Calculus Bovis Sativus up-regulates hepatic protein 2 (Mrp2) and Mrp4 in 17α-ethynylestradiol-induced cholestasis via a regulatory effect on ER signaling

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Abstract

OBJECTIVE: To investigate the pathway through which Calculus Bovis Sativus (CBS) up-regulates hepatic multidrug resistance-associated protein 2 (Mrp2) and Mrp4 in 17α-ethynylestradiol (EE)-induced cholestasis.

METHODS: Five groups of rats were designed: control group, EE+ICI182780 group, EE group, EE+CBS 50 mg/kg group and EE + CBS 150 mg/kg group. CBS (50 and 150 mg·kg⁻¹·d⁻¹) was orally given to rats by gavage for five consecutive days in coadministration with EE. The levels of cholestasis biomarkers, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (TBIL) were determined by biochemical methods. The bile flow was measured. The histopathology of the liver tissue was evaluated. The expression of Mrp2, Mrp3, Mrp4, estrogen receptor α (ERα) and ERβ was determined by Western blotting.

RESULTS: CBS markedly improved EE-induced cholestasis. EE exposure significantly reduced hepatic Mrp2 and Mrp4 expression compared with the control group. EE also dramatically up-regulated the expression of Mrp3. Compared to the EE group, CBS notably up-regulated hepatic Mrp2 and Mrp4 but failed to influence the Mrp3 level significantly. ICI182780, an ER antagonist, showed similar beneficial effects as CBS. Decreased expression of Mrp2 and Mrp4 caused by EE was also restored by ICI182780. Additionally, EE significantly induced hepatic ERα expression, which was reversed by ICI182780 or CBS (150 mg/kg) treatment, suggesting that CBS exerted a moderate regulatory effect on ER signaling.

CONCLUSION: CBS up-regulated hepatic Mrp2 and Mrp4 expression in EE-induced cholestasis, which might be associated with its regulation of ER signaling.

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Keywords: Calculus Bovis; Ethynylestradiol; Receptors, estrogen; Multidrug resistance-associated proteins; ICI182780

INTRODUCTION

Cholestasis usually affects the function of bile acid and
leads to several diseases.⁶ A potent synthetic estrogen, 17α-ethynylestradiol (EE), is known to reduce bile
flow in experimental animals, representing a useful
model to assess the mechanism involved in intrahepatic
cholestasis.⁷ Due to an obvious decrease in bile salt-independent bile flow (BSIF) observed in rats, the
repression of the canalicular multidrug resistance-assoc-
ated protein 2 (Mrp2) by EE seemed to be a key
event in the pathogenesis of cholestasis.⁸ As an ATP-de-
pendent organic anion efflux pump, Mrp2 is mainly
located on the apical membrane of polarized epithelial
and endothelial cells, predominantly those in the liver,
kidney and intestine.⁹ In humans, Mrp2 mediates the
biliary excretion of organic anions (e.g., bilirubin glu-
curonides, GSH conjugates), contributing to the forma-
tion of BSIF. The absence of functional Mrp2 from the
canalicular membrane causes conjugated hyperbilir-
ubinemia, as observed in the hereditary disorder de-
scribed by Dubin and Johnson.⁴ Thus, preservation of
Mrp2 expression and function at the canalicular level is
relevant not only to BSIF formation but also to the bili-
ary elimination of endogenous compounds such as bili-
rubin glucuronides and xenobiotics. Moreover, hepatic
basolateral membrane efflux transporters—e.g., Mrp3
and Mrp4—also mediate the elimination of organic
anions and bile acids from hepatocytes into sinusoidal
blood. The up-regulation of efflux transporters in the
liver, such as Mrp2, Mrp3 and Mrp4, has been proven to
counteract to the relieved liver injury in different
cholestasis.⁵,⁶,⁷

Synthetic medicine and Traditional Chinese Medicine
(TCM), including ursodeoxycholic acid (UDCA), ri-
fampin, phenobarbital, Yin-Chen-Hao-Tang and Yin-Zhi-Huang, have been used for the treatment of
cholestasis in recent years.⁸ Among them, UDCA is the
standard treatment of primary biliary cirrhosis (PBC) and is also used for other cholestatic diseases.⁹
Unfortunately, most patients do not have a full re-
response to UDCA. Despite considerable progress, effec-
tive therapy for most cholestatic liver disease is lacking.¹⁰

Calculus Bovis Sativus (CBS) is a commonly used
TCM exerting various pharmacological activities such
as antispasmodic, anti-inflammation, fever-relieving
and gallbladder-repairing effects.¹¹ We have previously
demonstrated that CBS could efficiently alleviate exper-
imental intrahepatic cholestasis.¹²,¹³ However, the cellu-
lar signaling pathways involved have not been fully elu-
cidated. In this study, CBS effect on EE-induced cho-
lestasis and the mechanism behind the action were inves-
tigated with emphasis on the regulation of ER signaling.

MATERIALS AND METHODS

Chemicals and reagents
CBS (dissolved with 0.5% sodium carboxymethyl cellulose) was provided by Wuhan Jianmin Dapeng Phar-
maceuticals Co., Ltd. EE was purchased from Sigma-
Aldrich (St. Louis, MO; purity ≥ 98%). ICI182780
was purchased from Sigma-Aldrich (St. Louis, MO; pu-
urity > 98%). Alanine aminotransferase (ALT), aspartate
aminotransferase (AST), alkaline phosphatase (ALP)
and total bilirubin (TBIL) kits were purchased from Ji-
ancheng Institute of Biotechnology (Nanjing, China).
Mrp2, Mrp3, Mrp4, estrogen receptor α (ERα), and
estrogen receptor β (ERβ) antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA,
USA). Glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) antibody was purchased from Santa Cruz
Biotechnology Inc (Santa Cruz, CA, USA).

Animals
Thirty adult male Sprague-Dawley rats, 8 weeks of age
and weighing (250 ± 20 g) were obtained from the
Center of Experimental Animal of Hubei Province
(Wuhan, China). All experimental procedures were car-
ried out in strict accordance with the guideline of the
Council on Animal Care of Academia Sinica, and the
protocol was approved by the Ethics Committee of
Puai Hospital, Tongji Medical College, Huazhong Uni-
versity of Science and Technology, China.

Research design
Animals were housed under standard and controlled
conditions with 12-h light/dark cycles and were given
free access to standard laboratory chow and tap water.
After acclimatization for one week before the experi-
ment, the rats were randomized into five experimental
groups (n = 6): (a) control group, rats were given nor-
mal saline (NS, containing 0.5% sodium carboxymeth-
yl cellulose, 1 mL/100 g) orally once per day for con-
secutive five days with the coadministration of vehicle
(propylene glycol, solvent of EE, 0.25 mL/100 g, sub-
cutaneously). (b) EE + ICI182780 group, rats were giv-
en ICI182780 (4 mg/kg, subcutaneously) once per
day for five consecutive days with the coadministration
of EE (5 mg/kg, subcutaneously). (c) EE group,
rats were given NS (containing 0.5% sodium carboxymeth-
yl cellulose, 1 mL/100 g) orally once per day for five
consecutive days with the coadministration of EE (5 mg/kg, subcutaneously). (d) EE + CBS 50
mg/kg group, rats were intragastrically administered
CBS (50 mg/kg body weight) once per day for five
consecutive days with the coadministration of EE (5
mg/kg, subcutaneously). (e) EE + CBS 150 mg/kg
group, rats were intragastrically administered CBS
(150 mg/kg body weight) once per day for five con-
secutive days with the coadministration of EE (5 mg/
kg, subcutaneously).

Experimental procedure
After the last dose of agents, all rats were fasted for
12 h before they were anesthetized. Bile was collected
for the determination of bile flow. Blood samples
were collected from the abdominal aorta for biochemical as-
says. After the rats were decapitated, the livers were re-
moved for histological assessment and Western blot-
ing analysis.
**Bile flow determination**
The rats were anesthetized with pentobarbital sodium (30 mg/kg body weight, intraperitoneally). The bile duct was cannulated with a PE10 polyethylene tube (inner diameter, 0.3 mm; outer diameter, 0.5 mm; Yihong Sci and Tech Co., Ltd., Wuhan, China), and bile was collected for 2 h to evaluate the bile flow.

**Serology determination**
The collected blood samples were placed at room temperature for 4-8 h and then were centrifuged at 3000 g for 10 min at 4 °C to retrieve the serum. The serum ALT, AST, ALP activities and TBIL contents were determined using commercially available kits (Jiancheng Institute of Biotechnology, Nanjing, China) and the DU730 Nuclear Acid/Protein Analyzer.

**Histological examination**
Liver samples were derived from the central part of the right large lobe of the rats and then were fixed in 10% formaldehyde for 24 h and embedded in paraffin. The paraffin-embedded livers were cut into 5-μm-thick sections, stained with hematoxylin and eosin solution and examined under a microscope.

**Western blot analysis**
Western blot analysis of Mrp2, Mrp3 and Mrp4 protein was performed in the membrane protein fractions of the liver. The membrane protein was extracted using the membrane protein extraction kit (Beyotime Institute of Biotechnology, China) according to the manufacturer’s instructions. The protein concentration was measured by the bicinchoninic acid (BCA) assay. Aliquots of protein (100 μg) were denatured at 95 °C for 5 min, and proteins were resolved by standard 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transfer to a polyvinylidene fluoride membrane (Millipore). The membrane was blocked with 5% nonfat milk solution and then incubated overnight at 4 °C in solution containing 0.1% Tween 20, 5% nonfat milk and anti-Mrp2, anti-Mrp3, anti-Mrp4, anti-ERα, anti-ERβ and anti-GAPDH at dilutions of 1: 200, 1: 200, 1: 200, 1: 500, 1: 500 and 1: 1000, respectively. Immunoreactive bands were quantified using the Gel-Pro Analyzer software (Media-Cybernetics, Bethesda, MD, USA). GAPDH was used as an internal index.

**Statistical analysis**
Data were expressed as the mean ± standard deviation. The significant differences between groups were assessed with SPSS version 13.0 (IBM Corp, Armonk, NY, USA). One-way analysis of variance with least significant difference post hoc analysis were performed to test the differences between group means. A P < 0.05 was the significant level.

**RESULTS**

**Bile flow and biochemical markers in rat serum**
At the beginning, prior to treatment, there was no significant difference among groups (P > 0.05). At the end of the experiment (5 d), as shown in Figure 1A-D, the serum level of ALT, AST, ALP and TBIL in the EE group was significantly higher than that in the control group (n = 6, P < 0.01). The increased serum ALT, AST, ALP and TBIL levels induced by EE were significantly reduced by CBS (50 and 150 mg/kg) or ICI182780 treatment. Bile flow was significantly inhibited in the EE group compared with that in the control group, indicating intrahepatic cholestasis (P < 0.01). CBS (50 and 150 mg/kg) or ICI182780 treatment significantly accelerated the hindered bile flow compared with that in the EE group (P < 0.05, P < 0.01 and P < 0.05 respectively, Figure 1E).

**Histological changes**
The hepatic samples of the control group showed normal cellular structures with distinct hepatic cells and sinusoidal spaces. The samples of the EE group underwent necrotic and degenerative changes, as well as severe interlobular duct epithelial damages with neutrophil cell infiltration. Compared with the samples of the EE group, those of the EE + CBS 50 mg/kg and EE + CBS 150 mg/kg groups encountered mild interlobular duct epithelial damages, less neutrophil cell infiltration and subtle necrotic and degenerative variations (Figure 2).

**Hepatic Mrps expressions**
Western blot studies indicated that the hepatic Mrp2 and Mrp4 protein levels were significantly suppressed in the EE group (P < 0.05). Coadministration of CBS (50 and 150 mg/kg) or ICI182780 with EE in rats markedly increased the Mrp2 and Mrp4 protein levels compared with that in the EE group (P < 0.05). Additionally, EE-induced elevation of the Mrp3 protein level did not influence CBS treatment significantly (Figure 3).

**ERs protein expressions**
EE treatment significantly increased ERα protein expression (P < 0.05) compared with ERβ, suggesting the involvement of ERα in the pathogenesis of EE-induced cholestasis. CBS (150 mg/kg) or ICI182780 obviously decreased ERα protein expression compared with that in the EE group (P < 0.05, Figure 4).

**DISCUSSION**
In this study, CBS treatment attenuated the elevation of the level of serum ALT, AST, ALP and TBIL induced by EE. CBS treatment markedly accelerated the hindered bile flow compared with the EE group. Additionally, CBS treatment obviously improved the liver lesion induced by EE. Additionally, our data indicated...
The beneficial effect of ICI182780, an ER antagonist, against EE-induced cholestasis. More importantly, these beneficial effects of CBS might be mediated through ER signaling.
In all identified cholestasis, estrogen-induced cholestasis is a common form. Estrogen can inhibit bile acid transportation from hepatocytes into bile canaliculi by interfering with the Bsep and Mrp2 as well.15 EE-induced cholestasis is a classical model to gain mechanistic and therapeutic insights into this condition. It is well known that EE administration leads to a marked decrease in bile flow, including BSDF and BSIF, in rodents.16 The formation of BSDF and BSIF depend on canalicular Bsep and Mrp2, respectively. Upon EE treatment, the expression of canalicular Bsep and Mrp2 were remarkably repressed.17,18 Additionally, the other basolateral Mrps, Mrp3 and Mrp4, were regulated differently by EE in cholestasis.19,20 EE treatment increased the expression of hepatic Mrp3 transcriptionally and required the participation of ERα.21 The compensation of impaired Mrp2 function in the hepatocyte canalicular membrane by basolateral Mrp3-mediated efflux of substances serves as a hepatoprotective response in cholestasis.22 Additionally, this protective basolateral efflux is also mediated by Mrp4 in the hepatocyte basolateral membrane.23 In this study, CBS notably up-regulated hepatic Mrp2 and Mrp4 but failed to influence Mrp3, suggesting that CBS exerted a hepatoprotective effect on cholestasis through different regulation of canalicular transporters.

A posttranscriptional regulation seemed to mediate the down-regulation of the level of Mrp2 protein in EE-treated rats. In accordance with the reduction in the protein level of Mrp2, immunofluorescence showed that there was a marked decreased labeling of Mrp2 at the canalicular membrane with EE treatment.24 This phenomenon has been attributed to the endocytic internalization of Mrp2 from the canalicular...
domain into the intracellular domain under cholestatic conditions. This short-term dynamic change in the Mrp2 location is a highly regulated posttranslational event requiring various cellular signaling pathways.

In general, biological actions of estrogenic compounds are mediated by estrogen receptors (ERs). The two isoforms of ER, ERα and ERβ, are involved in estrogen signaling pathways of classical and non-classical pathways. The participation of ER activation in the development of estrogen-induced cholestasis was confirmed using ICI182780, a high-affinity ER antagonist. ICI182780 (also named Fulvestrant) is a steroidal selective ER antagonist with no agonist effects. It binds, blocks and causes ER degradation, resulting in complete abrogation of estrogen target gene transcription. Preclinical studies have confirmed the potential of ICI182780 to inhibit the growth of human breast cancer cell lines. Clinical studies also have demonstrated that ICI182780 is an effective treatment option in postmenopausal women with advanced breast cancer who have progressed on prior endocrine therapy. ICI182780 partially prevented the decrease in Mrp2 activity induced by E,17G and protected transporter de-localization induced by estrogen. The liver expresses predominantly ERα, and its expression is under multiple regulations. It has been demonstrated in mice that the repression of hepatic transporters and alterations of bile acid biosynthesis were mediated by the binding of EE to ERα, contributing to the development of EE-induced cholestasis. Additionally, tamoxifen, an ERα antagonist, obviously decreased serum ALP levels in PBC patients, representing novel treatment. All these results suggest the potential of the ER/ERα antagonist in the treatment of estrogen-induced cholestasis.

In our study, CBS treatment restored bile excretion and up-regulated Mrp2 and Mrp4 in EE-induced cholestasis. Additionally, our results indicated that ICI182780, an ER antagonist, exerted similar protective effects on EE-induced cholestasis, indicating the participation of ERs. CBS (150 mg/kg) also obviously reversed the induction of ERα by EE treatment, suggesting the moderate regulatory effect on ER signaling. In conclusion, our findings suggested that CBS could restore Mrp2 and Mrp4 expression in EE-induced cholestasis in rats, which might be due to its regulation of ER signaling. A limitation of our findings was the differences between species. Meanwhile, there are different signaling pathways, such as the Nrf2 signaling path-
way, that participate in the induction of Mrps in the liver.\(^6\)

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