However, PCN cotreatment induced significant gene expression of Cyp3a11, Sult2a1, and Ugt1a1, since PCN is a classic PXR agonist that targets these genes to facilitate bile acid detoxification (Staudinger et al., 2001; Xie et al., 2001; Chen et al., 2003; Kitada et al., 2003). LCA greatly inhibited the ability of Ntcp and Oatp1b2 to defend against excessive bile acids entering hepatocytes. OA also caused a decrease in the gene expression of these two uptake transporters in normal mice, whereas cotreatment of OA with LCA led 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 alpha-naphthylisothioceyanate (ANIT) and bile duct ligation, was proven 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 upregulated by LCA in an adaptive responsive way; however, OA cotreatment alleviated this upregulation, possibly due to the same reason found with 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 Konig, 2000). In this study, OA remarkably induced the expression of these three Mrps at both the mRNA and protein level 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 proven 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 Chromatin immunoprecipitation (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 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 antioxidative property of OA involving Nrf2-mediated upregulation of glutamate cysteine ligase, NAD(P)H quinone oxidoreductase 1, and heme oxygenase-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 mouse liver, as well as Nrf2 activation using luciferase reporter assay in HepG2 cells. Furthermore, the Nrf2 silencing experiment conducted in mouse primary cultured hepatocytes directly proved that upregulation of Mrp2, Mrp3, and Mrp4 by OA was mediated via Nrf2, which was consistent with our in vivo results. In addition, we demonstrated that the effects of OA on the gene expression of Oatp1b2 and Nicp in mouse primary cultured hepatocytes were not affected when Nrf2 was silenced, as shown in Supplemental Fig. 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 in 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 a previous study (Sato et al., 2007). Furthermore, it should be noted that OA is an FXR antagonist as manifested in HepG2 cells (Liu et al., 2008). In the current study, OA had little impact on the five key metabolism enzyme genes: Cyp7a1, Cyp3a11, Cyp2b10, Sult2a1, and Ugt1a1. However, PCN cotreatment induced significant gene expression of Cyp3a11, Sult2a1, and Ugt1a1, since PCN is a classic PXR agonist that targets these genes to facilitate bile acid detoxification (Staudinger et al., 2001; Xie et al., 2001; Chen et al., 2003; Kitada et al., 2003). LCA greatly inhibited the ability of Ntcp and Oatp1b2 to defend against excessive bile acids entering hepatocytes. OA also caused a decrease in the gene expression of these two uptake transporters in normal mice, whereas cotreatment of OA with LCA led 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 alpha-naphthylisothioceyanate (ANIT) and bile duct ligation, was proven 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 upregulated by LCA in an adaptive responsive way; however, OA cotreatment alleviated this upregulation, possibly due to the same reason found with 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 Konig, 2000). In this study, OA remarkably induced the expression of these three Mrps at both the mRNA and protein level with or without LCA treatment, indicating that this induction may contribute to the hepatoprotection of OA by enhancing bile acid output.

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In our study, OA at 20 mg/kg caused no statistical change in FXR target genes such as \textit{Bsep}; however, OA at doses of 90 mg/kg or above produced a significant \textit{Bsep} decrease (Liu et al., 2013; Lu et al., 2013), suggesting FXR antagonism may be one of the mechanisms by which OA produces cholestasis at high doses. In addition, the data from the human PXR reporter assay indicated that OA could cause moderate PXR agonism; however, an in vivo experiment showed no significant induction of PXR target genes \textit{Cyp3a11}, \textit{Sult2a1}, and \textit{Ugt1a1} by OA, which may be explained by the fact that a species difference exists in PXR agonism by OA, or OA at relatively low doses.

Fig. 5. In vitro evidence of the upregulation of the Nrf2-Mrps pathway by OA. (A–B) OA dose dependently and time dependently induced Mrp2, Mrp3, and Mrp4 in mouse primary cultured hepatocytes. qRT-PCR analysis was performed to measure the gene expression, and data are expressed as the 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 the 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 mouse primary hepatocytes. qRT-PCR analysis was performed to measure the gene expression, and the data are the mean ± S.E.M. (\(n = 3\)). \(^*P < 0.05\) versus vehicle; \(^#P < 0.05\) versus OA alone. con, negative control small interfering RNA (siRNA); siNrf2, siRNA targeting mouse Nrf2.

Fig. 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, and 25 M) or respective positive agonist SFN (10 \(\mu\)M), RIF (10 \(\mu\)M), or CDCA (100 \(\mu\)M) for 24 hours. Data are the mean ± S.E.M. (\(n = 4\)). \(^*P < 0.05\) versus vehicle.
used in our study may not be sufficient to activate PXR and induce significant target gene expression. CAR was not examined in the 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 females are more susceptible to cholestasis (Kaplan and Gershwin, 2005); thus, we used male mice only in the current study 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. In addition, without the use of Mrp knockout mice, we cannot directly demonstrate that Mrp transporters are effectors of OA hepatoprotection, and this issue also requires further study.

In summary, the current study clearly demonstrated that OA at relatively 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 Nr2f2-mediated upregulation of Mrp2, Mrp3, and Mrp4, suggesting that Nr2f2 may be a promising cholestasis therapeutic target, discovering more effective and safe Nr2f2 activators would be necessary.

Authorship Contributions

Participated in research design: Bi, Huang, Chen.
Contributed new reagents or analytic tools: Chen, Zhou, Deng.
Performed data analysis: Chen, Bi, Xu.
Wrote or contributed to the writing of the manuscript: Chen, Bi.

References


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