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Pregnane X receptor-mediated induction of Cyp3a by black cohosh

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Abstract

Black cohosh (BC) has been widely applied for the treatment of menopausal symptoms. However, increasing concerns about herb-drug interactions demand the need for studies on the influence of BC on cytochromes 450. Cyp3a11 in liver was induced by 7-fold in wild-type mice treated with 500mg/kg black cohosh for 28 days compared to the control group as assessed by quantitative real-time PCR; no difference was found in small intestine and kidney, suggesting that up-regulation of Cyp3a11 by black cohosh was liver-specific. Western blot, activity assays and pharmacokinetic analyses established dose- and time-dependent induction of Cyp3a11. To determine the mechanism of Cyp3a11 induction, including the role of pregnane X receptor (PXR) in vivo and in vitro respectively, *Pxr*-null, PXR-humanized and double transgenic CYP3A4/hPXR mice, and cell-based luciferase assays were employed, thus we established that mouse PXR played a direct role in the induction of Cyp3a11; human PXR was not activated by black cohosh. Overall, these findings demonstrate that induction of Cyp3a11 is liver-specific and involved only mouse PXR, not the human counterpart. Thus, the incidence of herb-drug interaction in the patients administered black cohosh may not be mediated by human PXR and CYP3A4.

Keywords

pregnane X receptor; CYP3A; black cohosh; pregnenolone 16 α -carbonitrile; midazolam

Introduction

Black cohosh is mainly used as a dietary supplement for the improvement of perimenopausal or postmenopausal symptoms, as well as other gynecological disorders. A number of preparations made from the root of black cohosh are on the market, such as Remifemin and BNO 1055 (Viereck et al., 2005). Black cohosh was originally thought to

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Declaration of Interest

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have estrogen-like action, but recent evidence failed to support this point of view (Viereck *et al.*, 2005). It was reported that black cohosh may act via the central nervous system (Burdette *et al.*, 2003) or by interacting with estrogen receptors with mixed agonistic (tissue-selective) and antagonistic properties (Viereck *et al.*, 2005). A comprehensive review of the literature on the role of black cohosh in the treatment of menopausal symptoms led to the conclusion that more studies were needed to confirm its efficacy (Shams *et al.*, 2010).

Side effects of black cohosh are rarely reported, however, a few individual cases on possible hepatotoxicity have raised safety issues (Chow *et al.*, 2008; Cohen *et al.*, 2004; Teschke 2009; Teschke *et al.*, 2009). The herb-drug interaction is also of great concern in the safety of black cohosh (Borrelli and Izzo 2009; Kennedy and Seely 2010; Tirona and Bailey 2006). Others explored the cell toxicity and inhibition of CYP enzymes by black cohosh, suggesting its clinical safety (Huang *et al.*, 2010). However, the *in vivo* investigations into the safety of black cohosh are warranted to determine the influence of this herb on drug metabolism and upstream mediators.

CYP3A4, the major P450 in human liver, is regulated by the nuclear receptor, pregnane X receptor (Lehmann *et al.*, 1998; Xie *et al.*, 2000) and is involved in the detoxification of many clinically used drugs (Wienkers and Heath 2005). In mice, Cyp3a11 is the major mouse counterpart for CYP3A4 and controlled by murine PXR (Xie *et al.*, 2000) a number of chemically unrelated endogenous and xenobiotic compounds, including steroids, bile acids, prescription drugs and herbal medicines, can activate PXR (Carnahan and Redinbo, 2005), thus it plays a vital role in pharmacology and physiology (Staudinger *et al.*, 2001; Wagner *et al.*, 2005; Stanley *et al.*, 2006; Fiorucci *et al.*, 2010). The cross-species between human PXR and murine PXR led to generation of the PXR-humanized mouse model (Xie *et al.*, 2000) and PXR/CYP3A4 double humanized transgenic model (Ma *et al.*, 2008) for the prediction of potential drug-drug interaction and toxicity mediated by the human PXR pathway. PXR ligands are structurally diverse, and as noted above, display species specificity. Rifampicin is an agonist for human PXR but does not activate mouse PXR, while pregnenolone-16 α -carbonitrile (PCN), a rodent-specific PXR agonist, does not activate human PXR. Triterpene glycosides, the major components in black cohosh, have a similar structure as steroids, such as the PXR activators PCN and dexamethasone. To investigate the potential function of black cohosh on PXR-mediated drug-drug interactions, ovariectomized wild-type, *Pxr*-null, PXR-humanized and PXR/CYP3A4 double humanized transgenic mice were treated with black cohosh extracts, including 8% triterpene glycosides for 28 days. Black cohosh activated the mouse PXR but had no influence on human PXR, suggesting the possible lack of herb-drug interactions on drug metabolism involving PXR and CYP3A4.

Materials and methods

Chemicals

Extracts of black cohosh, actein and 23-epi-26-deoxyactein were obtained from Planta Analytica, LLC (Danbury, CT, USA). Rifampicin (Rif), pregnenolone 16 α -carbonitrile (PCN), ketoconazole, midazolam, 1'-hydroxymidazolam (1'-OH midazolam), 4-hydroxymidazolam (4-OH midazolam), nicotinamide adenine dinucleotide phosphate (NADPH), corn oil and sodium carboxymethyl cellulose (CMC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals and treatment

Female C57BL/6J wild-type (Jackson Laboratory, Bar Harbor, Maine), *Pxr*-null (Staudinger *et al.*, 2001), PXR-humanized (hPXR) (Ma *et al.*, 2007a), and human CYP3A4 and PXR

transgenic (Tg3A4/hPXR) (Ma *et al.*, 2008a) mice were housed under controlled conditions and fed purified diet (AIN-93G, Dyets Inc., Bethlehem, PA, USA) with free access to water. All animal experiments were performed under guidelines approved by the National Cancer Institute Animal Care and Use Committee.

Bilateral ovaries were removed under isoflurane anesthesia. Briefly, two longitudinal incisions (around 0.5cm) were cut respectively in the midline area of the back and the ovaries were removed. After surgery, mice were allowed one week for recovery before treatment. All mice (7- to 9-weeks-old) were fed with synthetic purified diet without phytoestrogen for 7 days before treatment. The extract of black cohosh, suspended in 0.5% CMC solution, PCN (10 mg/kg) or Rif (10 mg/kg) used as positive controls, suspended in corn oil, was intragastrically administrated to wild-type, *Pxr*-null, hPXR or Tg3A4/hPXR mice. hPXR and Tg3A4/hPXR mice were gavaged BC for 28 days or treated with PCN or Rif for 3 days. Wild-type mice, were treated with black cohosh for the dose-dependence experiments, were separated into four groups, vehicle group (ig 100 ml/kg 0.5%CMC), low-dosage group (ig 10 mg/kg BC), middle-dosage group (ig 100 mg/kg BC) and high-dosage group (ig 500mg/kg BC). For the time-dependence experiments, wild-type or *Pxr*-null mice were treated with vehicle (ig 100 ml/kg 0.5% CMC) or high-dosage BC (ig 500 mg/kg BC) for the different time intervals, one-day, three-day, seven-day, 14-day and 28-day.

Assessment of liver function

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were measured to evaluate the toxicity of BC. In brief, 1 μ l of serum was mixed with 200 μ l of AST or ALT assay buffer (Catachem, Inc., Bridgeport, CT, USA) in a 96-well microplate, and the oxidation of NADH to NAD⁺ was monitored at 340 nm for 5 min.

Quantitative real-time PCR (qPCR) analysis

Complementary DNA (cDNA) was synthesized from 1 μ g total mRNA using SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). qPCR was performed on the Applied Biosystems Prism 7900HT Sequence Detection System. Reactions were conducted in a 10 μ l volume of 25 ng cDNA, 300 nM of each primer and 5 μ l of SYBER Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) at 95 °C for 10 min, 95 °C for 3 sec and 60 °C for 30 sec for 40 cycles. The primer sets were listed in the Supplemental Table 1. The levels of cDNA were quantitated by the comparative threshold cycle method using β -actin as an internal standard.

Preparation liver microsomes and western blot analysis

One ml of liver homogenate from 300 mg tissue, prepared in 4 °C buffer (50 mM Tris-HCl, 150 mM KCl and 1 mM EDTA), was centrifuged at 9 000g for 20 min at 4 °C, and the supernatant was spun at 100 000g for 60 min at 4 °C. The pellet was suspended in the 4 °C buffer with 20% glycerol. Protein concentrations were determined using a BCA Protein Assay Kit. To access the amount of target protein, microsome was subjected to electrophoresis on a 4–15% SDS-PAGE and transferred to a PVDF membrane. The target protein was specifically bound to the primary antibody of anti-rat CYP3A1/2 mAb (Goldfarb *et al.*, 1993), anti-rat CYP2C polyclonal Ab (Nakajima *et al.*, 1994), anti-rat CYP1A1/2 mAb (Nakajima *et al.*, 1994) and the internal control Grp78 and detected with chemiluminescent agent (Thermo Scientific, Rockford, IL, USA) after incubating with corresponding secondary antibody (Cell Signaling, Danvers, MA, USA).

Detection of Cyp3a activity

Midazolam was used as a relatively specific substrate of Cyp3a enzymes, producing 1'-OH midazolam and 4-OH midazolam as the primary metabolites. Ketoconazole (1 μ M) was used as an inhibitor of Cyp3a. The incubation initiated by addition of 20 μ l 20 mM NADPH to the reaction mixture after 5-minute preincubation in 400 μ l 96-well plates containing a series concentrations of midazolam, at 0.1, 0.25, 0.5, 5, 25, 50 μ M, with 0.5 mg/ml liver microsomes and Tris buffer for 10 min. Hepatic microsomes were prepared from mice treated without or with 10 mg/kg, 100mg/kg and 500 mg/kg BC for 28 days, and the *Pxr*-null mice treated without or with 500 mg/kg BC for 28 days. The reactions were terminated by the addition of 200 μ l acetonitrile with 0.04 μ g/ml phenacetin as internal standard for LC-MS/MS analysis as described previously (Ma et al, 2008). To measure the inhibition of BC on the activity of Cyp3a, incubation of midazolam with or without 5 μ M, 25 μ M and 100 μ M BC dissolved in DMSO added to the reaction mixtures was performed.

Pharmacokinetic analysis

All the pharmacokinetic studies were performed in the wild-type mice. After a 14-day treatment of mice with 500 mg/kg BC or 3-day treatment of mice with 10 mg/kg PCN or 3-day co-treatment with 10 mg/kg PCN or 500 mg/kg BC (PB), blood samples were collected into heparinized tubes from the post-orbital venous plexus veins at 2, 5, 10, 20, 30, 60, 90, 120 min post intravenous injection of midazolam 0.3mg/kg and centrifuged at 8 000 rpm for 5 min at 4°C. Plasma was obtained and stored at -80°C until assay. Urine samples were collected at 0–30, 30–90 and 90–120 min intervals after intravenous injection of midazolam 0.3 mg/kg to 14-day treatment of mice with 500 mg/kg BC and the volume of urine was measured immediately, and then stored at -80°C until assay.

LC-MS/MS analysis of plasma and urine samples

Plasma (50 μ l) was mixed with 150 μ l of phosphate-buffered saline and 3ml of methyl t-butyl ether containing 20 μ l of 0.04 μ g/ml phenacetin. The supernatant was taken out and dried with N₂ after centrifugation at 3 000 rpm for 5 min at 4 °C. The residue was dissolved in 80 μ l of 70% methanol and 30% H₂O containing 0.1% formic acid for LC-MS/MS analysis as described previously (Ma et al, 2008). Urine (10 μ l) was mixed with 900 μ l of 100 mM sodium acetate (pH 4.75) containing 40 U glucuronidase (Sigma, St. Louis, USA) and incubated at 37°C overnight before adding 500 μ l 1M NaOH to stop the reaction. The mixtures were extracted using 3ml methyl t-butyl ether containing 20 μ l of 0.04 μ g/ml phenacetin. After centrifugation, the supernatant was dried and reconstituted with 80 μ l of 70% methanol and 30% H₂O containing 0.1% formic acid for LC-MS/MS analysis as described previously (Ma et al, 2008).

Transient transfection assays

HepG2 cells (purchased from ATCC, Manassas, VA, USA) were plated in 24-well plates (5 $\times$ 10⁵cells/well) and cultured in phenol red free Dulbecco's Modification of Eagle's Medium (DMEM) with 10% charcoal stripped fetal bovine serum (Gemini Bio-products, CA, USA). Twenty-four hours later, cells were cultured in phenol red free DMEM containing 2% charcoal stripped fetal bovine serum, and then cotransfected with 50 ng murine PXR, and 200 ng DR3-luciferase reporter (this construct is a pGL4-TK vector with three copies of DR3: AGGTCA_{nnn}AGGTCA; provided by Grace Guo, University of Kansas Medical Center, Kansas, USA) (Pacyniak et al., 2007) and 10 ng internal control plasmid pRL-SV40. After 24h posttransfection, the cells were treated with 0.1% dimethyl sulfoxide (DMSO), 10 μ M PCN, 10 μ g/ml, 25 μ g/ml or 50 μ g/ml BC, 10 μ M or 50 μ M actein and 10 μ M or 50 μ M 23-epi-26-deoxyactein. After 24 h treatment, the cells were

harvested and the dual luciferase assay was performed according to the Promega's protocol on the Veritas Microplate Luminometer (Turner Biosystem, CA, USA).

Statistical analysis

Results were expressed as means $\pm$ standard deviation. Differences between different groups were examined using student t-test.

Results

The toxicity of BC on liver

To determine the toxicity of BC, two main biochemical markers of hepatic toxicity, ALT and AST, were measured in serum of wild-type, *Pxr*-null, Tg3A4/hPXR and hPXR mice with or without 28-day 500mg/kg BC treatment (Supplemental Fig. 1). There was no difference in serum ALT and AST between untreated and treated wild-type mice, and no significant difference in *Pxr*-null and Tg3A4/hPXR mice, while AST was elevated up to 3-fold in hPXR mice with no change in ALT. Although AST increased in hPXR mice, its elevated value did not reach to levels indicative of significant hepatotoxicity.

Gene expression in liver, small intestine and kidney

Messenger RNAs (mRNAs) for various xenobiotic transporters and xenobiotic-metabolizing enzymes were quantified in liver, small intestine and kidney from BC-treated wild-type mice; PCN-treated wild-type mice were used as the positive control. The mRNAs encoding the phase I enzymes, Cyp3a11, Cyp3a13, Cyp2c29, Cyp1a2, Cyp2e1, Cyp2b9, the phase II Ugt1a1, and the transporters, Oatp2 and Mdr1a in the liver, kidney and small intestine were measured using qPCR. Only Cyp3a11, Cyp2c29, and Oatp2 increased in the liver of the 28-day BC treated mice (Fig. 2) while the expression of other genes did not change (Supplemental Fig. 2). In the kidney or small intestine, the expressions of these genes were not different from those in the untreated mice (Supplemental Figs. 3–4). Cyp3a11, Cyp2c29 and Oatp2 mRNAs were increased in the livers (Fig. 2), and Cyp3a11, Cyp3a13 and Cyp2c29 elevated in the small intestine of 3-day PCN treated mice (Supplemental Fig. 5) although there was no change in the kidney except a small increase in Cyp1a2 mRNA (Supplemental Fig. 6).

Elevated expression of Cyp3a11, Cyp2c29, Cyp1a2 and Oatp2 mRNAs in BC-treated wild-type mice revealed a dose-response relationship. Compared to the vehicle-treated group, Cyp3a11 mRNA level was elevated 2-fold and 7-fold in wild-type mice treated with 100 mg/kg and 500 mg/kg BC, respectively, while there was no change in the 10 mg/kg extract-treated group (Fig. 2A). Induction of Cyp3a11 mRNA in the highest dosage treated group was comparable with that in the 10 mg/kg PCN treated group. The increase of Cyp2c29 mRNA in the 500 mg/kg BC treated group was up to 2-fold, with only a small elevation observed in the 100 mg/kg BC treated group. Compared with the unchanged level of Cyp1a2 mRNA in the 10 mg/kg PCN-treated mice, an elevation of Cyp1a2 mRNA was observed in the 500 mg/kg BC-treated mice. Oatp2 mRNA levels increased 2-fold in the 500 mg/kg BC-treated mice. Interestingly, there was no change observed of expression level of Cyp3a11, Cyp2c29, Cyp1a2 and Oatp2 mRNAs in the *Pxr*-null mice treated with BC treatment.

Time-dependent profiles for the induction of Cyp3a11 and Cyp2c29 mRNAs revealed a significant increase of Cyp3a11 mRNA approaching to maximal levels at day 14; similarly, an increase of 2-fold of Cyp2c29 mRNA was observed from day 3 to day 28, and a transient increase in Cyp1a2 mRNA at day 3 (Fig. 2B). However, the expression profiles of these genes in the *Pxr*-null mice behaved differently except for Cyp1a2. Although there was a statistical decrease at day 14 for Cyp3a11 mRNA or at day 1 for Cyp2c29 mRNA, and

increased expression of Cyp1a2 mRNA at day 3, there was no time-specific change of these three mRNAs. The effect of BC on the human PXR and CYP3A4 was determined using hPXR or Tg3A4/hPXR mice revealing no change in the expression of Cyp3a11 and human CYP3A4 (Fig. 2C).

Protein expression in liver

Cyp3a, Cyp2c and Cyp1a were detected by immunoblot analysis. As shown in Fig. 3, there were two bands for Cyp3a or Cyp2c detected near 50kDa. For Cyp3a, the upper band was the main component while the lower band was a female-specific Cyp3a enzyme according to earlier studies (Sakuma *et al.*, 2000). Both bands showed a dose-dependent and time-dependent induction by BC in wild-type mice and no time-dependent tendency increase in the *Pxr*-null mice (Fig. 3A and 3B). Because of the multiple members of Cyp2c subfamily (Wang *et al.*, 2004), these two bands cannot be discriminated by polyclonal antibody against Cyp2c. Thus, the total contribution of the two bands corresponding to Cyp2c was used to evaluate the change of Cyp2c. The induction of Cyp2c protein was modest in the wild-type mice, which is consistent with mRNA results. No change of Cyp2c was observed in the *Pxr*-null mice. The amount of Cyp1a was not influenced by BC in either wild-type or *Pxr*-null mice. In agreement with the mRNA levels, the Cyp3a and CYP3A4 proteins were not influenced by BC in hPXR or Tg3A4/hPXR mice (Fig. 3C).

Activity assay for Cyp3a

The midazolam-mediated activity of the main induced enzyme Cyp3a was consistent with the results from qPCR and western blot (Fig. 4A). An increase in 1'-OH midazolam was detected from the range of 0.10 μ M to 50 μ M of midazolam in the wild-type mice from the 28-day middle or high dose BC treatment groups; the significant elevation at 50 μ M noted in the high dose group was lowered 2-fold by co-incubation with ketoconazole, a specific inhibitor of Cyp3a. Similarly, the production of 4-OH midazolam was also enhanced at high concentrations of midazolam (Fig. 4B). The production of 1'-OH midazolam and 4-OH midazolam in the *Pxr*-null mice after 28-day 500 mg/kg BC treatment was similar with that in the non-treated mice (Fig. 4C and Fig. 4D).

Pharmacokinetics of midazolam

The mean plasma concentration–time profile of midazolam in wild-type mice was evaluated (Fig. 5). Pharmacokinetic parameters of midazolam and 1'-OH midazolam were estimated as shown in Table 1. In the BC treated mice, a significantly decreasing concentration of midazolam was noted in the elimination phase at the 30, 60 and 90 min time points (Fig. 5A-1) resulting in a decreasing AUC and an increasing CL. The $t_{1/2}$ or MRT was not changed and no different pharmacokinetic behavior of midazolam was revealed in PCN- or PB-treated mice. On the other hand, T_{max} and C_{max} values for 1'-OH midazolam were changed in PCN-treated mice. Specifically, T_{max} decreased to 8.3 min from 20 min in PCN-treated mice compared with vehicle-treated mice and C_{max} increased by 1.3-fold. T_{max} of 1'-OH midazolam was also earlier in the BC- and PB-treated mice accompanied by an unexpected decrease in C_{max} and AUC. In addition, the concentrations of 1'-OH midazolam and 4-OH midazolam behaved in a totally reverse pattern compared with the parent substrate of Cyp3a in BC-treated mice (Fig. 5A-2, A-3). A similar phenomenon was also revealed when mice were treated with PB (Fig. 5B-2). To explain this contrary result, urine was collected at 30, 90 and 120 min after intravenous injection of midazolam (0.3 mg/kg). The urinary accumulation of unconjugated 1'-OH midazolam in the 28-day BC treated mice was dramatically elevated at all time points (Fig. 6A). Thus, the accelerated excretion of midazolam metabolites by BC resulted in the reverse pattern in BC- or PB-treated mice. Furthermore, in the activity assay with hepatic microsomes incubated with low or high

concentrations of BC, a dose-specific relationship for the inhibition effect of BC on Cyp3a was observed (Fig. 6B).

Activation of murine PXR by BC

To determine whether BC can activate PXR, a DR3-tk-luciferase construct was cotransfected into HepG2 cells with or without a construct expressing mouse PXR (Fig. 7). PCN, a prototypical inducer of mouse PXR, did not activate the DR3-tk-luciferase reporter in mouse Hepa cells but did activate the reporter in HepG2 cells (data not shown). There was no difference in luciferase activity in the presence of PCN, BC, actein or 23-epi-26-deoxyactein without mouse PXR transfection compared to the DMSO-treated HepG2 cells. There was a significant increase in the presence of 50µg/ml BC and 50µM 23-epi-26-deoxyactein in HepG2 cells transfected with mouse PXR. Thus, HepG2 cells were used as the platform for testing the activation of BC on murine PXR. In the presence of mouse PXR, the expression of DR3-tk-luciferase was activated dose-dependently at a concentration of 10 µg/ml, 25µg/ml or 50 µg/ml by BC, respectively, compared to that of DMSO treated group. The potency of BC was relatively low compared with the induction of PCN. However, the effective ligand among the low multiple components in BC was likely also relatively lower than the concentration of PCN used in this assay. The individual compounds in BC, actein and 23-epi-26-deoxyactein, showed less than 1.5-fold activation of the luciferase reporter. Overall, these results indicated that the induction of Cyp3a was mediated by mouse PXR while the fold-activation suggested that the collective contributions of individual compounds, actein and 23-epi-26-deoxyactein, may not be responsible for the activation of mouse PXR by BC.

Discussion

This study revealed that BC exhibited a species-specific induction of Cyp3a through nuclear receptor PXR. There are a number of studies reporting induction of Cyp3a by activation of PXR with herbs, such as hypericum perforatum (St. John's Wort), ginkgo biloba, coleus forskohlii, commiphora mukul, kava kava (Brobst *et al.*, 2004; Ding and Staudinger 2005; Li *et al.*, 2009; Moore *et al.*, 2000; Raucy 2003; Yeung *et al.*, 2008). Considering the widespread use of BC as a dietary supplement, the influence of BC on P450s was examined. In a clinical study (Gurley *et al.*, 2006; Gurley *et al.*, 2005), healthy young women, not in the peri or post-menopausal stage, were chosen for the induction and inhibition experiments to assess the effect of BC on CYP3A4, CYP1A2, CYP2D6 and CYP2E1. Furthermore, gender differences in the induction of P450 suggests that the endogenous steroid hormones play a role in the regulation of P450s (Anakk *et al.*, 2004; Hernandez *et al.*, 2006; Hernandez *et al.*, 2009; Sierra-Santoyo *et al.*, 2000; Yamada *et al.*, 2002). Black cohosh is mainly used as a dietary supplement in the population of perimenopausal or postmenopausal women. Considering the specific consumers and the influence of endogenous hormones, therefore, ovariectomized mice were chosen for studying the influence of BC on P450s.

CYP3A4 is the predominant P450 expressed in human liver and the main P450 contributing to drug metabolism. Since Cyp3a11 is its homolog in mouse, the expression of Cyp3a11 in liver, small intestine and kidney was determined revealing that Cyp3a11 was only elevated in liver and not in small intestine and kidney by BC, thus indicating liver-specific induction of Cyp3a11 by BC. This was confirmed at the mRNA, protein and activity level in wild-type mice treated with different doses of BC and different times. Results showed a dose and time-dependent induction of Cyp3a11, which was similar with the results from the well-known mouse PXR activator, PCN. To evaluate the functional significance of induction, Cyp3a11 enzyme activity was examined in vivo and in vitro. Hydroxylation of midazolam, a specific substrate of Cyp3a, was elevated dramatically in hepatic microsomes from BC-treated mice. In vivo, the well-known Cyp3a inducer, PCN, was also selected as a positive control in the

pharmacokinetic study. Its influence on the pharmacokinetic behavior of midazolam itself or its metabolites, such as $T_{\max}$ and $C_{\max}$, reflected the induction of Cyp3a by PCN. In the PCN-treated mice, the $T_{\max}$ of 1'-OH midazolam was earlier while $C_{\max}$ was elevated as expected. Thus, an early $T_{\max}$ is an indicator of the induction of Cyp3a. $T_{\max}$ of 1'-OH midazolam occurred earlier in the BC treated mice, which indicated that conversion of midazolam to 1'-OH midazolam by Cyp3a was faster in the BC-treated mice. The induced activity of Cyp3a in BC-treated mice also supported this view. In addition, the pharmacokinetic profile of midazolam indicated that concentrations in the elimination phase in BC-treated mice were significant lower than in the untreated mice. However, a series of concentrations of midazolam were incubated with hepatic microsomes in the presence or absence of the BC extract revealing that BC could inhibit the hydroxylation activity of microsomes in a dose-dependent manner. Because of the inhibition of BC on Cyp3a, the pharmacokinetic parameters, such as AUC and CL of midazolam, did not show significant changes between BC-treated and untreated mice other than the concentrations in the elimination phase. On the other hand, concentrations of the hydroxylation metabolites of midazolam, 1'-OH midazolam and 4-OH midazolam, were dramatically lower in BC-treated mice than that in untreated mice. Since the main pathway of elimination of the metabolites is renal, (Heizmann *et al.*, 1983), urine was collected after injection of midazolam to the mice after a 14-day BC treatment. The urinary accumulation of hydroxylated midazolam in the BC treated mice was much higher than that in the untreated mice. Since the striking urinary excretion of hydroxylation metabolites of midazolam, the induction of Cyp3a cannot be determined from the 1'-OH midazolam and 4-OH midazolam in BC-treated mice. Even though the increased dialysis of kidney in BC treated mice covered the increase of hydroxylation of midazolam in plasma, $T_{\max}$ and the *in vitro* evidence indicates that BC up-regulates the expression of Cyp3a and its activity. Only Cyp3a11, Cyp2c29 and Oatp2 were increased in the livers of 28-day BC-treated mice with no change in the kidney or small intestine although the induction of Cyp3a and Cyp2c in the liver and small intestine both occurred in the PCN-treated mice. The reason for this liver-specific induction is not known. Cyp3a11, Cyp2c29 and Oatp2 are the target genes of PXR or CAR (Knight *et al.*, 2008; Stedman *et al.*, 2005; Wagner *et al.*, 2005; Xie *et al.*, 2004), thus indicating that the induction of Cyp3a11 by BC may act through activation of PXR or CAR.

To elucidate the mechanism of induction of Cyp3a, *Pxr*-null mice were employed. The normal developmental and physiological characteristics of *Pxr*-null mice render them a reliable model to investigate the function of mouse PXR (Kliwer *et al.*, 1998; Ma *et al.*, 2008b). Interestingly, the basal level of Cyp3a11 in *Pxr*-null mice was higher than that in wild-type mice (Staudinger *et al.*, 2001) while the increased basal Cyp3a11 expression was not inducible by the PXR dependent pathway but by a PXR independent pathway. Thus in *Pxr*-null mice, the lack of induction of Cyp3a11 expression was caused by loss of PXR and not by saturated levels of Cyp3a11. Therefore, the *Pxr*-null mouse is a good model to investigate the PXR mediated pathways. Data from qPCR analysis, western blot and activity assays showed that there was no change in *Cyp3a* expression in this mouse line thus suggesting that the induction of Cyp3a by BC was mediated by PXR. In addition, an *in vitro* cell-based reporter assay supported this conclusion. Changes in luciferase activity were not observed in the presence of PCN without mouse PXR cotransfection compared with the DMSO treated group. Furthermore, the trace levels of hCAR in HepG2 cell is insufficient to activate CAR target genes (Sueyoshi *et al.*, 1999). Therefore, we exclude the effect of human PXR and CAR on the luciferase assay. The data showed that BC treatment resulted in a significant activation of mouse PXR compared to the control group although this induction was less robust than that of positive control, PCN. The percentage of actein or 23-epi-26-deoxyactein, main triterpene compounds in BC (Avula *et al.*, 2009; Jiang *et al.*, 2008), was about 2.5% or 1.6% respectively (data not shown). The relatively low amount of individual compounds in the BC extract compared to the concentration of PCN may be

responsible for explaining this phenomenon while other possible components of BC should also be investigated.

The effect of BC on human PXR was also examined because of the well-known species differences in ligand specificity of PXR (Gonzalez 2007; Willson and Kliewer 2002). Tg3A4/hPXR mice and hPXR mice are useful models to investigate the species-specific effects of PXR (Gonzalez 2007; Ma et al., 2008a). In Tg3A4/hPXR mice, BC did not change the expression level of CYP3A4 after a 28-day treatment, while in hPXR mice *Cyp3a11* mRNA was just statistically increased 1.5-fold. This indicates that there is no ligand in the components of BC extract that activate human PXR resulting in the induction of its target gene CYP3A4, thus suggesting that in clinical practice, BC may not induce the expression of CYP3A4 in pre or post menopausal women.

It is noteworthy that activation of PXR can also be involved in the hepatotoxicity of certain drugs (Cheng *et al.*, 2009; Guo *et al.*, 2004; Teng and Piquette-Miller 2007). Since PXR target genes are responsible for the metabolism of xenobiotics, the induction of PXR target genes may lead to the hepatic protection or toxicity. In this study, although there was no significant increase of ALT in the *Pxr*-null and hPXR mice after BC treatment, a slight tendency for increased serum enzymes was noted in both lines thus indicated that the induction of mouse PXR may mediate the hepatic protection against BC. A recent review about the safety of BC indicates that no direct evidence supports the hepatotoxicity of BC in humans but that attention should be paid to causal toxicity reports (Teschke 2010). The different dosages of BC used in these reports (Lude *et al.*, 2007; Mazzanti *et al.*, 2008) may result in the different conclusions about the hepatotoxicity of BC. This is an interesting point for further investigation.

In summary, mouse PXR is involved in the induction of Cyp3a by black cohosh with no influence on human CYP3A4 as revealed using the hPXR/CYP3A4 double transgenic mice, thus suggesting the clinical safety of black cohosh on drug-drug interaction and drug metabolism. CYP3A or PXR-associated interaction may not be likely to occur, not all. Because PXR can be involved in drug-drug interactions and hepatotoxicity, the findings in this study provide clues for further investigation of BC safety.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Chemical Structure of actein, 23-epi-26-deoxyactein and pregnenolone 16 α -carbonitrile.
Actein or 23-epi-26-deoxyactein is one of components in the extraction of black cohosh.
Pregnenolone 16 α -carbonitrile is a well-know murine PXR activator.

Fig. 2.

Quantitative real-time PCR analysis of mRNA from PXR target genes in the livers of the wild-type (WT), *Pxr*-null, hPXR and Tg3A4/hPXR mice. (A) Effect of BC on PXR target genes in liver of wild-type and *Pxr*-null mice in the dose-response experiment. Mice were treated orally without or with 10 mg/kg, 100 mg/kg and 500 mg/kg BC for 28 days, or 10 mg/kg PCN for 3 days as positive control. The expression levels of *Cyp3a11*, *Cyp2c29*, *Cyp1a2* and *Oatp2* mRNAs were measured. BC treated group and PCN treated groups were divided by dash line. Statistical analysis of comparison of treatment to control was calculated upon each control separately. (B) Effect of BC on PXR target genes in liver of wild-type and *Pxr*-null mice in the time-course experiment. Mice were treated orally with or without 500 mg/kg BC for 1 day, 3 days, 7 days, 14 days and 28 days. The expression levels of *Cyp3a11*, *Cyp2c29* and *Cyp1a2* mRNAs were measured. (C) Effect of BC on PXR target genes in liver of hPXR or Tg3A4/hPXR mice. Mice were treated orally with or without 500 mg/kg BC for 28 days, or 10 mg/kg Rif for 3 days as positive control. The mRNA expression levels were quantitated by $\Delta\Delta C_t$ method using β -actin as internal control. The relative fold of expression level in treated mice was expressed as mean $\pm$ SD, $n=4-5$, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$ compared with untreated mice. Pn, *Pxr*-null.

Fig. 3.

Western blot analysis of pooled hepatic microsomes prepared from wild-type (WT), *Pxr*-null, hPXR and Tg3A4/hPXR mice treated with BC. HLM, human liver microsomes. (A) dose dependent profile of Cyp3a, Cyp2c and Cyp1a in wild-type mice without or with 10 mg/kg, 100 mg/kg or 500 mg/kg BC for 28 days. (B) time dependent profile of pooled hepatic microsomes in wild-type (B-1) or *Pxr*-null (B-2) mice treated with 500 mg/kg BC. (C) effect of BC on CYP3A4 in Tg3A4/hPXR mice or Cyp3a in hPXR mice. Rifampicin was used as a positive control.

Fig. 4.

In vitro metabolism of midazolam to 1'-OH midazolam (A, C) or 4-OH midazolam (B,D) versus concentration of midazolam profiles following incubation of a series concentrations of midazolam, 0.1, 0.25, 0.5, 5, 25, 50 uM, with 0.5mg/ml liver microsomes from wild-type (A,B) or *Pxr*-null (C,D) mice treated with or without 10 mg/kg, 100 mg/kg and 500 mg/kg BC for 28 days. The upper right panel shows the metabolic profiles of 1'-OH midazolam or 4-OH midazolam following incubation with human liver microsomes. 4-OH Midazolam was not detectable when the concentrations of midazolam were below 0.5 uM. Human liver microsomes were used as a positive control for testing the metabolism of midazolam. Ketoconazole (1 uM) was used as an inhibitor of Cyp3a. Data were expressed as mean $\pm$ SD, n=4-5, *, p<0.05, **, p<0.01, ***, p<0.001 compared with microsomes from untreated mice.

Fig. 5.

Plasma concentration versus time profiles of midazolam and its hydroxylation metabolites following a single intravenous dose of 0.3mg/kg in wild-type (WT) mice (A) treated with 500 mg/kg BC for 14 days or (B) treated with 10 mg/kg PCN only or combining with 500 mg/kg BC for 3 days. Data were expressed as means $\pm$ SD, n=3, *, p<0.05, **, p<0.01, ***, p<0.001, compared with untreated mice. (A-1) plasma concentration versus time plot of midazolam (A-2) plasma concentration versus time plot of 1'-OH midazolam (A-3) plasma concentration versus time plot of 4-OHmidazolam. 4-OH midazolam was not detectable in the wild-type mice treated with 500 mg/kg BC. (B-1) plasma concentration versus time plot of midazolam (B-2) plasma concentration versus time plot of 1'-OH midazolam

Fig. 6.

(A) Cumulative renal levels of 1'-OH midazolam at different periods following a single intravenous dose of 0.3mg/kg midazolam in wild-type (WT) mice treated with 500 mg/kg BC for 14 days. (B) Inhibition of BC on the activity of Cyp3a. Incubation of midazolam with 0, 5, 25, 100uM BC dissolved in DMSO was performed in 400 ul 96-well plate containing a series concentration of midazolam, at 0.1, 0.25, 0.5, 5, 25, 50 uM, with 0.5 mg/ml liver microsomes in Tris buffer for 10 min. The reaction was terminated by 200 ul acetonitrile with 0.04 ug/ml phenacetin as internal standard for LC-MS/MS analysis. Data were expressed as mean±SD, n=3, *, p<0.05, **, p<0.01, ***, p<0.001 compared with untreated mice (A) or DMSO group (B).

Fig. 7.

Effect of BC on murine PXR-mediated Cyp3a reporter gene activation. HepG2 cells were cotransfected with 50 ng murine PXR, 200ng DR3-luciferase reporter and 10ng internal control plasmid pRL-SV40. After 24h post-transfection, cells were treated with 0.1% dimethyl sulfoxide (DMSO), 10uM PCN, 10ug/ml, 25 ug/ml or 50ug/ml BC, 10uM or 50uM actein and 10um or 50 uM 23-epi-26-deoxyactein. After 24 h treatment, cells were harvested and the dual luciferase assay was performed. The relative folds of luciferase activity in the PCN, BC, Actein and 23-epi-26-deoxyactein (Epi actein) were expressed as mean $\pm$ SD, n=3, *, p<0.05, **, p<0.01, ***, p<0.001, compared with DMSO treated cells.

Pharmacokinetic parameters for midazolam and 1'-OH midazolam after single intravenous administration of midazolam (0.3mg/kg) in wild-type mice. Vehicle is corn oil and CMC.

Table 1

Parameters	14-day treatment			3-day treatment		
	CMC	BC	Vehicle	PCN	PB	
Midazolam						
AUC ₀₋₉₀ (nmol·min/L)	8850±2350	6130±1120	1990±176	2210±119	2200±258	
AUC _{0-∞} (nmol·min/L)	8980±2360	6190±1134	2290±104	2520±193	2520±351	
CL (ml/min)	105 ± 24	149 ± 27	393 ± 18	358 ± 28	363 ± 51	
MRT (min)	15.3 ± 2.5	13.6 ± 0.5	36.4 ± 3.6	35.1 ± 10.9	36.3 ± 10.5	
t _{1/2} (min)	17.8 ± 1.1	16.2 ± 1.1	28.5 ± 4.9	31.1 ± 12.6	26.7 ± 11.8	
1'-OH Midazolam						
T _{max} (min)	23.3 ± 11.5	8.3 ± 2.9*	20.0 ± 0.0	8.3 ± 2.9**	10.0 ± 0.0***	
C _{max} (nmol/L)	333 ± 51	196 ± 38*	314 ± 49	398 ± 39*	91 ± 30** ††	
AUC ₀₋₉₀ (nmol·min/L)	21400±3810	8110±781**	17400±1950	15700±927	3510 ± 396*** †††	
AUC _{0-∞} (nmol·min/L)	28900±3040	10200 ±2720**	19700±2470	16300±764	3710±453*** †††	

Data were expressed as mean ± SD, n=3,

* p<0.05,

** p<0.01,

*** p<0.001, compared with CMC group or Vehicle group;

†† p<0.01,

††† p<0.001, compared with PCN group.