Hepatoprotective Effects of Schisandra sphenanthera Extract against Lithocholic Acid–Induced Cholestasis in Male Mice Are Associated with Activation of the Pregnane X Receptor Pathway and Promotion of Liver Regeneration

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ABSTRACT

We previously reported that the ethanol extract of Schisandra sphenanthera [Wuzhi (WZ) tablet] significantly protects against acetaminophen-induced hepatotoxicity. However, whether WZ exerts a protective effect against cholestasis remains unclear. In this study, the protective effect of WZ on lithocholic acid (LCA)–induced intrahepatic cholestasis in mice was characterized and the involved mechanisms were investigated. WZ pretreatment (350 mg/kg) with LCA significantly reversed liver necrosis and decreased serum alanine aminotransferase activity. More importantly, serum total bile acids and total bilirubin were also remarkably reduced. Quantitative reverse-transcription polymerase chain reaction and Western blot analysis showed that hepatic expression of pregnane X receptor (PXR) target genes such as CYP3A11 and UDP-glucuronosyltransferase (UGT) 1A1 were significantly increased by WZ treatment. Luciferase assays performed in LS174T cells illustrated that WZ extract and its six bioactive lignans could all activate human PXR. In addition, WZ treatment significantly promoted liver regeneration via inhibition of p53/p21 to induce cell proliferation–associated proteins such as cyclin D1 and proliferating cell nuclear antigen. In conclusion, WZ has a protective effect against LCA-induced intrahepatic cholestasis, partially owing to activation of the PXR pathway and promotion of liver regeneration.

Introduction

Cholestasis is a clinical syndrome with toxic bile acid retention that is caused by obstruction of bile formation or secretion. Various liver diseases develop from cholestasis, including biliary atresia, primary sclerosing cholangitis, and primary biliary cirrhosis (Trauner et al., 1998). Lithocholic acid (LCA) is a toxic, hydrophobic secondary bile acid primarily formed in the intestine by bacterial 7α-dehydroxylation of chenodeoxycholic acid (Ridlon et al., 2006). Elevated levels of LCA are found in humans with cholestasis (Carey et al., 1966). Rodents treated with LCA can develop cholestasis and liver damage, and rats have thus been used as an applied model of intrahepatic cholestasis (Ceryak et al., 1998; Lucangioli et al., 2009; Wang et al., 2012b). The therapeutic strategy against cholestasis is mainly focused on improving elimination of excess toxic bile acids. However, ursodeoxycholic acid is currently the only Food and Drug Administration–approved drug to treat cholestasis, although its efficacy is limited to early stages of primary biliary cirrhosis (Ishizaki et al., 2005).

A number of metabolizing enzymes and transporters participate in bile acid elimination, including cytochrome (CYP) 3A, UDP-glucuronosyltransferase (UGT) 1A1, Sulfortransferase (SULT) 2A1, bile salt export pump (BSEP), multidrug resistance-associated protein (MRP) 2, MRP3, MRP4, sodium taurocholate cotransporting polypeptide (NTCP), organic anion transporter polypeptide 2 (Oatp2), and organic solute transporter β. Nuclear receptors and transcription factors, such as pregnane X receptor (PXR), farnesoid X receptor, constitutive androstane receptor, and nuclear factor E2–related factor, can regulate these genes and are considered potential therapeutic targets of cholestasis (Yu et al., 2002; Rizzo et al., 2005; Tan et al., 2010; Zollner et al., 2010). Agonists of these factors may have potential preventive effects against cholestasis (Zollner et al., 2010; Chen et al., 2014). PXR and its human homolog (steroid and xenobiotic receptor) are known to induce CYP3A and UGT1A1 expression (Goodwin et al., 1999; Chen et al., 2003). In rats, pregnenolone-16α-carbonitrile (PCN) is reported to act as a PXR agonist and can prevent LCA-induced hepatotoxicity (Staudinger et al., 2014).
2001b; Xie et al., 2001). In PXR null mice, PCN failed to prevent LCA-induced cholestasis and liver damage. In addition, glycyrrhizin, a PXR agonist, has been demonstrated to protect against LCA-induced cholestasis (Wang et al., 2012b). These findings suggest that PXR plays an important role in bile acid elimination and its agonists may have potential protective effects against cholestasis. In addition, the p38/p21 pathway plays an important role in liver regeneration after liver injury (Wu et al., 1996). Several cell cycle–related proteins, such as cyclin D1 (CCND1) and proliferating cell nuclear antigen (PCNA), are regulated by p21, which may explain their therapeutic effects in treating cholestatic liver damage (Yang et al., 2011).

**Schisandra sphenanthera**, the dried ripe fruit of *S. sphenanthera* Rehd. et Wils, is widely used as a tonic and a nutritive food in many countries. The Wuzhi (WZ) tablet, containing 7.5 mg schisandrin A (SinA) per tablet, is a preparation of the ethanol extract of *S. sphenanthera*. In China, WZ is prescribed to protect liver function in patients with chronic hepatitis and liver dysfunction (registration number Z20025766). In our previous study, we determined the chemical fingerprint of WZ by using high-performance liquid chromatography with photodiode array detection analysis; we also determined the relative amounts of its major active lignans, including SinA, schisandrin B (SinB), schisandrin C (SinC), schisandrol A (SoLA), schisandrol B (SoLB), and schisandrin A (SinA) (Qin et al., 2014). Previous studies demonstrated that WZ protects against a number of hepatotoxins that induce acute liver damage, including carbon tetrachloride and acetaminophen (Zhu et al., 2000; Xie et al., 2010; Bi et al., 2013). On the basis of these data, we hypothesized that WZ may have potential hepatoprotective effects against cholestasis. This study aimed to investigate the protective effects of WZ against LCA-induced cholestasis in mice and to explore the potential mechanisms.

**Materials and Methods**

**Chemicals and Reagents.** The WZ tablets were manufactured by Fenglue Pharmaceutical Company (Guangxi, China) and were quantified to 7.5 mg SinA per tablet. WZ extract prepared by ethanol extraction was performed as described previously and quantified by the concentration of SinA in it (Bi et al., 2013; Qin et al., 2013; Fan et al., 2014). SinA, SinB, SinC, SoLA, SoLB, and SinA were all produced by Shanghai Winherb Medical Science and Technology Development Co., Ltd. (Shanghai, China). LCA (purity >98%) was purchased from Aladdin Company (Shanghai, China). PCN was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The pS5-human pregnane X receptor (hPXR) expression vector was generously provided by Dr. Steven Kliewer (University of Texas Southwestern Medical Center, Dallas, TX) (Lehmann et al., 1998). The pGL3-CYP3AA-xenobiotic responsive enhancer module (XREM) luciferase reporter construct containing the basal promoter (−362/+/53) with the proximal PXR response element (ER6) and the distal XREM (−7836/−7208) of the CYP3AA gene 5′-flanking region inserted to pGL3-basic reporter vector was generously provided by Dr. Jeff Staudinger (University of Kansas, Lawrence, KS) (Goodwin et al., 1999). The pRL-TK *Rovylechus rufus* control vector was obtained from Promega (Madison, WI). All other chemicals and solvents were commercially available and of analytical grade.

**Animals and Treatment.** Adult male C57BL/6 mice, weighing 21–25 g, were purchased from Guangdong Animal Experimental Centre (Guangzhou, China). Mice were maintained on standard laboratory chow and were allowed food and water ad libitum. The animal study protocols were approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University (Guangzhou, China). WZ (350 mg/kg) was dispersed in saline and administered orally twice a day for 7 days. The dose was calculated from the dose used in clinical practice, as described previously (Bi et al., 2013; Qin et al., 2013; Fan et al., 2014). PCN and LCA were dissolved in corn oil and injected intraperitoneally either once (PCN, 50 mg/kg) a day for 7 days or twice (LCA, 250 mg/kg) a day for 4 days, as described previously (Staudinger et al., 2001). For WZ and PCN protection, WZ or PCN was administered for 3 days. Subsequently, WZ and PCN treatment was continued for another 4 days, during which time animals were also injected with LCA (250 mg/kg per day) (Staudinger et al., 2001). Mice were euthanized 12 hours after the final LCA injection. Liver photographs were taken first, followed by collection of serum and liver samples, which were snap-frozen in liquid nitrogen and stored at −80°C until use. The full animal study methods are illustrated in (Supplemental Fig. 1).

**Histologic and Biochemical Evaluation.** Liver tissues fixed in neutral buffered formalin were embedded in paraffin, cut into sections, and stained with hematoxylin and eosin stain (H&E) according to a standard protocol. H&E-stained liver sections were examined using a Leica DM5000B microscope (Leica, Heidelberg, Germany). Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were analyzed using commercial kits (Kefang Biotech, Guangzhou, China) on a Beckman Synchro CX5 Clinical System (Beckman Coulter, Brea, CA). Serum total bile acids (TBAs) and total bilirubin levels were analyzed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. For histology evaluation, tissues were immediately fixed in formaldehyde, embedded in paraffin, sectioned, and stained with H&E.

**Quantitative Reverse-Transcription Polymerase Chain Reaction Analysis.** Total RNA from mice liver tissue or cultured HepG2 cells 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 by using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Biotech, Kyoto, Japan). Quantitative reverse-transcription polymerase chain reaction (PCR) analysis was performed using the SYBR Premix Ex Taq II (TaKaRa Biotech) on an Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The glycolaldehyde-3-phosphate dehydrogenase gene for mice was run for each sample to normalize expression. The gene-specific primers were obtained from a primer bank (Spandidos et al., 2010; Wang et al., 2012), and the sequences are listed in (Supplemental Table 1).

**Western Blot Analysis.** Proteins extracted from mice liver tissue were prepared using radioimmunoprecipitation assay lysis buffer (BioColor BioScience and Technology, Shanghai, China) and were determined by the BCA Protein Assay (Thermo Scientific, Rockford, IL). Forty micromgms of protein sample was separated in 8%–12% SDS-PAGE 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 CYP3A11 (L-14) and p21 (F-8) (Santa Cruz Biotechnology), UGT1A1 (ab62600) and p53 (bap240, ab26) (Abcam, Cambridge, MA), glycolaldehyde-3-phosphate dehydrogenase (14c10) (Cell Signaling Technologies, Danvers, MA), CDDN1 (AB20509b), and PCNA (AB20014) (Sangon, Shanghai, China). A secondary horseradish peroxidase–conjugated anti-rabbit, anti-mouse, or anti-goat IgG antibody (Santa Cruz Biotechnology) was subsequently applied, and then specific bands were visualized using an enhanced chemiluminescence detection kit (Engreer Biosystem, Beijing, China).

**Transient Transfection Assays.** LS174T cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 100 U penicillin/streptomycin. Cells were seeded in a 96-well plate at a density of 1.5 × 10^4 cells per well without antibiotics. For the PXR transactivation assay, each well contained 100 ng pGL3-CYP3A4-XREM-Luc, 50 ng pS5-g-hPXR, and 5 ng pRL-TK. The transfection procedure was followed by administration 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 fetal bovine serum. Transfected cells were then treated with SinA, SinB, SinC, SoLA, SoLB, SinA, or WZ extract (2.5, 10, 40 μM) or the respective positive agonist (10 μM rifampicin) for 24 hours. The concentrations of these ligands and WZ extract were chosen as described previously (Qin et al., 2010). Luciferase activity was assayed in a luminometer (Amersham Pharmacia Biotech, Little Chalfont, UK) using the Promega Dual-Luciferase Reporter Assay System according to the manufacturer’s instructions. Firefly luciferase activity was normalized to Renilla activity for each well.

**Statistical Analysis.** All values are expressed as means ± S.E.M. One-way analysis of variance, followed by the Dunnett’s multiple comparison post hoc test or the unpaired *t* test, was used for statistical analysis of data along with GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA). Differences with *P* values < 0.05 were considered significant.

**Results**

WZ Protects Against LCA-Induced Cholestasis and Liver Injury. Results of the morphologic and histologic evaluations illustrate...
that 4-day LCA treatment resulted in severe hepatic necrosis, diffuse vacuolization, infiltrating neutrophils, as well as gall bladder enlargement (Fig. 1). Mice pretreated with 350 mg/kg WZ or 50 mg/kg PCN abrogated LCA-induced liver damage, with liver appearance similar to the vehicle-treated group (Fig. 1).

Consistent with liver histopathology, LCA treatment caused a large increase in serum ALT, AST, and ALP levels to 4731, 4536, and 410 U/L, respectively (Fig. 2, A–C). Pretreatment with WZ reversed LCA-induced increases of ALT, AST, and ALP to 15%, 45%, and 38%, respectively (Fig. 2, A–C). Similarly, LCA treatment increased serum TBAs and serum total bilirubin levels to 947 and 27 μM, which was 8.2- and 3.8-fold higher than that of the vehicle-treated group, respectively (Fig. 2, D and E). Pretreatment with WZ reversed serum TBAs and total bilirubin levels to 23% and 56%, respectively (Fig. 2, D and E). Moreover, similar protective effects were also observed in the 50-mg/kg PCN pretreatment group. Taken together, these data demonstrate that WZ can protect against LCA-induced intrahepatic cholestasis and hepatotoxicity.

WZ Upregulates PXR Targeted Gene Expression Involved in Bile Acid Homeostasis. Figure 3 (A and B) illustrates that WZ alone significantly upregulated Cyp3a11, Ugt1a1, and Oatp2 mRNA levels, whereas the Cyp7a1 mRNA level was not affected. LCA also increased Cyp3a11 mRNA, whereas it inhibited Ugt1a1 and Cyp7a1 expression and had no significant effect on Oatp2. Interestingly, WZ pretreatment only significantly upregulated Ugt1a1, Oatp2, and Cyp7a1 expression, although no further induction effect was found for Cyp3a11 compared with the LCA-treated group. In addition, PCN as a PXR agonist performed a similar induction pattern in these genes.

Fig. 1. Hepatoprotective effects of WZ against LCA-induced cholestatic liver damage. (A) In situ photographs of representative livers. Gall bladders are marked by arrows and the apparent subcapsular necrotic foci in the liver can be seen in the LCA-treated group. (B) Representative H&E-stained liver sections (10 × 10). Areas of severe liver necrosis are indicated with arrows.

Fig. 2. Hepatoprotective effects of WZ against LCA-induced cholestatic liver damage. (A–E) Serum ALT (A), AST (B), and ALP (C) activity as well as serum TBAs (D) and total bilirubin (E) levels elevated by LCA were significantly reduced by pretreatment with 350 mg/kg WZ or 50 mg/kg PCN. Data are means ± S.E.M (n = 6–8). *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle; *P < 0.05, **P < 0.01, ***P < 0.001 versus LCA alone. Tbili, total bilirubin.
To confirm the above PCR results, we further determined the protein levels of CYP3A11 and UGT1A1 using Western blot analysis. As shown in Fig. 3 (D and E), the protein expression patterns of CYP3A11 and UGT1A1 were consistent with the mRNA levels.

Taken together, these data indicate that the protective effect of WZ on LCA-induced cholestasis may be due to its induction effect on hepatic Cyp3a11 and Ugt1a1, which results in accelerating detoxification of toxic bile acids.

**WZ and Its Lignans Activate hPXR in Human Cell Lines.** To further confirm whether WZ and its active ingredients can activate hPXR, the dual-luciferase reporter gene assay was performed in LS174T cells via transfection with reporter plasmids to determine WZ and the activation effects of its bioactive lignans on hPXR. As shown in Fig. 4, rifampicin (a classic hPXR agonist) significantly enhanced the luciferase activity of the hPXR reporter gene to a level 4.8-fold that of the control group, whereas WZ enhanced hPXR reporter gene activity, with a notable 3.8-fold elevation at 10 μM. Meanwhile, all six bioactive lignans (SinA, SinB, SinC, SolA, SolB, and SinA) showed a similar activation effect on hPXR at 10 and 40 μM. These results demonstrate that WZ and its main active lignans can activate hPXR, which may play a role in its hepatoprotective effects against LCA-induced cholestasis.

**WZ Suppresses the p53/p21 Signaling Pathway to Promote Liver Regeneration.** p53 and p21 levels were investigated to explore whether WZ can block p53/p21 signaling to promote liver repair in mice after LCA-induced toxicity. Expression of p53 and p21 was slightly affected by WZ treatment but was dramatically elevated by LCA administration compared with the vehicle-treated group. In addition, WZ pretreatment strikingly reversed the elevation of p53 and p21 by LCA treatment. Furthermore, levels of cell cycle–regulating protein CCND1 and PCNA expression were enhanced by WZ administration, compared with that of the vehicle-treated group. CCND1 and PCNA expression were also slightly induced by LCA treatment (Fig. 5), whereas WZ preconditioning further induced expression levels compared with the LCA-treated group. Interestingly, PCN showed effects on CCND1 and PCNA protein expression, similar to WZ. Taken together, these findings suggest that WZ can inhibit the p53/p21 pathway and induce CCND1/PCNA-mediated liver regeneration during cholestatic liver injury.
which produce more hydrophilic, less toxic metabolites that are more hydroxylation and phase II conjugation by metabolizing enzymes, UGT1A1, CCND1 and PCNA, is significantly weaker than PCN.

This may be explained by the fact that the induction effects of WZ on the mRNA levels of PCN returned most of the above index to a normal level. This may be because the hepatoprotective effect of WZ against LCA-induced cholestasis was possibly due to PXR-mediated upregulation of CYP3A11 and UGT1A1, as well as promotion of p53/p21-mediated liver regeneration.

In this study, the WZ dosage was 700 mg/kg per day, which is converted from the clinical dose. We also previously observed an obvious hepatoprotective effect of WZ against acetaminophen-induced liver damage at this dose (Bi et al., 2013; Fan et al., 2014). LCA-induced liver damage as well as TBAs, total bilirubin levels, and serum ALT, AST, and ALP were significantly altered by LCA treatment, although WZ pretreatment reversed these changes (Supplemental Fig. 2). However, WZ treatment alone could not affect expression of any of these genes, which suggests that the reversal effect on gene expression by WZ may affect other CYP7A1 modulators that counteract the regulation effect of PXR. This result is crucial and indicates that WZ would not affect normal bile acid synthesis, which suggests the safety of WZ pretreatment elevated transcription of CYP7A1. This study revealed that WZ treatment could significantly upregulate liver UGT1A1, which may due to the observed reversal effect on serum total bilirubin by WZ pretreatment. Since PXR is a modulator of UGT1A1 (Chen et al., 2003), we assume that the induction effect of WZ on UGT1A1 is also via the activation of PXR.

We thus further studied whether WZ and its active components can activate PXR. We found that WZ, together with its six main components (SinA, SinB, SinC, SolA, SolB, and Smn), could activate hPXR, which was consistent with previous report (Mu et al., 2006). Therefore, we assume that WZ produces a hepatoprotective effect against LCA-induced cholestasis by activating PXR and subsequently inducing CYP3A11 and UGT1A1, resulting in the acceleration of LCA detoxification.

CYP7A1 is the rate-limiting enzyme during bile acid synthesis and plays a crucial role in bile acid homeostasis. CYP7A1 could be negatively regulated by PXR agonists (Staudinger et al., 2001; Li and Chiang, 2005). Interestingly, no significant regulation of WZ on hepatic CYP7A1 was found without LCA treatment. On the contrary, WZ pretreatment elevated transcription of CYP7A1 compared with the LCA-treated group. This may be due to the net regulation of CYP7A1; WZ may affect other CYP7A1 modulators that counteract the regulation effect of PXR. This result is crucial and indicates that WZ would not affect normal bile acid synthesis, which suggests the safety of WZ treatment on normal status.

In addition to the genes mentioned above, a variety of metabolism or transporter genes (e.g., Mrp2, Mrp3, Mrp4, Bsep, Ntcp, Mdr2, and organic solute transporters a and b) are involved in bile acid homeostasis. We observed several genes in which hepatic mRNA levels were significantly altered by LCA treatment, although WZ pretreatment reversed these changes (Supplemental Fig. 2). However, WZ treatment alone could not affect expression of any of these genes, which suggests the reversal effect on gene expression by WZ pretreatment with LCA was attributable to reduced toxic bile acid retention, which can cause alteration in expression of these genes.

We previously found a significant induction effect of WZ on several proteins related to liver regeneration after acetaminophen-induced hepatotoxicity (Fan et al., 2014); thus, we also investigated p53/p21-related liver regeneration pathways. LCA treatment caused a noticeable elevation in p53/p21 expression as well as a slight upregulation of PCNA and CCND1. As expected, WZ pretreatment displayed significant inhibition of p53/p21 expression, along with induction of PCNA and CCND1. Moreover, PCN pretreatment exhibited a similar regulation pattern, which suggests the potential role of PXR in liver regeneration.
This is further supported by a previous report that the liver regeneration process was delayed in PXR-deficient mice after a partial hepatectomy (Dai et al., 2008). Interestingly, the p53 pathway could also negatively regulate PXR activity (Eliai et al., 2013), indicating the possibility that WZ activates PXR via inhibition of the p53/p21 pathway.

In conclusion, this study clearly demonstrates that WZ S. sphenanthera extract could prevent LCA-induced intrahepatic cholestasis and hepatotoxicity. Furthermore, this hepatoprotective effect is possible due to activation of the PXR pathway and promotion of liver regeneration.

Authorship Contributions

Participated in research design: H. Zeng, Huang, Bi.


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