ABSTRACT

The effect of breast cancer resistance protein (Bcrp/Abcg2) on the disposition of phytoestrogens daidzein, genistein, and coumestrol was investigated using Bcrp−/− mice. Expression of the genes for either mouse Bcrp or human BCRP in MDCK II cells induced apically directed transport of the three phytoestrogens, whereas their transcellular transport was identical in mock and LLC-PK1 cells expressing mouse Mdr1a. After oral administration, the plasma levels of daidzein and genistein were increased in Bcrp−/− mice, but only a minimal change was observed for coumestrol. At steady state, tissue-to-plasma concentration ratios of the three phytoestrogens in the brain and testis of wild-type mice were very small and similar to those of [14C]inulin, whereas those were significantly increased in the brain and testis of Bcrp−/− mice. The largest increases were observed with genistein (9.2- and 5.8-fold in the brain and testis, respectively). The distributions of genistein in the epididymis and fetus, but not the ovary, were also increased in Bcrp−/− mice. The Bcrp protein was localized in the luminal membrane of the endothelial cells in the testis and the body of the epididymis and in both the luminal and abluminal side of ducts in the head of the epididymis. These results suggest that Bcrp limits the oral availability and distribution into the brain and testis, epididymis, and fetus of phytoestrogens.

Phytoestrogens are plant compounds that produce estrogen-like activity in mammals. Daidzein and genistein are two major isoflavonoids included in soy-based food and are the most commonly consumed phytoestrogens. Coumestrol, an isoflavone present in high concentrations in red clover, is known as the most potent estrogen among phytoestrogens (Mueller et al., 2004). Because of their estrogenic activity, phytoestrogens have been proposed as alternative agents for the treatment of postmenopausal disease; this concept has been supported by some clinical studies (Cassidy et al., 1995; Watanabe et al., 2000). Furthermore, epidemiological studies suggest that the low incidences of prostate, breast, and colon cancers and coronary disease in Asian populations are associated with the high consumption of isoflavonoids, a group of phytoestrogens highly included in soy-based diets (Adlercreutz, 1995). Based on these findings, phytoestrogens are believed to be beneficial for human health and are widely consumed in food and food supplements. On the other hand, warnings against the excessive consumption of phytoestrogens have been issued, because some adverse effects of phytoestrogens have been reported. For example, perinatal and neonatal exposure to genistein caused abnormalities in the ovary and vagina, reduced size of the testis and prostate, and caused the suppression of sexual behavior in rodents (Delclos et al., 2001; Wisniewski et al., 2003; Kyselova et al., 2004). Suppressive effects on sexual behavior were also reported for coumestrol in rats (Whitten et al., 2002).

The disposition of phytoestrogens has been well studied for genistein and daidzein. These exist naturally in the glycoside forms. Upon ingestion, they are hydrolyzed by bacterial β-glycosidase and are absorbed mainly as an aglycon (Setchell et al., 2002). Genistein and daidzein are predominantly metabolized to the glucuronide conjugates in the intestine and liver, followed by excretion into the bile (Chen et al., 2005). In the intestinal lumen, the glucuronide conjugates are hydrolyzed by bacterial β-glucuronidase and reabsorbed (Sfakianos et al., 1997). Thus, both genistein and daidzein undergo enterohepatic circulation. Tissue and fetal distributions of genistein have been investigated in rats (Chang et al., 2000; Doerge et al., 2001). Very low distributions of

**ABBREVIATIONS:** Bcrp, breast cancer resistance protein; mBcrp, mouse breast cancer resistance protein, hBCRP, human breast cancer resistance protein; GFP, green fluorescent protein; L-Mdr1a, LLC-PK1 cells expressing mouse Mdr1a; AUC, area under the curve; Kp, tissue-to-plasma concentration ratio; PCR, polymerase chain reaction; BSA, bovine serum albumin; PBS, phosphate-buffered saline; LC, liquid chromatography.
genistein were observed in the brain and testis, whereas moderate and high distributions were found in the other hormone target organs (prostate, ovary, and uterus) (Chang et al., 2000). Furthermore, in pregnant rats exposed to genistein, serum genistein concentrations were approximately 5 times less in fetuses than in maternal rats (Doerge et al., 2001). These findings suggest that penetration of genistein into the brain, testis, and fetus are limited by the blood-brain, -testis, and -placental barriers, attenuating the toxicological effects of genistein on the development of the brain, testis, and fetus.

Here we investigated the role of the breast cancer resistance protein (Bcrp/Abcg2) in the disposition of phytoestrogens. Bcrp is a member of the ATP-binding cassette transporter family and mediates the efflux transport of endo- and xenobiotics. Bcrp is expressed in various normal tissues (Maliepaard et al., 2001), and cumulative in vivo studies, particularly using Bcrp−/− mice, revealed important roles for this protein in the site of absorption and in clearance organs (van Herwaarden et al., 2003; Breedveld et al., 2004; Mizuno et al., 2004; Hirano et al., 2005). In addition, BCRP is also expressed in various tissue barriers, such as those in the brain, testis, and placenta (Jonker et al., 2002; Zhang et al., 2003; Bart et al., 2004). In these tissue barriers, Bcrp is expressed in the plasma membranes facing circulating blood and has its protective role against xenobiotics. Indeed, it was shown using Bcrp−/− mice that Bcrp restricts the penetration of imatinib and topotecan into the brain, testis, and epididymis. Female mice used in the fetus distribution study were at 2 weeks’ body weight) were used in the ovary distribution study. Pregnant female mice (15–16 weeks old and 24–28 g) were orally administered at a dose of 30 μmol/kg. Blood was collected from tail vein at appropriate time points and was centrifuged at 4°C and 1000g for 5 min to obtain plasma.

Tissue and Fetus Distribution. Under urethane anesthesia (1.25 g/kg, i.p.), the right jugular vein was cannulated with a polyethylene tube (PE-50; BD Biosciences, San Jose, CA). Compounds were solubilized at a concentration of 1.25 mM in 10% dimethyl sulfoxide/90% saline containing 2 mM NaOH and continuously infused through the cannula at a dose rate of 5 μmol/h/kg. Blood was collected from the left jugular vein at 60, 80, 100, and 120 min and centrifuged at 4°C and 1000g for 5 min to obtain plasma. Immediately after the last blood sampling, mice were sacrificed by cervical dislocation, and tissues or fetus was collected. PBS was added to tissues or fetus and homogenized from the slopes of the time profiles of apical-to-basal and basal-to-apical transport. Flux ratios were obtained by dividing the efflux rates in the basal-to-apical direction by those in the apical-to-basal direction.

Materials and Methods

Materials and Animals

Daidzein, genistein, and coumestrol were purchased from Sigma-Aldrich (St. Louis, MO), and 14C-labeled inulin was from Moravek Biochemicals (Brea, CA). All of the other chemicals were commercially available and of reagent grade. Wild-type FVB mice and Bcrp−/− mice (Jonker et al., 2002) were used in the present study. Male mice (9–17 weeks old and 23–32 g body weight) were used in the studies of oral administration and tissue distribution (brain, testis, and epididymis). Female mice (15–16 weeks old and 24–28 g body weight) were used in the ovary distribution study. Pregnant female mice used in the fetus distribution study were at 2 weeks' gestation and weighed 35 to 48 g. All of these animals were maintained under controlled temperature with a light/dark cycle. Food and water were available ad libitum except for the oral administration study in which mice were fasted for approximately 12 h before administration.

Transcellular Transport Study

The transcellular transport study was performed as reported previously with minor modifications (Matsushima et al., 2005). In brief, MDCK II cells were seeded in the 24-well Transwell (Corning, Cambridge, MA) at a density of 1.4 × 10^5 cells/well and grown for 3 days in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich). The cells were infected with the recombinant adenovirus harboring expression vector for green fluorescent protein (GFP), mouse Bcrp (mBcrp) or human BCRP (hBCRP) at 200 multiplicity of infection. The details of the construction of these recombinant adenoviruses were described in a previous report (Kondo et al., 2004). After 2 days of culture, the cells were used for transport studies. The cells were preincubated in Krebs-Henseleit buffer (142 mM NaCl, 23.8 mM Na2CO3, 4.83 mM KCl, 0.96 mM KH2PO4, 1.20 mM MgSO4, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl2, pH 7.4) at 37°C for 30 min, and transport experiments were initiated by replacing the medium on one side of the cell monolayer with Krebs-Henseleit buffer containing 3 μM test compounds. At appropriate times, 100-μl aliquots were taken from the opposite side of the cell monolayer and replaced with 100 μl of buffer.

In vitro transport by mMdr1a was examined using Mdr1a-expressing LLC-PK1 cells (L-Mdr1a). L-Mdr1a was established previously (Smith et al., 1998). L-Mdr1a and parent LLC-PK1 cells were seeded in the 24-well Transwell at a density of 4.8 × 10^5 cells/well and grown in medium 199 (Invitrogen) with 10% fetal bovine serum and 1% antibiotic-antimycotic solution. Medium was changed on the second day, and cells were subjected to the transport study on the fourth day. The procedures of the transport study were the same as those used for mBcrp and hBCRP. Efflux rates were calculated from the slopes of the time profiles of apical-to-basal and basal-to-apical transport. Flux ratios were obtained by dividing the efflux rates in the basal-to-apical direction by those in the apical-to-basal direction.

Animal Experiments

Oral Administration. Animals were fasted 12 h before administration. Compounds were suspended in 0.5% methylcellulose and orally administered at a dose of 30 μmol/kg. Blood was collected from tail vein at appropriate time points and was centrifuged at 4°C and 1000g for 5 min to obtain plasma.

[14C]Inulin Distribution. Inulin cannot penetrate cellular membrane, therefore [14C]Inulin was used as a marker of space outside of barriers in the brain and testis. Under urethane anesthesia (1.25 g/kg, i.p.), the right jugular vein was cannulated with a polyethylene tube (PE-50; BD Biosciences, San Jose, CA). Compounds were solubilized at a concentration of 1.25 mM in 10% dimethyl sulfoxide/90% saline containing 2 mM NaOH and continuously infused through the cannula at a dose rate of 5 μmol/h/kg. Blood was collected from the left jugular vein at 60, 80, 100, and 120 min and centrifuged at 4°C and 1000g for 5 min to obtain plasma. Immediately after the last blood sampling, mice were sacrificed by cervical dislocation, and tissues or fetus was collected. PBS was added to tissues or fetus and homogenized to make 20% homogenate for brain and testis, 10% homogenate for epididymis, 5% homogenate for ovary, and 33% homogenate for fetus. All of the samples were stored at ~80°C until use.

[14C]Inulin Distribution. Inulin cannot penetrate cellular membrane, therefore [14C]Inulin was used as a marker of space outside of barriers in the brain and testis. Under urethane anesthesia (1.25 g/kg, i.p.), [14C]Inulin was administered intravenously from the right jugular vein (50 μCi/kg). At 10-min postdose, blood was collected from the left jugular vein, and mice were sacrificed by cervical dislocation, and then the brain, testis, and epididymis were collected. Blood was centrifuged at 4°C and 1000g for 5 min, and plasma was obtained. Plasma (10 μl) was mixed with 8 ml of Hionic-Fluor (PerkinElmer Life and Analytical Sciences, Waltham, MA), and the radioactivity was measured with a liquid scintillation counter (LS 6000SE, Beckman Coulter, Fullerton, CA). Tissues were mixed with 400 μl of hydrogen peroxide and 800 μl of 2-propanol and left for 1 h at room temperature. Subsequently, 1 ml of Soluene 350 (PerkinElmer Life and Analytical Sciences) was added and incubated at 55°C for 4 h to solubilize the tissues. 10 ml of Hionic-Fluor was added and then subjected to a liquid scintillation counter (LS 6000SE).
LC/Mass Spectrometry Analysis
Samples were precipitated with two (for in vitro samples) or three (for in vivo samples) volumes of acetonitrile and centrifuged at 4°C and 15,000g for 10 min. After the evaporation of supernatants, the pellets were reconstituted with 10% acetonitrile/90% water and subjected to LC/mass spectrometry analysis. LCMS-2010 EV equipped with a Prominence LC system (Shimadzu, Kyoto, Japan) was used for the analysis. Samples were separated on a CAPCELL PAK C18 MGII column (3 μm, 2 × 50 mm; Shiseido, Tokyo, Japan) in a binary gradient mode. Mobile phase A was 0.05% formic acid, and mobile phase B was acetonitrile. For the analysis of daidzein and genistein, the concentration of mobile phase B was initially 18%, linearly increased up to 60% over 1.5 min, kept at 60% for a further 1 min, and finally re-equilibrated at 18% for 2.5 min. The total run time was 5 min. Daidzein and genistein were eluted at 3.0 and 3.3 min, respectively. For the analysis of coumestrol, the concentration of mobile phase B was initially 25%, linearly increased up to 90% over 1.5 min, kept at 90% for a further 1 min, and finally re-equilibrated at 25% for 3 min. Coumestrol was eluted at 2.7 min. Daidzein, genistein, and coumestrol were detected at mass-to-charge ratios of 255, 269, and 267, respectively, under negative electron-spray ionization mode. The interface voltage was −3.5 kV and the nebulizer gas (N2) flow was 1.5 L/min. The heat block and curved desolvation line temperatures were 200 and 150°C, respectively.

Quantification of mRNA Level of BCRP in Mouse and Human Epididymis
Mice were anesthetized with ether and sacrificed by exsanguination from the femoral artery and vein. Immediately after sacrifice, the epididymis was collected. Total RNA from mouse and human epididymis was isolated from these tissues using ISOGEN (Wako Pure Chemical Industries, Tokyo, Japan). Total RNA of human epididymis was purchased from BioChain Institute (Hayward, CA). Total RNA from mouse and human epididymis was converted to cDNA using random primer and avian myeloblastosis virus reverse transcriptase. Real-time PCR was performed with a QuantiTect SYBR Green PCR Kit (QiAGEN, Valencia, CA) and a LightCycler system (Roche Diagnostics, Mannheim, Germany). PCR primers were as follows: mouse Bcrp: forward, AAATGGGACCACTCACAATCTG; reverse, CCCATACAAGGCTCATCTTG; human BCRP: forward, CAGGTCCTGTGGTTCAATGCTACA; reverse, TCCATATCGTGGAATGCTGAGG; mouse GAPDH: forward, ATGGTCGCAATGTGACTCTG; reverse, ATGGACTGTTGCTGATGACC; and human GAPDH: forward, AATGGACCCCTCTGAGAGC; reverse, TCCACGATCTACGAGGCG. External standard curves were generated by dilution of the target PCR product purified by agarose gel electrophoresis. The absolute concentration of the external standard was measured with PicoGreen dsDNA Quantification Reagent (Molecular Probes, Eugene, OR).

Pharmacokinetic Analysis
Area under the curve (AUC) from 0 to 240 min after oral administration was calculated by trapezoidal method. Tissue-to-plasma or fetus-to-plasma concentration ratios (Kp values) were calculated by dividing tissue or fetus concentrations by plasma concentrations at 120-min postdose.

Immunohistochemical Analysis
Frozen sections of the testis and epididymis were prepared from FVB wild-type and Bcrp−/− mice and fixed to glass slides in methanol (−20°C). The sections were incubated in 1% Triton X-100 for 30 min at room temperature and subsequently washed with PBS three times. The sections were then incubated in PBS containing 5% bovine serum albumin (BSA-PBS) to block nonspecific protein binding. After washing with PBS three times, the sections were incubated with 1:40 dilution of anti-Bcrp monoclonal antibody (BXP-53; Signet Laboratories, Dedham, MA) in BSA-PBS at 4°C overnight. After washing with PBS three times, the sections were incubated with secondary antibody (Alexa 488 anti-rat IgG; Molecular Probes, Eugene, OR) and Topro3 (Molecular Probes) in BSA-PBS for 1 h at room temperature. The sections were mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA).

Statistical Analysis
Statistical analysis for significant differences was performed using the two-tailed Student’s t test. A probability of <0.05 was considered to be statistically significant.

Results
In Vitro Transport of Phytoestrogens by mBcrp, hBCRP, and Mdr1a. The transport activities of daidzein, genistein, and coumestrol by mBcrp, hBCRP, and mMdr1a were investigated using transporter expressing cell systems (MDCK II/mBcrp, MDCK II/hBCRP, and L-Mdr1a). In those systems, mBcrp, hBCRP, and Mdr1a are localized in the apical membrane and direct the transcellular transport of their substrates in the apical direction (Smith et al., 1998; Matsushima et al., 2005). In MDCK II/mBcrp and MDCK II/hBCRP, the permeability of basal-to-apical direction was greater than that of apical-to-basal direction for all three phytoestrogens, whereas the transcellular transport was almost identical in both directions in MDCK II/GFP (Table 1). The flux ratios were higher in MDCK II/mBcrp and MDCK II/hBCRP than those in MDCK II/GFP, suggesting the three phytoestrogens are the substrate of mBcrp and hBCRP. In LLC-PK1 and L-Mdr1a, the permeability of basal-to-apical direction was slightly higher than that of apical-to-basal direction; however, the flux ratios were almost identical between the two cell systems, suggesting the three phytoestrogens are not the substrate of mMdr1a (Fig. 2 and Table 1).

Effects of Bcrp on the In Vivo Disposition of Phytoestrogens. Plasma levels of daidzein, genistein, and coumestrol after oral administration were compared between wild-type and Bcrp−/− mice (Fig. 3). Daidzein and genistein exhibited significantly higher plasma exposure in Bcrp−/− mice than in wild-type mice (Fig. 3A). The area under the curve over 4 h (AUC0−240 min) was 3.7- and 2.0-fold greater for daidzein and genistein, respectively, than the corresponding control values (Fig. 3B). The plasma exposure of coumestrol was very low compared with the other phytoestrogens, and no significant difference was observed in the AUC0−240 min of coumestrol between the wild-type and Bcrp−/− mice, although significant changes were observed in plasma concentrations at several time points.

Tissue distributions of the phytoestrogens were determined at steady state achieved by a constant intravenous infusion (Fig. 4). Plasma concentrations were almost constant between 60 and 120 min, indicating that plasma concentrations reached a plateau at 60 min (Fig. 4A). The plasma concentrations of daidzein were significantly higher in Bcrp−/− mice than that in wild-type mice, but no significant changes were observed for genistein and coumestrol. The Kp values of brain and testis were significantly increased in Bcrp−/− mice for all of the three phytoestrogens (Fig. 4B). The fold increases in the Kp values of the brain were 5.6, 9.2, and 3.9 and those in the testis were 5.8, 5.8, and 4.1 for daidzein, genistein, and coumestrol, respectively. The Kp values of [14C]inulin were investigated to estimate the volume of
the capillary space in the brain and testis. The $K_p$ values of $[14C]$inulin were $0.011 \pm 0.004$ and $0.031 \pm 0.011$ (mean ± S.E., n = 3) for the brain and testis, respectively (indicated by a broken line in Fig. 4B). In addition, the effects of Bcrp on the distributions of genistein in the epididymis and ovary were also investigated (Fig. 5). The $K_p$ value of the epididy-

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound and Cell Line</th>
<th>Permeability</th>
<th>Flux Ratio</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>A to B (μl/h/well)</td>
<td>B to A (μl/h/well)</td>
</tr>
<tr>
<td><strong>Daidzein</strong></td>
<td></td>
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<tr>
<td>MDCKII/GFP</td>
<td>17.4 ± 0.2</td>
<td>23.7 ± 0.3</td>
</tr>
<tr>
<td>MDCKII/hBCRP</td>
<td>12.9 ± 0.7</td>
<td>53.2 ± 4.1</td>
</tr>
<tr>
<td>MDCKII/mBcrp</td>
<td>14.7 ± 0.3</td>
<td>44.1 ± 0.8</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>26.7 ± 1.2</td>
<td>49.9 ± 1.0</td>
</tr>
<tr>
<td>L-Mdr1a</td>
<td>27.9 ± 0.8</td>
<td>47.3 ± 1.0</td>
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<tr>
<td><strong>Genistein</strong></td>
<td></td>
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<tr>
<td>MDCKII/GFP</td>
<td>13.0 ± 0.3</td>
<td>15.7 ± 0.4</td>
</tr>
<tr>
<td>MDCKII/hBCRP</td>
<td>12.2 ± 0.8</td>
<td>28.8 ± 1.0</td>
</tr>
<tr>
<td>MDCKII/mBcrp</td>
<td>12.5 ± 0.5</td>
<td>25.6 ± 0.3</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>54.0 ± 2.3</td>
<td>77.5 ± 0.8</td>
</tr>
<tr>
<td>L-Mdr1a</td>
<td>54.2 ± 1.1</td>
<td>76.0 ± 1.8</td>
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<tr>
<td><strong>Coumestrol</strong></td>
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<tr>
<td>MDCKII/GFP</td>
<td>2.80 ± 0.04</td>
<td>4.06 ± 0.18</td>
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<tr>
<td>MDCKII/hBCRP</td>
<td>3.13 ± 0.15</td>
<td>10.2 ± 0.6</td>
</tr>
<tr>
<td>MDCKII/mBcrp</td>
<td>3.29 ± 0.23</td>
<td>8.75 ± 0.41</td>
</tr>
<tr>
<td>LLC-PK1</td>
<td>11.3 ± 0.4</td>
<td>18.7 ± 0.4</td>
</tr>
<tr>
<td>L-Mdr1a</td>
<td>18.9 ± 0.5</td>
<td>30.9 ± 0.6</td>
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mis was increased in $Bcrp^{-/-}$ mice with a fold increase of 2.5, whereas the $K_p$ value of the ovary was unchanged.

**Localization of Bcrp in Mouse Testis and Epididymis, and mRNA Expression of BCRP in the Human Epididymis.** Immunohistochemical analysis was performed to identify the membrane localization of Bcrp protein in the testis and epididymis using the anti-Bcrp antibody (BXP-53) (Fig. 6). In the testis of wild-type mice, Bcrp was detected

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**Fig. 2.** The transcellular transport of daidzein (A), genistein (B), and coumestrol (C) across monolayers of control and mMdr1a-expressing LLC-PK1 cells. Transport in the apical-to-basal direction is represented by ○ and that in the basal-to-apical direction by ●. Data are represented by means ± S.E. of triplicate experiments.

**Fig. 3.** Comparison of the plasma concentrations of daidzein, genistein, and coumestrol after oral administration (30 μmol/kg) of daidzein, genistein, or coumestrol between wild-type and $Bcrp^{-/-}$ mice. A, the time profiles of plasma concentrations. B, plasma AUC from time 0 to 240 min. Data are represented by means ± S.E. of three or four mice. Asterisks represent statistically significant differences between wild-type and $Bcrp^{-/-}$ mice: *, $P < 0.05$; **, $P < 0.01$. 
only in the endothelial cells (Fig. 6A) and specifically in the luminal membrane (Fig. 6C). In the epididymis, the localization of Bcrp differed between the head and body regions (Fig. 6D). In the head (Fig. 6D, left), Bcrp was detected in both the luminal and abluminal sides of ducts, whereas in the body (Fig. 6D, right), Bcrp staining was observed in the endothelial cells. Immunofluorescence by BXP-53 was diminished in Bcrp/H11002/H11002 mice, indicating that the signals were associated specifically with Bcrp. We also examined the mRNA expression level of BCRP in the human epididymis. The ratio of BCRP mRNA to that of GAPDH was $1.16 \times 10^{-2}$ and was similar to that found in the mouse epididymis ($1.42 \times 10^{-2}$).

Role of Bcrp on the Accumulation of Genistein in the Fetus and Fetus Brain. Role of Bcrp on the fetal distribution of genistein was investigated in pregnant mice. Genistein was given to pregnant mice by constant intravenous infusion, and the plasma concentrations were similar between wild-type and $Bcrp^{-/-}$ mice (Fig. 7A). The fetus-to-maternal plasma concentration ratio was 1.8-fold greater in $Bcrp^{-/-}$ mice than controls (Fig. 7B). Brain-to-whole body concentration ratios were compared between wild-type and $Bcrp^{-/-}$ fetus to evaluate the Bcrp function in fetal blood-brain barrier. Brain-to-whole body concentration ratio was 1.4-fold increased in $Bcrp^{-/-}$ mice (Fig. 7C).

Discussion

In the present study, the role of Bcrp in limiting oral absorption of the phytoestrogens and their penetration into the brain, testis, epididymis, and fetus was investigated using $Bcrp^{-/-}$ mice. We tested three phytoestrogens, daidzein, genistein, and coumestrol. Daidzein and genistein are two major isoflavonoids in soy-based meal and are the most frequently ingested phytoestrogens. Coumestrol is known to be the most potent phytoestrogen. We found that all three phytoestrogens are substrates of Bcrp (Fig. 1). We also investigated the transport of the phytoestrogens by mMdr1a, which exhibits overlapped tissue distribution with Bcrp, and has been shown to limit the oral availability and tissue distributions of a variety of compounds. However, it was found that

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A Plasma concentrations

![Image of plasma concentrations with graphs showing measurements for Daidzein, Genistein, and Coumestrol.

B Kp

![Image of Kp values showing comparisons between Wild-type and Bcrp mice for Brain and Testis.]
all three phytoestrogens were not the substrate of Mdr1a (Fig. 2).

After oral administration, the plasma AUC of daidzein and genistein was significantly increased in Bcrp<sup>−/−</sup> mice (Fig. 3, A and B). For coumestrol, the plasma concentrations exhibited a significant change in Bcrp<sup>−/−</sup> mice only at early time points, and thus, the AUC did not exhibit a statistically significant change. There are three potential sites to account for the increase in the plasma concentration after oral administration: an increase in intestinal absorption, and a decrease in intestinal and hepatic extraction. When given intravenously, the change in the plasma concentrations of phytoestrogens between wild-type and Bcrp<sup>−/−</sup> mice were marginal (Fig. 4). This is reasonable because the major elimination pathway of phytoestrogens from the systemic circulation is glucuronidation. Impaired Bcrp hardly affects the hepatic first-pass effect for phytoestrogens. Glucuronidation may be part of the mechanism limiting oral availability of the phytoestrogens as reported for quercetin (Crespy et al., 1999). We have reported that the intestinal glucuronidation activity of 4-methylumbelliferone exhibited no change in Bcrp<sup>−/−</sup> mice (Enokizono et al., 2007). Therefore, the enhanced plasma exposure after oral administration in Bcrp<sup>−/−</sup> mice is probably due to an increased intestinal absorption. The effect of Bcrp on the oral availability of coumestrol was minimal, whereas the in vitro transport activity by mBcrp

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Fig. 6. Immunohistochemical analysis of Bcrp in the testis and epididymis. Localizations of Bcrp protein and DNA are shown by green and blue staining, respectively. A, testis of wild-type mice. B, testis of Bcrp<sup>−/−</sup> mouse. C, endothelial cells in the testis of wild-type mice. D, epididymis of wild-type mouse. E, epididymis of Bcrp<sup>−/−</sup> mouse. In D and E, the white lines discriminate the head (left) from the body (right).

Fig. 7. Comparison of the distributions of genistein into the fetuses and fetal brain between wild-type and Bcrp<sup>−/−</sup> mice. Genistein was intravenously infused to pregnant mice at a dose rate of 5 μmol/h/kg, and the distributions into the fetuses and fetal brain were measured at 120 min. A, the time profiles of plasma concentrations. B, fetus-to-maternal plasma concentration ratios. C, brain-to-whole body concentration ratios in fetal mice. Data are represented by means ± S.E. of three mice. Asterisks represent statistically significant differences between wild-type and Bcrp<sup>−/−</sup> mice: **, P < 0.01.
was similar among the three phytoestrogens (Fig. 1). This indicates the smaller contribution of Bcrp to the intestinal absorption of coumestrol. The contribution of paracellular transport and intestinal metabolism may be greater for coumestrol than the other phytoestrogens. Indeed, tranacellular transport of coumestrol in the apical-to-basal direction in control cells (MDCKII/GFP and LLC-PK1) was lowest among the three phytoestrogens, suggesting the lowest tranacellular transport of coumestrol. The mechanism governing the increased plasma concentrations of daidzein during intravenous infusion in Bcrp−/− mice remains unknown. Impaired urinary excretion could be one possible mechanism because daidzein also undergoes urinary excretion: approximately 10% of the dose after oral administration (Bayer et al., 2001). The $K_p$ values of brain and testis of all three phytoestrogens were significantly increased in Bcrp−/− mice (Fig. 4B). Considering that the $K_p$ values of the phytoestrogens and [14C]inulin were similar in wild-type mice, the brain and testis distribution of the phytoestrogens is almost completely limited by Bcrp. Bcrp has been identified on the luminal membrane of both the human and mouse brain capillary endothelial cells that form the blood-brain barrier (Cooray et al., 2002; Lee et al., 2005), whereas there is an interspecies difference in membrane localization in the testis. Bcrp is localized on both the luminal side of the endothelial cells and the apical membrane of myoid cells in the human testis (Bart et al., 2004), whereas Bcrp expression is restricted to the luminal membrane of capillary-like structures in the mouse testis (Fig. 6; A and C). It is generally considered that the Sertoli and myoid cells form the blood-testis barrier, but endothelial cell-cell junctions are more leaky in rats (Dym and Fawcett, 1970). However, from the present results, testicular endothelial cells evidently have an adequate barrier function against phytoestrogens, at least in mice.

In addition to the testis, we investigated the role of Bcrp in other reproductive organs, the epididymis and ovary. The epididymis is divided into three segments (head, body, and tail). Sperm formed in the testis enter the head and finally reach the tail. During this transition, they undergo maturation and are finally stored in the tail region. It was found that the distribution of genistein was also increased in the epididymis of Bcrp−/− mice (Fig. 5). Therefore, we propose that Bcrp limits the penetration of genistein into the epididymis. The membrane localization of Bcrp was regionally dependent in the epididymis. Bcrp was mainly localized in capillary-like structures in the body (Fig. 6D, left), whereas Bcrp was found both in the luminal and abluminal membranes of the ducts in the head (Fig. 6D, right). Bcrp in the capillary-like structure and the abluminal membranes of ducts may contribute to the reduced distribution of genistein in wild-type mice. The physiological role of the Bcrp in the luminal membranes of the ducts in the head is unknown. It may mediate the luminal secretion of some endogenous compounds. Unlike male reproductive organs, the ovaries did not exhibit any change in the tissue distribution of genistein (Fig. 5), although the Bcrp mRNA level in the ovaries is similar to that in the testis (Tanaka et al., 2005). The reason for the discrepancy between mRNA expression and functional activity in the ovaries remains unknown.

Fetal and newborn mice are more sensitive to estrogen than adults. The distribution of genistein in the fetus was increased in pregnant Bcrp−/− mice (Fig. 7B), suggesting that Bcrp limits the penetration of genistein into the fetus in the placenta. Furthermore, the brain-to-whole body concentration ratio was also increased in fetal Bcrp−/− mice (Fig. 7C). Therefore, fetal brain capillaries may develop a barrier function to some degree even at this stage. The smaller increase (1.4-fold) than that observed in adult mice (9.2-fold) suggests that the barrier function is still immature at this stage (Nico et al., 1999). Taken together, the exposure of phytoestrogens to the fetal brain is limited by Bcrp in the fetal blood-brain barrier, the placenta, and the maternal small intestine.

Estrogen plays a key role in the development of reproductive systems, and sexual differentiation and estrogenic chemicals are known to influence reproductive functions, such as reduced testicular weight, sperm counts, induction of the acrosome reaction in both human and mouse (Atanassova et al., 2000; Adeoya-Osiguwa et al., 2003; Kyselova et al., 2004; Fraser et al., 2006), and adult sexual behavior, such as reduced mounting and ejaculation in males and reduced lordosis in females (Patisaul et al., 2004). Bcrp will prevent these adverse effects of phytoestrogens by limiting the exposure to the reproductive organs and brain.

In conclusion, we have demonstrated the importance of Bcrp in limiting the oral availability of phytoestrogens and their penetration into the brain and male reproductive organs. In addition, Bcrp also limited the exposure of the mouse fetus to phytoestrogens by extruding them to the blood from the placenta. These results indicate the important roles of Bcrp in protecting the body from the adverse effects of phytoestrogens on sexual behavior and spermogenesis.

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References


