Expression of the breast cancer resistance protein (Bcrp1/Abcg2) in tissues from pregnant mice: effects of pregnancy and correlations with nuclear receptors

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Wang, Honggang; Xiaohui Wu, Kelly Hudkins, Andrei Mikheev, Huixia Zhang, Anshul Gupta, Jashvant D. Unadkat, and Qingcheng Mao. Expression of the breast cancer resistance protein (Bcrp1/Abcg2) in tissues from pregnant mice: effects of pregnancy and correlations with nuclear receptors. *Am J Physiol Endocrinol Metab* 291: E1295–E1304, 2006. doi:10.1152/ajpendo.00193.2006.—The breast cancer resistance protein (BCRP) plays an important role in drug disposition, including limiting drug penetration across the placental barrier. Our goal was to investigate the effects of pregnancy on Bcrp1 expression in pregnant mice. We examined Bcrp1 expression in placenta, kidney, liver, and small intestine at various gestational ages. Bcrp1 protein levels peaked at gestation day (gd) 15 in placenta, at gd 10 and 15 in kidney, and at gd 15 in liver; however, Bcrp1 protein levels in small intestine did not change significantly with gestational ages. Immunohistochemistry analysis revealed that the cellular localization of Bcrp1 in placenta, kidney, liver, and small intestine was not influenced by pregnancy. Bcrp1 mRNA levels were analyzed by quantitative real-time RT-PCR. In general, the effects of pregnancy on Bcrp1 protein somewhat lagged behind the effects on Bcrp1 mRNA. To further investigate the possible roles of nuclear receptors in the regulation of the Bcrp1 gene during pregnancy, we examined mRNA levels of aryl hydrocarbon receptor (AhR), hypoxia-inducible factor 1α (HIF1α), estrogen receptor α (ERα), estrogen receptor β (ERβ), and progesterone receptor and compared them with those of Bcrp1. Bcrp1 mRNA was significantly correlated with mRNA of AhR, HIF1α, and ERβ in placenta, with mRNA of HIF1α in kidney, and with mRNA of AhR and ERα in liver. These data suggest that Bcrp1 expression in mouse tissues can be altered by pregnancy in a gestational age-dependent manner. Such effects are likely mediated by certain nuclear receptors through a transcriptional mechanism.

Bcrp1; Abcg2; pregnant mice; tissue expression; nuclear receptors

BREAST CANCER RESISTANCE PROTEIN (BCRP; gene symbol Abcg2) is an ATP-binding cassette (ABC) efflux transporter with a broad range of substrate specificity (5, 21). BCRP is prominently expressed in the apical membrane of placental syncytiotrophoblasts, in the epithelium of the small intestine, and in the liver canicular membrane (20). This pattern of tissue distribution implies that BCRP plays an important role in drug disposition, including limiting drug penetration across the placental barrier and reducing absorption of drugs from the small intestine (21). Indeed, Bcrp1, the murine homolog of human BCRP, has been shown to significantly alter fetal distribution of topotecan, a BCRP substrate. The fetus-to-

plasma ratio of topotecan was increased twofold in pregnant mice treated with the BCRP inhibitor GF-120918 compared with the vehicle treatment controls (14). Inhibition of BCRP by GF-120918 also increased the oral bioavailability of topotecan from 40 to 97% in patients with cancer (18).

Pregnant women often need to take medications, as many suffer from complications, including epilepsy, hypertension, or pregnancy-induced conditions such as nausea and gestational diabetes. As a result, a major concern is the transfer of drugs across the placental barrier leading to potential toxicity to the fetus. Transports expressed in placenta play a crucial role in the distribution of drugs across the maternal-fetal barrier (8, 19, 37, 40). ABC efflux transporters P-glycoprotein (P-gp), multidrug resistance protein 2 (MRP2), and BCRP are highly expressed in the apical syncytiotrophoblast membrane of the placenta (20, 32, 34). Therefore, these transporters are expected to play an important role in the efflux of medications or xenobiotics from the fetal compartment to the maternal circulation, thus protecting the fetus from toxicity.

A growing number of studies have shown that pregnancy can significantly alter the expression and function of ABC drug transporters in tissues important for drug disposition. For example, expression of P-gp in human placenta at early gestational stages (13–14 wk) was 2–4.5 times greater than that at late gestational stages (38–41 wk; see Refs. 9, 22, and 35). In contrast, expression of MRP2 in human placenta significantly increased with gestational ages (26). Expression and function of hepatic Mrp2 of pregnant rats decreased to 50% of that of nonpregnant controls (2). Thus pregnancy is expected to affect drug distribution across the placental barrier, as well as absorption and elimination of drugs, by altering the expression and function of drug transporters in tissues important for drug disposition. Although a variety of studies regarding the effects of pregnancy on expression and function of P-gp and MRP2 have already been published, little is yet known about BCRP with this regard. A recent study (22) showed that BCRP expression in human placenta did not change during pregnancy. However, these studies were conducted with limited tissue samples, and substantial variation in BCRP expression was observed. Most recently, a more detailed analysis using a larger number of tissue samples (25) illustrated that BCRP expression in human placenta at preterm (28 ± 1 wk, 15 placentas) was approximately two times greater than that at

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term (39 ± 2 wk, 29 placentas). Yasuda et al. (39) also showed that Bcrp1 expression in rat placenta at gestation day (gd) 14 was significantly greater than that at gd 20 (term in rat is ~21 days). However, neither the effects of pregnancy on BCRP expression at earlier gestational ages nor the effects of pregnancy on BCRP expression in other tissues are currently known.

Therefore, in the present study, we systematically analyzed Bcrp1 expression in placenta from pregnant mice at various gestational ages to determine whether Bcrp1 expression in mouse placenta is altered during pregnancy. Liver, kidney, and small intestine are the major organs in which transporters play a crucial role in the absorption and elimination of drugs. Therefore, we also examined Bcrp1 expression in these tissues from pregnant mice and compared this with Bcrp1 expression in nonpregnant controls. We also analyzed mRNA expression of nuclear receptors aryl hydrocarbon receptor (AhR), hypoxia-inducible factor 1 (HIF1α), estrogen receptor α (ERα), estrogen receptor β (ERβ), and progesterone receptor (PR) to determine whether there are any correlations between mRNA expression of Bcrp1 and the nuclear receptors. Such nuclear receptors were selected because recent studies suggest that they are likely to be involved in the regulation of the BCRP gene (6, 7, 17, 38). The results obtained from these studies provide new insights into the regulation of BCRP expression by pregnancy.

**MATERIALS AND METHODS**

**Animals.** FVB wild-type mice were purchased from Taconic (Hudson, NY). Pregnant and nonpregnant mice were cared for in accordance with the United States Public Health Service policy for the Care and Use of Laboratory Animals. The animal studies were approved by the Institutional Animal Care and Use Committee at the University of Washington. The mice had free access to food (a standard diet) and water and were maintained on a 12:12-h automatically timed light-dark cycle at 18–20°C. The animal experiment procedure was essentially the same as described previously (23). Briefly, male mice of 7–9 wk of age, weighting 20–30 g, were mated with female mice of the same age and weight. Female mice demonstrating sperm plug were housed in new cages. Gestational age was calculated based on the estimated time of insemination (presence of sperm plug as gd 0). Progress of pregnancy in these female mice was regularly monitored by visual inspection and by measuring the increase in body weight. On gd 10, 15, and 19 (term in mice is ~20–21 days), pregnant mice were killed under anesthesia (pentobarbital sodium), and then tissues including placenta, liver, kidney, and small intestine were collected. Age- and weight-matched nonpregnant female mice were used as controls (gd 0). Tissues were snap-frozen in liquid N2 and stored at −80°C until use.

**Protein sample preparation, SDS-PAGE, and immunoblotting.** Approximately 10 mg of tissues were thawed on ice and mixed with 1 ml of ice-cold homogenization buffer containing 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 10 mM KH2PO4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease-inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). S-9 fractions were isolated from all the tissues using a standard protocol as described previously (3, 29). Briefly, after homogenization, tissue homogenates were centrifuged at 600 g for 5 min at 4°C. The supernatant was transferred to a new centrifuge tube and subjected to a centrifugation at 10,000 g for 15 min at 4°C. The S-9 fraction (supernatant) was then harvested. Aliquots of S-9 fractions were frozen at −80°C until use. For small intestine, crude membranes were also prepared as described previously (28). Protein concentrations were determined with the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA) using BSA as standard.

For immunoblotting, S-9 fractions or crude membranes were mixed with Laemmli buffer without heating. Proteins were separated on 9% SDS-polyacrylamide minigels in a Bio-Rad Mini-protein II electrophoresis cell and transferred to Immuno-P nitrocellulose membranes (Millipore, Billerica, MA). Nonspecific protein binding on the blot was blocked by incubation of the blot overnight in TBS-T buffer [10 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, and 0.05% (vol/vol) Tween 20] containing 5% mouse serum (Jackson ImmunoResearch, West Grove, PA), followed by incubation in 5% fat-free milk for 2 h. The blot was then incubated with Bcrp1-specific rat monoclonal antibody (mAb) BXP-9 (Kamiya Biomedical, Seattle, WA) for 1 h. Dilution ratios of BXP-9 for protein samples from placenta, kidney, liver, and small intestine were 1:200, 1:50, 1:150 and 1:100, respectively. The blot was washed three times with Tris-buffered saline-Tween 20 (TBS-T) and incubated with horseradish peroxidase (HRP)-conjugated affinity mouse anti-rat IgG (Jackson ImmunoResearch) for 1 h at 1:20,000, 1:5,000, 1:40,000 and 1:30,000 dilution for protein samples from placenta, kidney, liver, and small intestine, respectively. For detection of β-actin, mAb against β-actin (AC-15; Sigma, St. Louis, MO) was used as the primary antibody at 1:50,000 dilution and goat anti-mouse HRP conjugate antibody (Bio-Rad) as the secondary antibody at 1:25,000 dilution. The blot was developed and relative Bcrp1 protein levels determined by densitometric analysis as described previously (38). β-Actin was used as an internal control.

**Immunohistochemistry.** Five-micrometer sections of frozen tissues were cut using cryostat, air-dried, and then fixed in acetone and then processed by an indirect avidin biotin immunoperoxidase technique as described previously (1, 12). Briefly, the sections were blocked using an avidin biotin blocking kit (Vector Laboratories, Burlingame, CA), and rat mAb BXP-9 was applied at 1:10 dilution for placenta, kidney, and small intestine or at 1:5 dilution for liver. The sections were then incubated overnight at 4°C. Subsequently, the slides were washed in PBS and then incubated sequentially with biotinylated anti-rat antibody (1:300 dilution) and HRP-conjugated avidin-biotin-complex (Vector Laboratories). The color reaction was developed using 3,3′-diaminobenzidine (Sigma), and counterstained with hematoxylin, dehydrated, and coverslipped. For all samples, substitution of the primary antibody with an irrelevant rat IgG or with PBS was used as negative controls.

**Total RNA isolation and quantitative real-time TaqMan RT-PCR.** The effects of pregnancy on mRNA expression of Bcrp1 and the nuclear receptors AhR, HIF1α, ERα, PR, or PRA were quantified by TaqMan real-time RT-PCR. Membrane PR1 and PR2 were also analyzed because our previous study suggested that nonclassical membrane-bound PRs could also be involved in the regulation of BCRP (38). Total RNA was isolated from mouse tissues using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The concentration of RNA was determined by measuring optical density at 260 nm. The ratios of optical density at 260 to 280 nm of all RNA samples were determined to be between 1.7 and 2.0 to ensure that all RNA samples are highly pure. RNA integrity was examined by agarose gel electrophoresis. Single-strand cDNA was then synthesized from 4 μg of purified total RNA using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA), all in a volume of 50 μl. Real-time PCR reactions were performed using a TaqMan universal PCR master mix on the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The TaqMan probes and primers for mouse genes Bcrp1 (Mm00496364_m1), AhR (Mm00478932_m1), HIF1α (Mm00468869_m1), ERα (Mm00433149_m1), ERβ (Mm00599819_m1), PR (Mm00435625_m1), membrane PR1 (Mm00443985_m1), membrane PR2 (Mm01283155_m1), or β-actin (Mm00690793_s1) were assay-on-demand gene expression products purchased from Applied Biosystems. These primers have been validated by the manufacturer for detection of the above genes using real-time RT-PCR. The primers for PR are designed to amplify both PRα and PRβ simultaneously. Reactions were carried out in triplicate.
in a MicroAmp optical 96-well plate in a total volume of 20 μL/well. Each reaction mixture contained 10 μL of 2× TaqMan universal PCR master mix, 6.5 μL of sterile Millipore water, 1 μL of primer and probe mixture, and 2.5 μL (40 ng) of reverse transcription products. PCR conditions were as follows: 50°C for 2 min; 95°C for 10 min; 95°C for 15 s, and 60°C for 1 min (40 cycles). Quantification of relative mRNA levels was carried out by determining the threshold cycle (C_T), which is defined as the cycle at which the 6-carboxyfluorescein reporter fluorescence exceeds 10 times the SD of the mean baseline emission for cycles 3–10. β-Actin was used as an internal standard. To compare the relative mRNA levels of a specific gene at different gestational ages or in different tissues, a mixture of total RNA from placenta, liver, kidney, and small intestine was prepared and used as a calibrator for all the real-time PCR experiments. The mRNA levels of each test gene were normalized to β-actin, according to the following formula: C_T (test gene) − C_T (β-actin) = ΔC_T. Thereafter, the relative mRNA levels of each gene were calculated using the ∆∆C_T method: ΔC_T (test gene) − ΔC_T (test gene in the calibrator) = ∆∆C_T (test gene). The degree of changes of mRNA levels of Bcrp1, AhR, HIF1α, ERα, ERβ, PR, membrane PR1, or membrane PR2 was expressed as 2^{−∆∆C_T}.

Because of the potentially variable expression of β-actin in different tissues, we also measured Bcrp1 mRNA levels in different tissues using an absolute quantitation method. Briefly, single-strand cDNA was synthesized from 0.3 μg of purified total RNA isolated from mouse tissues using a high-capacity cDNA archive kit (Applied Biosystems) in a volume of 30 μL. Full-length double-strand Bcrp1 cDNA was then amplified using conventional PCR under the following conditions: 5 μL of 10× Pfu buffer, 0.1 μM forward primer, 0.1 μM reverse primer, 0.25 mM dNTP, 1 μL Pfu polymerase (Stratagene, La Jolla, CA), and 2 μL of single-strand cDNA from mouse placenta in a final volume of 50 μL. Conventional PCR conditions were 95°C for 5 min, followed by 95°C for 1 min, 58°C for 1 min, and 72°C for 2.5 min (35 cycles), and a final extension reaction for 7 min at 72°C. Forward and reverse primers for Bcrp1 were 5′-AAGGAAAAGC-GGGCCGCGATGTCCAGTAA-TGACCGTGTAGTA-3′ and 5′-GGGATCCTAAGATATCGTTTAAAGAACA-AATT-3′, respectively. The PCR product was purified using a High Pure PCR Product Kit (Roche) and quantified by measuring optical density at 260 nm. To perform absolute quantitation, single-strand cDNA samples prepared from all mouse tissues were subjected to real-time PCR as described above, along with a series of dilutions of the PCR product (double-strand Bcrp1 cDNA used as template) to generate a standard curve. The standard curve produced a linear relationship between C_T and the logarithm of the amounts of Bcrp1 cDNA (data not shown), allowing quantitation of Bcrp1 in mouse tissues based on their C_T values. The C_T values used for calculation were at a linear range of the standard curve. The levels of Bcrp1 mRNA were expressed as picomoles of Bcrp1 cDNA per milligram total RNA.

Standard analysis. Data were analyzed for statistical significance using one-way ANOVA analysis. Correlations between mRNA expression of Bcrp1 and nuclear receptors were assessed by Spearman rank analysis (SPSS version 10.0; SPSS, Chicago, IL) and expressed by the corresponding correlation coefficient r. Differences with P values of <0.05 were considered statistically significant.

RESULTS

Effects of Pregnancy on Bcrp1 Protein Expression in Mouse Tissues. We first examined whether pregnancy can affect Bcrp1 protein expression. Bcrp1 protein expression was analyzed by immunoblotting of S-9 fractions isolated from mouse tissues. For small intestine, crude membranes were also used. Relative protein levels were determined by densitometric analysis of the immunoblots. In placenta, Bcrp1 protein could be readily detected at all gestation days (Fig. 1A), and Bcrp1 protein levels at gd 15 were approximately four times greater than those at gd 10 and gd 19. Bcrp1 protein levels at gd 19 were slightly higher than those at gd 10, but the difference was not statistically significant (Fig. 1A). In kidney, Bcrp1 protein could also be readily detected, and its levels at gd 10 and gd 15 were approximately three times higher than those at gd 0 and gd 19. Bcrp1 protein expression was not significantly different between gd 0 and gd 19 and between gd 10 and gd 15 (Fig. 1B). Bcrp1 protein levels in liver peaked at gd 15, which were approximately two to three times greater than those at gd 0, gd 15, and gd 19, and no statistically significant differences in Bcrp1 protein were found between gd 0, gd 10, and gd 19 (Fig. 1C). We could not detect Bcrp1 protein in S-9 fractions from small intestine (data not shown); however, Bcrp1 protein expression could clearly be demonstrated in crude membranes isolated from small intestine (Fig. 1D). Although Bcrp1 protein expression in small intestine seems to progressively increase with gestational ages (Fig. 1D), no statistically significant differences were observed using one-way ANOVA analysis.
**Immunohistochemistry.** The proper cellular localization in tissues is essential for transporters to perform their in vivo transport function. Therefore, we also examined whether tissue localization of Bcrp1 in pregnant mice is altered by pregnancy. In placenta, Bcrp1 staining was mainly found in the syncytiotrophoblast, the epithelial lining of the chorionic villi. There was also strong positive staining of some apparent vascular structures within the myometrium (Fig. 2, A2–A4). In kidney, Bcrp1 staining was mainly observed in the renal proximal tubules (Fig. 2, B2–B4). In liver, Bcrp1 staining was primarily at the apical surface of the hepatocytes (Fig. 2, C2–C4). In small intestine, Bcrp1 staining was mainly detected in the apical membranes of epithelial cells of small intestinal villi. Also in cells located within the lamina propria (the core of the villi) staining was strongly positive for Bcrp1 (Fig. 2, D2–D4). In all the tissues, Bcrp1 staining was not observed in negative controls. Overall, the cellular localization of Bcrp1 in placenta, kidney, liver, and small intestine does not appear to be changed over the course of pregnancy. The pattern of Bcrp1 localization in kidney, liver, and small intestine of pregnant mice is similar to that previously reported for nonpregnant mice (13). Although not intended for quantitative determination of Bcrp1, Bcrp1 staining in placenta at gd 15 (Fig. 2A3) seems to be significantly stronger than that at gd 10 (Fig. 2A2) and gd 19 (Fig. 2A4), which is consistent with the immunoblot data (Fig. 1A). The same appears to be true for liver, when Bcrp1 staining at gd 15 (Fig. 2C3) is compared with that at gd 0 (Fig. 2C2) and gd 19 (Fig. 2C4).

**Effects of pregnancy on Bcrp1 mRNA expression in mouse tissues.** We then examined whether the effects of pregnancy on Bcrp1 protein were due to changes of Bcrp1 mRNA levels. Bcrp1 mRNA in mouse tissues can be readily detected by real-time RT-PCR at 23–28 cycles. Also, in the same type tissues, β-actin mRNA levels did not significantly change with gestational ages (data not shown). The Bcrp1 mRNA levels normalized to β-actin are shown in Fig. 3A. In placenta, Bcrp1 mRNA levels at gd 10 were not significantly different from those at gd 15; however, Bcrp1 mRNA levels at gd 19 were significantly decreased by ~30–40% compared with those at gd 10 and gd 15. In kidney, Bcrp1 mRNA levels at gd 10 were

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**Fig. 2.** Immunohistochemistry of Bcrp1 in mouse tissues. Localization of Bcrp1 in placenta (A1–A4), kidney (B1–B4), liver (C1–C4), and small intestine (D1–D4) from pregnant mice at indicated gestation days was determined by immunohistochemistry. Selected areas of tissues are shown, and Bcrp1 protein staining is indicated in brown and by arrows. Images shown are representative results obtained in typical experiments. Three or four sets of tissues at each gestation day were analyzed for kidney and small intestine or placenta and liver, respectively. NC, negative control.
approximately two times greater than those at gd 0, and Bcrp1 mRNA levels were comparable at gd 0, gd 15, and gd 19. In liver, the highest levels of Bcrp1 mRNA were at gd 10, which were approximately two to three times higher than those at gd 0. Bcrp1 mRNA levels in liver were not significantly different at gd 0, gd 15, and gd 19. Similarly, the highest expression of Bcrp1 mRNA in small intestine was observed at gd 10, which was approximately two to three times greater than that at gd 0. Again, no statistically significant differences in Bcrp1 mRNA were found between gd 0, gd 15, and gd 19 in small intestine.

We noticed significant variability in the expression of β-actin mRNA in different tissues. Based on the Ct values obtained, whereas the levels of β-actin mRNA in kidney, liver, and small intestine were comparable, its levels in placenta were ~10 times greater than those in other tissues, regardless of gestational ages (data not shown). Therefore, to compare the levels of Bcrp1 mRNA in different tissues, we performed absolute real-time PCR quantitation using double-strand full-length Bcrp1 cDNA as a template. The double-strand Bcrp1 cDNA was amplified from total RNA isolated from mouse placenta by conventional PCR. The absolute Bcrp1 mRNA levels in placenta were comparable to those in kidney at all gestation days (Fig. 3B). The levels of Bcrp1 mRNA in liver and small intestine were significantly lower than those in placenta and kidney (Fig. 3B). In each tissue, the effect of pregnancy on Bcrp1 mRNA illustrated by absolute quantitation (Fig. 3B) was similar to those demonstrated by normalization to β-actin (Fig. 3A).

**Effects of pregnancy on mRNA expression of AhR, HIF1α, ERα, ERβ, PR, and membrane PR1 or membrane PR2 in mouse tissues.** To investigate the possible mechanisms by which Bcrp1 gene is regulated by pregnancy, we examined the effects of pregnancy on mRNA levels of nuclear receptors AhR, HIF1α, ERα, ERβ, or PR as well as membrane PR1 and membrane PR2 using quantitative real-time RT-PCR. These genes were selected because they have been implicated in the regulation of BCRP (6, 7, 17, 38).

First, mRNA levels of AhR in placenta at gd 15 were approximately two times higher than those at gd 10 and gd 19. No statistically significant changes of AhR mRNA in kidney with gestation days were found. In liver, AhR mRNA levels at gd 10 and gd 15 increased by ~80% compared with those at gd 0, and then AhR mRNA levels at gd 19 decreased to approximately the same levels as gd 0. In small intestine, AhR mRNA levels first increased approximately fourfold at gd 10 compared with gd 0, and then decreased at gd 15 and gd 19 to the same levels as gd 0. In general, liver seems to express higher levels of AhR mRNA than kidney and small intestine (Fig. 4A).
In placenta, HIF1α mRNA levels at gd 15 increased approximately twofold compared with those at gd 10; however, HIF1α mRNA levels at gd 19 decreased to the same levels as gd 10. In kidney, HIF1α mRNA expression at gd 10 increased approximately twofold compared with that at gd 0, and then HIF1α mRNA levels at gd 15 and gd 19 decreased to the same levels as gd 0. In liver, HIF1α mRNA expression increased progressively 2.3-, 2.9-, and 5.1-fold at gd 10, gd 15, and gd 19, respectively, compared with gd 0. No significant changes of HIF1α mRNA were observed in small intestine with gestational ages. HIF1α mRNA expression in kidney and liver appears to be much greater than that in small intestine (Fig. 4B).

The levels of ERα mRNA in placenta at gd 10 were approximately three times higher than those at gd 15 and gd 19. Hepatic ERα mRNA at gd 10 and gd 15 increased ~2.5-fold and 1.8-fold, respectively, compared with gd 0. Hepatic ERα mRNA levels at gd 0 and gd 19 were comparable. There were no significant changes of ERα mRNA in kidney and small intestine with gestational ages. Hepatic expression of ERα mRNA appears to be much higher than renal and intestinal expression (Fig. 5A).

ERβ mRNA levels in placenta at gd 15 were approximately two times greater than those at gd 10 and gd 19. No significant difference in placental ERβ mRNA between gd 10 and gd 19 was noticed. Also, we did not observe any significant changes of ERβ mRNA in kidney with gestational ages. ERβ mRNA was barely detectable in liver, and a weak signal was detected only at gd 10 in small intestine (Fig. 5B).

Finally, relative changes of mRNA expression of PR and two nonclassical membrane PRs were analyzed (Fig. 6). Placental expression of PR mRNA at gd 10 was approximately two and three times greater than that at gd 15 and gd 19, respectively. In kidney, there were no significant changes in PR mRNA with gestational ages. PR mRNA was barely detectable in liver and small intestine.

Hepatic mRNA expression of membrane PR1 at gd 10, gd 15, and gd 19 increased 3.1-, 1.6- and 2-fold, respectively, compared with that at gd 0. No significant changes in mRNA of membrane PR1 with gestational ages were observed in
placenta, kidney, and small intestine. Expression of membrane PR1 mRNA in liver was generally greater than that in kidney and small intestine at all gestation days. For membrane PR2, no significant changes of mRNA expression with gestational ages were observed in all the tissues. Like membrane PR1, much greater expression of membrane PR2 mRNA was noticed in liver than in kidney and small intestine at all gestation days. Membrane PR2 mRNA levels in placenta were much higher than those in other tissues.

Correlations of expression of Bcrp1 mRNA with nuclear receptors in mouse tissues. Finally, we investigated whether expression of Bcrp1 mRNA was correlated with that of nuclear receptors, membrane PR1, or membrane PR2 in mouse tissues. Data obtained with Spearman rank analysis are summarized in Table 1. Significant correlations exist between Bcrp1 mRNA and HIF1α mRNA (Fig. 7A), between Bcrp1 mRNA and AhR mRNA (Fig. 7B), and between Bcrp1 mRNA and ERβ mRNA (Fig. 7C) in placenta; between Bcrp1 mRNA and AhR mRNA (Fig. 7D) and between Bcrp1 mRNA and ERα mRNA (Fig. 7E) in liver; and between Bcrp1 mRNA and HIF1α mRNA in kidney (Fig. 7F). We did not find any correlations between mRNA of Bcrp1 and any of the nuclear receptors in small intestine (Table 1). There were also no correlations of Bcrp1 mRNA with that of PR, membrane PR1, or membrane PR2 in all the tissues. These data suggest that it is possible that Bcrp1 expression in some mouse tissues is regulated by a transcriptional mechanism through certain nuclear receptors during pregnancy.

**Table 1. Correlations between mRNA of Bcrp1 and nuclear receptors**

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<th>Correlation</th>
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<tr>
<td>Bcrp1/HIF1α</td>
<td>−0.27</td>
<td>0.31</td>
</tr>
<tr>
<td>Bcrp1/ERα</td>
<td>−0.15</td>
<td>0.58</td>
</tr>
<tr>
<td>Bcrp1/ERβ</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bcrp1/PR</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bcrp1/membrane PR1</td>
<td>0.34</td>
<td>0.20</td>
</tr>
<tr>
<td>Bcrp1/membrane PR2</td>
<td>0.16</td>
<td>0.57</td>
</tr>
</tbody>
</table>

Bcrp, breast cancer resistance protein; AhR, aryl hydrocarbon receptor; ER, estrogen receptor; HIF, hypoxia-inducible factor; PR, progesterone receptor; r_s, Spearman rank correlation coefficient. ND, cannot be reliably determined by real-time PCR. *Significant correlations.

**Fig. 7. Correlations between mRNA of Bcrp1 and nuclear receptors.** Significant correlations between HIF1α and Bcrp1 mRNA in placenta (n = 24; A), between AhR and Bcrp1 mRNA in placenta (n = 24; B), between ERβ and Bcrp1 mRNA in placenta (n = 24; C), between AhR and Bcrp1 mRNA in liver (n = 16; D), between ERα and Bcrp1 mRNA in liver (n = 16; E), and between HIF1α and Bcrp1 mRNA in kidney (n = 16; F). In each type of tissue, the lowest relative mRNA level of a specific gene was set as 1, and all other mRNA levels of the same gene were normalized to the lowest level. Such normalized values were used to plot all the graphics in this figure.

DISCUSSION

Our results indicate that pregnancy can significantly alter Bcrp1 expression in mouse tissues such as placenta, kidney, and liver. Bcrp1 protein levels in kidney and liver of pregnant mice were generally increased at midgestational ages (gd 10 and/or gd 15) compared with nonpregnant female controls (gd 0) or term pregnancy (gd 19; Fig. 1, B and C). Bcrp1 protein expression in placenta peaked at gd 15 compared with gd 10 and gd 19 (Fig. 1A). However, intestinal Bcrp1 protein expression did not significantly change during pregnancy, although there seems to be a progressive increase with gestational ages...
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(Fig. 1D). These data are consistent with two recent studies with respect to the effects of pregnancy on BCRP expression in placenta. Yasuda et al. (39) showed significantly higher expression of rat Bcrp1 in placenta at gd 14 than at gd 20. Likewise, BCRP expression (protein and mRNA) in human placenta at midgestational stages (28 ± 1 wk) was reported to be significantly greater than that at term (39 ± 2 wk; see Ref. 25). In addition, we found that, although pregnancy can clearly affect Bcrp1 expression in certain mouse tissues, it does not appear to change cellular localization of the transporter in these tissues (Fig. 2). These results and those published by others (25, 39) support the notion that BCRP exerts its maximum protective function for fetus, and tissues such as kidney and liver, at midgestational ages. Therefore, the fetus may be particularly vulnerable to potential toxicity of BCRP substrate drugs in early pregnancy.

The molecular mechanisms of the dependence of Bcrp1 expression on gestational ages, and tissue-specific regulation of Bcrp1 expression by pregnancy, are currently unknown but may be related to the drastic changes in concentrations of pregnancy-specific hormones during pregnancy and the differential exposure of these hormones to different tissues. We have shown that treatment of BeWo cells with progesterone significantly increased BCRP expression in this model human placental cell line. A combination of progesterone with 17β-estradiol at concentrations of early or preterm pregnancy further increased BCRP expression compared with progesterone treatment alone; however, further increase of 17β-estradiol concentrations to the levels of term pregnancy decreased, rather than increased, BCRP expression (38). Therefore, the combined effects of progesterone and 17β-estradiol at concentrations of term pregnancy may contribute to the decrease of Bcrp1 expression in mouse placenta at term pregnancy.

The effects of pregnancy on Bcrp1 mRNA were next compared with the effects on Bcrp1 protein. Both Bcrp1 protein and mRNA data support the conclusion that, in general, pregnant mice at midgestational ages express significantly higher levels of Bcrp1 in placenta, kidney, and liver compared with nonpregnant female controls or term pregnancy. Nevertheless, expression of Bcrp1 protein somewhat lagged behind that of Bcrp1 mRNA. For example, Bcrp1 protein expression in placenta at gd 10 was significantly lower than at gd 15 (Fig. 1A); however, there was no significant difference in Bcrp1 mRNA between gd 10 and gd 15 (Fig. 3). In liver, the highest Bcrp1 mRNA expression was at gd 10, but the highest Bcrp1 protein expression was at gd 15. Likewise, Bcrp1 mRNA levels in small intestine at gd 10 were significantly higher than those at gd 15 and gd 19 (Fig. 3). However, Bcrp1 protein levels did not decrease at gd 15 and gd 19 compared with gd 10 (Fig. 1D). This phenomenon is possibly attributable to the fact that translation and synthesis of protein always lag behind synthesis of mRNA. The correlation between Bcrp1 mRNA and protein may also be affected by posttranscriptional factors such as translational efficiency and/or half-life of Bcrp1 protein. In small intestine, Bcrp1 protein may be particularly stable relative to Bcrp1 mRNA. Collectively, these data suggest that regulation of Bcrp1 expression by pregnancy through a transcriptional mechanism is possible, but other possibilities such as posttranscriptional regulation cannot be excluded.

Our study suggests high expression of Bcrp1 in mouse kidney, regardless of gestational ages, and that Bcrp1 expression in liver and small intestine was significantly lower (Fig. 3). This pattern of tissue distribution is similar to that reported in previous studies (36). However, in contrast to high-level expression in mouse kidney and only moderate expression in mouse placenta, as previously reported (36), we found that Bcrp1 expression in mouse placenta was also quite substantial and comparable to that in mouse kidney (Fig. 3B). Similarly, high-level expression of BCRP was also reported in human placenta (20). Although Bcrp1 protein can be readily visualized in kidney and placenta by immunohistochemistry (Fig. 2), staining for Bcrp1 in liver required a relatively higher antibody concentration, indicating that Bcrp1 expression in liver was indeed lower than that in kidney and placenta. This may reflect the fact that adult female mice express significantly lower amounts of Bcrp1 in liver than adult male mice (24). Bcrp1 expression in small intestine also appears to be relatively low, since unlike placenta, kidney, or liver, Bcrp1 protein could hardly be detected by immunoblotting in S-9 fractions isolated from small intestine (data not shown).

In the present study, we have for the first time systematically analyzed mRNA expression of AhR, HIF1α, EROα, ERβ, or PR in mouse tissues at different gestation days (Figs. 4–6). Clearly, expression of the nuclear receptors in various tissues was also altered by pregnancy. For example, expression of PR mRNA in mouse placenta was significantly decreased over gestational ages (Fig. 6A), which is consistent with the human data of a previous study in which PR expression (both protein and mRNA) was shown to be much higher in first-trimester human placenta than in term placenta (31). The mechanisms by which the expression of nuclear receptors is regulated by pregnancy are not known but may be related to pregnancy-specific hormones, since some of these nuclear receptors such as AhR and HIF1α have already been shown to be regulated by pregnancy-specific hormones such as progesterone and 17β-estradiol (4, 10, 31). When compared with Bcrp1 expression, we found significant correlations of Bcrp1 mRNA with HIF1α, AhR, and ERβ mRNA in placenta, with HIF1α mRNA in kidney, and with AhR and EROα mRNA in liver (Table 1 and Fig. 7). Regulation of gene expression through nuclear receptors has been reported for various ABC transporters, including P-gp and MRP2, and two major factors have been appreciated from these studies. First, gene expression of ABC transporters can be regulated upon response to environmental or physiological stress, such as heat shock (16), hypoxia (17), inflammation (27) or ultraviolet irradiation (11). Second, multiple nuclear receptors can contribute to the regulation of ABC transporters, either independently or cooperatively (15, 30). Pregnancy is one of the major physiologically stressful events during which hormone levels are drastically changed and hypoxia in certain tissues can be induced. Therefore, based on the fact that Bcrp1 mRNA is significantly correlated with mRNA of AhR, HIF1α, EROα, or ERβ in various mouse tissues during pregnancy, we hypothesize that regulation of Bcrp1 expression by these nuclear receptors during pregnancy is possible. Previous studies have indeed indicated that AhR, HIF1α, EROα, or ERβ may be involved in the regulation of BCRP expression (6, 7, 17, 38). Further studies will be necessary to confirm this hypothesis. Although our previous study indicated that PRB may play a role in the regulation of BCRP in BeWo cells (38), we did not observe any correlations between mRNA of Bcrp1 and PR. Because the design of
primers does not allow amplification of PR$_A$ and PR$_B$ separately, correlations between mRNA of Berp1 and PR$_A$ or PR$_B$ could have been masked. Likewise, no correlations were observed between mRNA of Berp1 and membrane PR1 or membrane PR2.

The relationship between changes in the expression of nuclear receptors and Berp1 appears to be tissue specific. For example, significant correlations between mRNA of HIF1$\alpha$ and Berp1 were observed in placenta and kidney, but not in liver and small intestine (Table 1 and Fig. 7). These results indicate that pregnancy differentially regulates the expression of nuclear receptors, and hence Berp1, in a tissue-specific manner. If such tissue-specific regulation of nuclear receptors by pregnancy does occur in humans, our findings could have important consequences for Berp1 function. For example, hypoxia occurred during pregnancy may play a particularly important role in modulating BCRP function in the placenta, but not in liver and small intestine. Likewise, ligands for AhR may only affect BCRP function in placenta and liver, but not in small intestine. Caution should be taken when extrapolating our data to kidney in humans, since unlike in mice, there is no significant renal expression of BCRP in humans. It has been reported that there is cross talk between AhR and estrogen receptors, and the continued presence of estrogen is required to maintain high levels of AhR expression (33). Our finding that both AhR mRNA and ER$\beta$ mRNA are significantly correlated with Berp1 mRNA in mouse placenta suggests that interaction between AhR and ER$\beta$ may occur in the placenta, and the two nuclear receptors cooperatively regulate Berp1 expression in this particular tissue. Similarly, our results indicate that interaction between AhR and ER$\alpha$ may also occur in the liver.

In summary, Berp1 expression in placenta, kidney, and liver of pregnant mice was significantly increased at midgestational ages compared with early or term pregnancy. These results suggest that not only fetal distribution, but also systemic exposure to drugs that are BCRP substrates, could be influenced by pregnancy, particularly at midgestational stages. Studies are now in progress in our laboratory to confirm this hypothesis. Besides significant clinical implications, these data are particularly valuable for laying out experimental guidelines for the design of in vivo pharmacokinetic studies of BCRP substrate drugs in pregnancy using mice as animal models.

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GRANTS

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REFERENCES


