Characterization of Organic Anion Transporting Polypeptide 1b2-null Mice: Essential Role in Hepatic Uptake/Toxicity of Phalloidin and Microcystin-LR

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The liver-specific importer organic anion transporting polypeptide 1b2 (Oatp1b2, Slco1b2, also known as Oatp4 and Lst-1) and its human orthologs OATP1B1/1B3 transport a large variety of chemicals. Oatp1b2-null mice were engineered by homologous recombination and their phenotype was characterized. Oatp1b2 protein was absent in livers of Oatp1b2-null mice. Oatp1b2-null mice develop normally and breed well. However, adult Oatp1b2-null mice had moderate conjugated hyperbilirubinemia. Compared with wild-types, Oatp1b2-null mice had similar hepatic messenger RNA expression of most transporters examined except a higher Oatp1a4 but lower organic anion transporter 2. Intraarterial injection of the mushroom toxin phalloidin (an Oatp1b2-specific substrate identified in vitro) caused cholestasis in wild-type mice but not in Oatp1b2-null mice. Hepatic uptake of fluorescence-labeled phalloidin was absent in Oatp1b2-null mice. Three hours after administration of microcystin-LR (a blue-green algae toxin), the binding of microcystin-LR to hepatic protein phosphatase 1/2a was much lower in Oatp1b2-null mice compared with wild-type mice. In contrast, Oatp1b2-null mice were transiently protected from decrease in bile flow induced by estradiol-17β-D-glucuronide, a common substrate for Oatps. Oatp1b2-null mice were completely resistant to the hepatotoxicity induced by phalloidin and microcystin-LR, but were similarly sensitive to α-amanitin–induced hepatotoxicity compared with wild-type mice. In conclusion, Oatp1b2-null mice display altered basic physiology and markedly decreased hepatic uptake/toxicity of phalloidin and microcystin-LR. Oatp1b2-null mice are useful in elucidating the role of Oatp1b2 and its human orthologs OATP1B1/1B3 in hepatic uptake and systemic disposition of toxic chemicals and therapeutic drugs.

Key Words: Oatp1b2; liver; knockout; mice; phalloidin; microcystin.

Many transporters are responsible for uptake of endogenous and exogenous chemicals into liver, including the organic anion transporting polypeptides (Oatps), Na+-taurocholate-cotransporting polypeptide (Ntcp), organic anion transporters (Oats), and organic cation transporters (Klaassen and Lu, 2007). Among these transporters, the Oatps are thought to be responsible for the majority of hepatic uptake of chemicals, whereas Ntcp mainly transports bile acids into liver (Trauner and Boyer, 2003). Oats and organic cation transporters are predominantly expressed in kidney and are thought to be less important in liver. Though the name Oat implies selective transport of organic anions, certain Oatps can also transport organic cations and neutral compounds (Hagenbuch and Meier, 2004; Konig et al., 2006). Rodent Oatp1a1, 1a4, 1b2, and 2b1, as well as human OATP1B1, 1B3, and 2B1 are highly expressed in liver (Cheng et al., 2005; Choudhuri et al., 2000; Hagenbuch et al., 2000; Li et al., 2002; Ogura et al., 2000). The mouse/rat liver-specific importer Oatp1b2 (also known as Oatp4, Lst-1, gene symbol SLC1b2), first cloned in our laboratory (Choudhuri et al., 2000; Ogura et al., 2000), is orthologous to human OATP1B1 (also known as OATP-2) and OATP1B3 (also known as OATP-8) (Hagenbuch and Meier, 2003).

The organic anionic dye dibromosulfophthalein (DBSP) disulfonate is rapidly extracted from the circulation by hepatocytes, and is almost entirely eliminated in bile. Thus, clearance of DBSP is used to determine liver function (Dhumeaux et al., 1974). The nonmetabolizable feature of DBSP makes it an excellent chemical to characterize the pure role of transporters in hepatic uptake and biliary excretion of chemicals.

Certain chemicals selectively accumulate in liver to produce hepatotoxicity, such as the mushroom toxins phalloidin and amanitin (in *Amanita phalloides*) as well as the blue-green algae toxin microcystin-LR. It was proposed that these toxic cyclic peptides are transported into hepatocytes by the same multispecific transport systems that mediate hepatic uptake of
bile acids (Eriksson et al., 1990; Kronke et al., 1986). However, recent cellular studies showed that rat Oatp1b2 as well as human OATP1B1 and OATP1B3, but not other Oatps (Oatp1a1, Oatp1a4, OATP2B1) or Ntcp, transport phalloidin (Meier-Abt et al., 2004) and microcystin-LR (Fischer et al., 2005), whereas rat Ntcp and human OATP1B3 may mediate hepatic uptake of α-amanitin (Gundala et al., 2004; Letschert et al., 2006). Accidental ingestion of Amanita phalloides and environmental pollution of microcystin are important clinical causes of liver injury (Bischoff, 2001; Faulstich and Wieland, 1996). The in vivo importance of Oatp1b2 and OATP1B1/1B3 in mediating the hepatic uptake and toxicity of these three toxins remains to be elucidated.

Intrahepatic cholestasis of pregnancy (ICP) is a benign disease for the mother but associated with an increased perinatal morbidity and mortality. Hypersensitivity to elevated estrogens, particularly estrogen glucuronide, is implicated as a major cause of ICP (Fagan, 1999). In rodents, estradiol-17β-D-glucuronide (E2;17βG), the major estrogen glucuronide in vivo, induces an immediate, profound, but reversible inhibition of bile flow (Meyers et al., 1980). Many of the functionally characterized Oatps are able to transport E2;17βG in vitro (Hagenbuch and Meier, 2003); thus, the importance of a single Oatp, namely Oatp1b2 herein, in mediating E2;17βG-induced cholestasis remains to be determined.

In humans, functional polymorphisms of OATP1B1 (with decreased uptake activity) are associated with elevated blood levels of a number of therapeutic drugs, such as the cholesterol-lowering drugs statins (pravastatin, pitavastatin, and simvastatin) (Ho et al., 2007; leiiri et al., 2007; Pasanen et al., 2006), the antidiabetic drugs nateglinide and repaglinide (Niemi et al., 2005a; Zhang et al., 2006), as well as the H 1-receptor antagonist fexofenadine (Niemi et al., 2005b).

We hypothesize that Oatp1b2 has an important and unique role in hepatic uptake of chemicals, and that the loss of its function cannot be compensated by other hepatic importers. We engineered Oatp1b2-null mice by homologous recombination and characterized their phenotype. Oatp1b2-null mice develop normally and breed well. However, Oatp1b2-null mice have changes in blood chemistry. To characterize the role of Oatp1b2 in hepatic uptake and systemic disposition of chemicals, we investigated the pharmacokinetics and/or hepatotoxicity of five chemicals, namely DBSP (a non-metabolizable xenobiotic), phalloidin and microcystin-LR (in vitro specific substrates for Oatp1b2), α-amanitin, and E2;17βG (a common endogenous substrate for Oatps).

MATERIALS AND METHODS

Chemicals and reagents. Phalloidin, fluorescein isothiocyanate–labeled phalloidin (phalloidin-FITC, PS282), α-amanitin, microcystin-LR, and E2;17βG were purchased from Sigma, Inc. (St Louis, MO). DBSP was acquired from SERB Laboratories (Paris, France). Analytical kits for total and direct bilirubin, bile acids, alanine transaminase (ALT), alkaline phosphatase (ALP), and urea nitrogen were obtained from Wako Chemicals USA, Inc. (Richmond, VA) and Pointe Scientific Inc. (Canton, MI), respectively.

Development of Oatp1b2-null mouse. The mouse Oatp1b2 cDNA and gene were cloned and characterized in our laboratory (Ogura et al., 2000). The targeting vector for the disruption of Oatp1b2 gene was created by cloning part of the mouse Oatp1b2 gene (intron 1 through part of intron 3) into the pKO scrambler NTKV-1902 (Stratagene, La Jolla, CA).

The 5′ fragment of the targeting construct was generated and subcloned as two separate fragments: 5′ fragment no. 1 (more upstream of the two) and 5′ fragment no. 2 (more downstream of the two). The 5′ fragment #1 was PCR-amplified as a ~1.08-kb HindIII–HindIII fragment which included sequences spanning from intron 1 through exon 2. The 5′ fragment no. 2 was generated as a HindIII–ClaI fragment, and was first subcloned into pBluescript. PCR primers were designed to introduce ClaI and BamHI sites, and the 5′ fragment no. 2 was amplified as a ~1.3-kb HindIII–ClaI fragment, which included sequences spanning from exon 2 through part of exon 3. The longer 3′ fragment of the targeting construct was generated as a BamHI–KpnI fragment. The final nonlinearized targeting vector (Fig. 1A) had a neo-gene cassette that was flanked by a short arm (HindIII–ClaI; ~2.4-kb, containing intron 1 through part of exon 3) and a long arm (BamHI–KpnI; –6 kb, containing the rest of exon 3 and part of intron 3) of the mouse Oatp1b2 gene.

Upon Nol linearization, the linearized targeting vector was about 14-kb long; it contained the long arm (~7.4 kb) that included the neo cassette, short arm (~2.4 kb), and ~3.8-kb vector-derived sequence, which included the thymidine kinase (TK) gene sequence. The nucleotide (nt) positions of the major segments in the final targeting vector are as follows: Myeloid Cells-1 promoter: 12–300; TK: 377–1507; TK polyA: 1523–2019; Mouse Oatp1b2 gene 5′-end (intron 1 through part of exon 3): 2068–4489; lacO: 4490–4524; bgh polyA–Neomycin phosphotransferase (neo)–phosphoglucerase kinase promoter: 4552–6155; lpsP: 6171–6205; Mouse Oatp1b2 gene 3′-end (the rest of exon 3 and part of intron 3): 6214–9902 (this region contains about 3.5-kb unsequenced part of intron 3 and also an AccI site); Nol site: 9945. Figure 1B (upper panel) shows the targeting strategy through homologous recombination.

The Nol-linearized targeting vector was electroporated into W9.5 ES cell line (129SJ). Upon selection with 300 mg/ml G418 and 2 mM gancyclovir, 216 colonies were available upon request. Western blot (Fig. 1B; lower panel, right). PCR genotyping protocol is available upon request.

Treatment of animals. Mice were maintained at an American Animal Associations Laboratory Animal Care-accredited facility at the University of Kansas Medical Center (KUMC). Age-matched C57BL/6 and 129Sv mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA) and allowed at least one week to adapt to the environment before being used for the experiments. Mice were housed at an ambient temperature of 22°C with alternating 12-h light/dark cycles, and allowed water and rodent chow
ad libitum (Teklad 8604; Harlan, Indianapolis, IN). All animal procedures in this study were approved by the Institutional Animal Care and Use Committee of KUMC.

In the initial studies on the toxicity of mushroom toxins, phalloidin, and α-amanitin, adult male Oatp1b2-null mice in the mixed genetic background were used; age-matched male C57BL/6 and 129Sv mice were used as wild-type controls. The dosages of phalloidin and α-amanitin were selected based on literature and pilot experiments. Mice were injected ip with phalloidin (2.5 mg/kg in saline) or saline (10 ml/kg). Blood and liver samples were collected 6-h postinjection under pentobarbital anesthesia. Liver samples were fixed in 10% neutral formalin, processed by standard histopathological techniques, and liver sections (5 μm) stained with hematoxylin–eosin were examined under light microscopy. In a second study, mice were injected ip with α-amanitin (1.0 mg/kg in saline) or saline (10 ml/kg), and blood and liver samples were collected 16 h later under pentobarbital anesthesia. In all the other experiments, Oatp1b2-null mice backcrossed to C57BL/6 mice for six generations were used, and age-matched C57BL/6 mice were used as wild-type controls.

**FIG. 1.** Generation and identification of Oatp1b2-null mice. (A) Schematic structure of targeting vector. (B) Upper panel, targeting strategy through homologous recombination; Lower panel, left: PCR genotyping of Oatp1b2-null and wild-type mice. Lanes 1–3, Oatp1b2-null; lane 4, wild-type; lane 5, 100 bp DNA ladder. Lower panel, right: Western blot detection of protein expression of Oatp1b2 in liver of wild-type and Oatp1b2-null mice.
In the study of blood chemistry (Table 1) and hepatic expression of transporters, blood samples of adult (3 months old) male and female Oatp1b2-null and C57BL/6 mice were drawn from the carotid artery via a cannula under anesthesia. Mouse serum samples (0.5 ml) were analyzed by Physicians Reference Laboratory (Overland Park, KS). Mouse tissues were collected and weighed. Liver samples were used for preparation of total RNA and protein samples.

In the study of microcystin-LR–induced hepatotoxicity, male adult Oatp1b2-null and C57BL/6 mice were injected ip with microcystin-LR (120 μg/kg in saline) or saline (10 ml/kg). Mouse blood and liver samples were collected 20-h postinjection under pentobarbital anesthesia.

**Binary excretion and pharmacokinetic study.** Mice were anesthetized by ip injection of ketamine (100 mg/kg)–midazolam (5 mg/kg), and their body temperatures were maintained at 37°C by means of heating pads. Subsequently, the right carotid artery was cannulated with PE-10 tubing, and the common bile duct was cannulated with the shaft of a 30-gauge needle attached to PE-10 tubing. Bile samples were collected in 15-min periods into preweighed tubes for five periods (15 min prior to until 60 min after injection). After the first bile-collecting period, the test chemical was injected into the carotid cannula, and 25–30 μl of blood were taken at 2-, 7.5-, 22.5-, 37.5-, and 52.5-min postinjection into heparinized tubes. The volumes of bile samples were measured gravimetrically with 1.0 used as the specific gravity of bile. One-hour postinjection, the livers were harvested.

DBSP (120 μmol/kg) was dissolved in pathogen-free water and injected at 10 ml/kg. Plasma and bile samples were alkalized by being admixed with an equal volume of 0.1N NaOH, and DBSP concentrations were quantified spectrophotometrically (Gregus and Klaassen, 1982). A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE), which only requires 1 μl of sample for measurement, allowed an accurate quantification of plasma levels of DBSP.

For the study of phalloidin-induced cholestasis, adult male Oatp1b2-null and C57BL/6 mice were injected intra-arterially with phalloidin 0.4 mg/kg (0.32 μmol/kg phalloidin–FITC). One h after phalloidin injection, livers were harvested and slices of liver samples were frozen on dry ice and stored at –80°C for detection of phalloidin-FITC in mouse liver. For the study of E217G-induced decrease in bile flow, adult female Oatp1b2-null and C57BL/6 mice were injected intra-arterially with E217G (24 μmol/kg in 10 ml/kg of 0.02N NaOH).

**Detection of phalloidin-FITC in mouse liver.** Frozen liver sections (5 μm) were fixed with 4% paraformaldehyde for 5 min and washed with phosphate buffered saline containing 0.1% Triton X-100. Sections were air dried and mounted in Prolong Gold with 4’,6-diamidino-2-phenylindole (DAPI, nuclear stain) (Invitrogen Corp., Carlsbad, CA). Images were captured on an Olympus BX41 fluorescent microscope with a DP70 camera and DP Controller software. Each fluorescent channel (for FITC and DAPI staining) was acquired sequentially and then merged to create the final image. Images were cropped and brightness and contrast were adjusted under uniform conditions in Adobe Photoshop CS2 (San Jose, CA).

**Development of peptide specific antibodies to detect mouse Oatp1b2 protein.** A 25 amino acid long peptide epitope specific for mouse Oatp1b2 (protein_id NP_065241), CNPPEVPNNYGCSVPSDEKSETPL (666–689) was designed using DS Gene software (Accelys Inc., San Diego, CA). This peptide epitope was synthesized and conjugated with maleimide-activated keyhole limpet hemocyanin and used to produce affinity-purified rabbit polyclonal antibodies (Bethyl Laboratories, Montgomery, TX).

**Western blot analysis of Oatp1b2 protein.** Protein expression of Oatp1b2 in crude membrane fractions prepared from livers of wild-type and Oatp1b2-null mice was determined by Western blot (Chen et al., 2005). Protein concentrations were determined with Bradford reagent. Briefly, protein samples (50 μg) were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes, and filters were probed with a polyclonal antibody against mouse Oatp1b2. Signals were visualized with horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ).

**Western blot detection of microcystin-LR in nuclear or cytosolic extracts.** Adult male C57BL/6 and Oatp1b2-null mice were injected ip with microcystin-LR (60 μg/kg) and livers were removed 3 h after microcystin administration. Livers were homogenized in a buffer (25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.6, 1.5mM ethylenediaminetetra-acetic acid, 10% glycercol, 1mM dithiotreitol, 0.1 mg/ml phenylmethylsulfonyl fluoride, 0.5M KCl, and 1× protease inhibitor cocktail). After being incubated on ice for 1 h, the homogenates were centrifuged at 100,000 × g for 30 min at 4°C. The resultant supernatant was stored at –80°C. Protein concentrations were determined with Bradford reagent. A total of 20 μg of protein from nuclear or cytosolic extracts were boiled for 2 min and loaded to 10% SDS–PAGE gels. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes and filters probed with a primary anti-microcystin monoclonal antibody (Mouse IgG1, clone MC10E7, AXXORA, LLC, San Diego, CA). Signals were visualized with horseradish peroxidase–conjugated secondary antibody.

**RNA extraction.** Total tissue RNA was extracted using RNA-Beet reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer’s protocol. Each RNA pellet was redissolved in 0.2 ml of diethyl pyrocarbonate-treated water. RNA concentrations were quantified by ultraviolet absorbance at 260 nm.
Branched DNA signal amplification assay. The branched DNA (bDNA) assay is a high-throughput and quantitative method for messenger RNA (mRNA) quantification (Canales et al., 2006), which has been used extensively in our laboratory. The mRNA of genes examined was quantified using Quantigene bDNA signal amplification kit (Panomics, Fremont, CA) with modifications (Leazer and Klaassen, 2003). The probes for all mouse genes determined herein have been reported previously (Buist and Klaassen, 2004; Cheng et al., 2005; Maher et al., 2005). Luminescence of the 96-well plate was quantified with a Synergy 2 Microplate reader (BioTek Instruments, Inc., Winooski, VT). The luminescence for each well is reported as relative light units (RLU) per 10 μg of total RNA.

Statistics. Data are presented as mean ± standard error. Differences between groups were determined by ANOVA, followed by Duncan’s multiple-range test with significance set at p < 0.05.

RESULTS

Hepatic Expression of Major Uptake and Efflux Transporters in Oatp1b2-null Mice

To investigate whether knockout of Oatp1b2 causes compensatory alterations in hepatic expression of other transporters, we quantified hepatic mRNA expression of major uptake and efflux transporters. There is no gender difference in hepatic mRNA expression of Oatp1b2 in wild-type mice (Fig. 2). In Oatp1b2-null mice, the truncated Oatp1b2 mRNA was detected, but with much lower RLUs compared with wild-type mice. Hepatic mRNA expression of most transporters remained unchanged, namely Oatp1a1 (also known as Oatp1), Oatp2b1, Ntcp, bile salt export pump, adenosine triphosphate binding cassette g5/g8, multidrug resistance protein 2, multidrug resistance–associated protein 2 (Mrp2), Mrp4, Mrp5, Mrp6, and breast cancer resistance protein (data not shown). However, compared with wild-type mice, male and female Oatp1b2-null mice had 2.5- and 1.1-fold higher hepatic mRNA expression of Oatp1a4 (also known as Oatp2) but 50 and 43% lower mRNA expression of Oat2, respectively (Fig. 2). Wild-type female mice had higher hepatic mRNA expression of Mrp3 than wild-type males. Oatp1b2-null males had 97% higher hepatic Mrp3 mRNA than wild-type males, in contrast to similar hepatic levels of Mrp3 transcripts in both genotypes of female mice (Fig. 2).

Alteration in Blood Chemistry in Oatp1b2-null Mice

Oatp1b2-null mice develop and breed well, and the relative liver/body weight ratio remained unchanged in both genders of Oatp1b2-null mice (data not shown). The blood chemistry of Oatp1b2-null mice remained generally normal with a few exceptions. Blood levels of glucose, urea nitrogen, creatinine, globulin, ALP, aspartate aminotransferase, lipase, creatine phosphokinase, calcium, phosphorus, potassium, and CO2 remained similar between wild-type and Oatp1b2-null mice (data not shown). In Oatp1b2-null females, the most notable finding was a 2.5-fold elevation in serum levels of total bilirubin, which was almost entirely due to an increase in conjugated bilirubin. In contrast, in Oatp1b2-null males, serum levels of unconjugated bilirubin tended to be higher (p = 0.067), compared with wild-type males. A change observed in Oatp1b2-null males was a 30% increase in serum levels of total cholesterol compared with wild-type males. In both genders of Oatp1b2-null mice, serum levels of total protein and albumin increased slightly. Additionally, slight but statistically significant changes in blood chemistry were detected in Oatp1b2-null females, including increased serum levels of ALT, sodium, and chloride, as well as decreased serum levels of amylase compared with wild-type females.
Plasma Levels and Biliary Excretion of DBSP in Oatp1b2-null Mice

After intra-arterial injection, plasma levels of DBSP decreased rapidly in wild-type mice but much slower in Oatp1b2-null mice (Fig. 3A). Consequently, plasma levels of DBSP remained 4.8-fold higher in Oatp1b2-null compared with wild-type mice at 52.5 min after DBSP administration. The area under curve of blood levels of DBSP is 4950 and 14,900 μmol/min/l in wild-type and Oatp1b2-null mice, respectively. DBSP did not alter bile flow in either genotype of mice (Fig. 3B). The biliary excretion of DBSP in wild-type mice was rapid: within 1 h after injection, more than 95% of the administered DBSP was excreted into bile. The initial biliary excretion of DBSP for the first and second 15-min collection period was 48% and 33% less in the Oatp1b2-null mice (Fig. 3C), and the 1-h cumulative biliary excretion of DBSP was 30% less (Fig. 3D) in Oatp1b2-null compared with wild-type mice.

Role of Oatp1b2 in Mediating the Decrease in Bile Flow
Induced by E217βG

E217βG, a common substrate for Oatp1a1, 1a4, and 1b2, was used to investigate the role of Oatp1b2 in hepatic uptake of common substrates for Oatps. There was no difference in the basal rate of bile flow between female wild-type and Oatp1b2-null mice (Fig. 4). Intra-arterial injection of E217βG into wild-type female mice induced a 78% decrease in bile flow 30-min postinjection (Fig. 4). The initial decrease in bile flow induced by E217βG was attenuated in Oatp1b2-null females; however, such protection faded gradually, resulting in similar bile flow between wild-type and Oatp1b2-null females 1 h after E217βG administration.

Role of Oatp1b2 in Mediating Hepatic Uptake of Phalloidin
and Phalloidin-Induced Cholestasis

Similar to females, there was no difference in the basal rate of bile flow between male wild-type and Oatp1b2-null mice (Fig. 5A). Wild-type mice had severe cholestasis 1 h after intra-arterial injection of phalloidin 400 μg/kg (containing 80 μg/kg phalloidin-FITC) (Fig. 5A). This phalloidin-induced decrease in bile flow was substantially attenuated in Oatp1b2-null males throughout the entire experimental period. One h after phalloidin administration, the rates of bile flow were 7 and 53% of the initial values in wild-type and Oatp1b2-null males, respectively. One-hour postinjection, phalloidin-FITC was

FIG. 3. Plasma levels (A), bile flow (B), and biliary excretion (C, D) of DBSP in male C57BL/6 and Oatp1b2-null mice. Mice were administered a bolus intra-arterial dose of DBSP (120 μmol/kg). Data are presented as mean ± standard error of four to five samples. *p < 0.05 compared with C57BL/6 group.

FIG. 4. Partial protection of Oatp1b2-null mice from estradiol-17β-D-glucuronide (E217βG)-induced decrease in bile flow. Female adult C57BL/6 and Oatp1b2-null mice were administered a bolus intra-arterial dose of E217βG (24 μmol/kg). Data are presented as mean ± standard error of four to five samples. *p < 0.05 compared with C57BL/6 group.
associated with the inner plasma membrane of all hepatocytes of wild-type mice (Fig. 5B), but was absent in hepatocytes of Oatp1b2-null mice (Fig. 5C), indicating a lack of hepatic uptake of phalloidin in Oatp1b2-null mice.

### Differential Roles of Oatp1b2 in Mediating Hepatotoxicity of Mushroom Toxins

Phalloidin induces a rapid disruption of hepatocytes. Serum levels of ALT were highly elevated in both strains of wild-type mice (Fig. 6A) 6 h after ip injection of phalloidin (2.5 mg/kg). By gross examination, the livers of phalloidin-treated wild-type mice were swollen and markedly hemorrhagic. Oatp1b2-null mice (in a mixed genetic background of C57BL/6 and 129Sv) were completely protected from phalloidin-induced hepatotoxicity as indicated by the lack of elevation in serum levels of ALT (Fig. 6A). Histological analysis showed that after phalloidin treatment, there was extensive hemorrhagic necrosis of the liver characterized by numerous nonfatty vacuoles in wild-type mouse liver (Fig. 6B), whereas Oatp1b2-null mouse liver remained normal (Fig. 6C). In a separate experiment, Oatp1b2-null mice remained active 24 h after being injected ip with 2.5 mg/kg of phalloidin, and there was no elevation of serum levels of ALT or urea nitrogen (data not shown), indicating that there was no delayed hepatotoxicity or injury to kidneys. In contrast, Oatp1b2-null mice were similarly sensitive to α-amanitin–induced hepatotoxicity compared with wild-type mice 16 h after ip injection of α-amanitin (1.0 mg/kg) indicated by similarly elevated serum levels of ALT in 3 groups of mice treated with α-amanitin (Fig. 6A).

### Protection of Oatp1b2-null Mice from Microcystin-LR–induced Hepatotoxicity

Three of six C57BL/6 mice died 20 h after ip injection of microcystin-LR (120 μg/kg), whereas all six Oatp1b2-null mice (backcrossed to C57BL/6 for six generations) survived. Microcystin-LR induced severe liver injury in C57BL/6 mice, evident by the markedly elevated serum levels of both ALT and ALP (Fig. 7A), indicating that both hepatocytes and cholangiocytes were injured. In contrast, serum levels of both ALT and ALP remained unchanged in Oatp1b2-null mice (Fig. 7A). Histological analysis showed that after microcystin-LR treatment, there was extensive hemorrhagic necrosis of the liver in wild-type mouse liver (Fig. 7B), whereas Oatp1b2-null mouse liver remained normal (Fig. 7C). Thus, Oatp1b2 is critical in mediating the hepatotoxicity of microcystin-LR. Additionally, there was no acute nephrotoxicity induced by
microcystin-LR in either genotype of mice as indicated by a lack of elevation in serum levels of urea nitrogen (data not shown).

After uptake into hepatocytes, microcystin-LR binds tightly to protein phosphatase 1/2a (PP1/2a) in mouse nuclei; the microcystin-PP1/2a adducts (at approximately 40 kDa) can be detected by Western blot (Guzman and Solter, 2002). As shown in Figure 7D, in microcystin-LR treated C57BL/6 mouse liver samples (lanes 1–2), the anti-microcystin antibody detected two strong bands (~40 kDa), which were absent in untreated mouse liver (lane 5). The two ~40-kDa bands were much weaker in Oatp1b2-null mouse livers (lanes 3–4) compared with C57BL/6 mouse livers (lanes 1–2).

**DISCUSSION**

The present study demonstrates that the first mice null for the liver-specific importer Oatp1b2 have been generated. Oatp1b2-null mice develop normally and breed well. However, Oatp1b2-null mice have moderately altered blood chemistry. Compared with wild-type mice, Oatp1b2-null mice have higher plasma levels and lower biliary excretion of DBSP after a bolus dose, as well as higher rates of bile flow after administration of phalloidin and E217betaG. Phalloidin and microcystin-LR cause severe hepatotoxicity in wild-type mice but not in Oatp1b2-null mice. However, Oatp1b2-null mice are similarly sensitive as wild-type mice to alpha-amanitin–induced hepatotoxicity.

Currently, the mechanism of apparent induction of Oatp1a4 in the liver of Oatp1b2-null mice remains unknown. Oatp1a4 is closely linked with Oatp1b2 on chromosome 6. Thus, the disruption of Oatp1b2 gene might result in a compensatory induction of its neighbor gene, Oatp1a4. Previous in vitro studies demonstrate that Oatp1a4 transports E217betaG, but not phalloidin or microcystin-LR (Fischer et al., 2005; Meier-Abt et al., 2004). Thus, the induction of Oatp1a4 in Oatp1b2-null mice may compensate for Oatp1b2 in hepatic uptake of E217betaG and other common substrates for Oatps, but has no effect on hepatic uptake or toxicity of phalloidin or microcystin-LR.

Upon uptake into hepatocytes, the nonmetabolizable organic dye, DBSP is rapidly excreted into bile without biotransformation. Thus, in Oatp1b2-null mice, a slower uptake of DBSP into liver results in a slower rate of biliary excretion of DBSP. Loss of Oatp1b2 will decrease the rate of biliary excretion of its substrates that do not undergo further biotransformation.

The degree of decrease in bile flow by E217betaG has been shown to be dose-dependent in the range of 8.5–21 μmol/kg iv (Meyers et al., 1980). Thus, the initial partial protection of the decrease in bile flow in Oatp1b2-null mice (Fig. 4) indicates that hepatic uptake of E217betaG is partially decreased in Oatp1b2-null mice at early time points, which is consistent with the fact that Oatp1a1 and Oatp1a4 also transport E217betaG. The lack of significant difference in bile flow between wild-type and Oatp1b2-null mice at a later time after E217betaG administration (1 h) suggests that Oatp1a1 and Oatp1a4 can...
compensate with time for Oatp1b2 in hepatic uptake of certain common Oatp substrates under physiological conditions.

The mushroom toxin phalloidin specifically causes hepatotoxicity after parenteral injection, and is only toxic to hepatocytes (Frimmer, 1987). Phalloidin cannot be degraded by peptidases or proteases in animals (Frimmer, 1987). The complete protection of phalloidin-induced hepatotoxicity in Oatp1b2-null mice (Fig. 6) is consistent with phalloidin being a specific substrate for rat Oatp1b2 in vitro (Meier-Abt et al., 2004). The lack of hepatic uptake of phalloidin in Oatp1b2-null mice is confirmed by the absence of phalloidin-FITC in Oatp1b2-null hepatocytes. The mild decrease in bile flow in Oatp1b2-null mice may be due to a membrane-disturbing effect of phalloidin, which is evident by a transient gastrointestinal discomfort after oral ingestion of phalloidin (Frimmer, 1987). Oral ingestion of phalloidin is not hepatotoxic due to the inability of intestinal cells to take up phalloidin (Petzinger et al., 1982). In contrast, α-amanitin, a cyclic peptide structurally similar to phalloidin, causes liver injury in humans after accidental ingestion of Amanita phalloides (Jaeger et al., 1993). Cellular studies indicate that OATP1B1 is the primary transporter for phalloidin, whereas OATP1B3 only weakly transports phalloidin (Fehrenbach et al., 2003; Meier-Abt et al., 2004). In contrast, α-amanitin is transported by OATP1B3 but not OATP1B1 (Letschert et al., 2006), and α-amanitin only weakly inhibits the uptake of phalloidin by OATP1B1 (Fehrenbach et al., 2003). Cellular studies indicate that the liver-specific importer rat Ntcp (Gundala et al., 2004) and human OATP1B3 (Letschert et al., 2006) transport α-amanitin. Thus, Ntcp and OATP1B3 may mediate hepatic uptake of α-amanitin in rodents and humans, respectively.

The present study demonstrates that Oatp1b2 is essential in mediating microcystin-LR–induced hepatotoxicity. After iv administration, the cyclic peptide microcystin-LR is highly concentrated and accumulates in mouse liver (Robinson et al., 1991); the inhibition of serine/threonine PP 1 and 2A by microcystin-LR causes hepatic hemorrhage and necrosis at high doses, and promotes liver cancer at low doses (Gehringer, 2004). The complete protection of Oatp1b2-null mice against microcystin-induced hepatotoxicity is consistent with the in vitro results of Oatp1b2 being the sole hepatic importer for microcystin-LR in rodents (Fischer et al., 2005). Both OATP1B1 and OATP1B3 can transport microcystin-LR (Fischer et al., 2005), and cells expressing OATP1B1/1B3 are highly sensitive to microcystin-LR cytotoxicity compared with wild-type cells (Komatsu et al., 2007; Monks et al., 2007), demonstrating a critical role of OATP1B1/B3 in mediating cellular uptake and toxicity of microcystin. Although microcystin-LR is selectively toxic to liver in vivo and hepatocytes in vitro, exposure of other types of cells to microcystin-LR at higher concentrations for a longer period of time results in cell death (Khan et al., 1995). In the present study, the binding of microcystin-LR to PP1/2a was attenuated, but not abolished, in livers of Oatp1b2-null mice, indicating...
that in addition to Oatp1b2-mediated transport, microcystin-LR can enter cells by passive diffusion or other mechanism(s) at a lower rate. Different from the non-cell-permeable phalloidin, microcystin-LR is bioavailable, and the hepatotoxicity of oral microcystin-LR markedly increases in aged mice, most likely due to increased intestinal absorption (Ito et al., 1997). The development of primary liver cancer in China has been linked to a long-term chronic exposure to microcystin as a result of contamination of drinking water (Ueno et al., 1996). Thus, the essential role of Oatp1b2 and OATP1B1/1B3 in mediating microcystin-LR hepatotoxicity has important clinical and public health implications.

Currently, the mechanism of a female-selective development of conjugated hyperbilirubinemia in Oatp1b2-null mice is unknown, but the data suggest that Oatp1b2 plays an important role in bilirubin homeostasis. Unconjugated bilirubin produced in splenic macrophages is released into blood, bound to albumin, and distributed to liver, where it is conjugated with glucuronic acid and excreted into bile via Mrp2 (Klaassen and Lu, 2007). Consequently, conjugated bilirubin is difficult to detect in normal mouse blood. Hyperbilirubinemia occurs during cholestasis. However, the female-selective conjugated hyperbilirubinemia is not due to cholestasis, because the rate of bile flow remains unchanged in Oatp1b2-null females compared with wild-type females (Fig. 4). Uridine diphosphate glucuronosyltransferase 1a1 (Ugt1a1), the sole enzyme glucuronidating bilirubin (Kamisako et al., 2000), is expressed at higher levels in female than male mouse liver (Buckley and Klaassen, 2007). Hepatic expression of Oatp1a1, which is able to transport bilirubin glucuronide (Kamisako et al., 2000), is lower in female than male mice (Cheng et al., 2005). In contrast, Mrp3, a basolateral exporter retro-transporting bilirubin glucuronides from liver into blood during cholestasis (Belinsky et al., 2000), is expressed at higher levels in females than males (Fig. 2). Bilirubin glucuronides formed within hepatocytes can be excreted into bile via Mrp2, or transported back into blood via Mrp3. In vitro studies demonstrate that multiple Oatps (Oatp1a1 and OATP1B1) are capable of transporting bilirubin glucuronides (Cui et al., 2001; Kamisako et al., 2000). OATP1B1 and OATP1B3 have high affinities for bilirubin glucuronides (Km at nanomolar concentrations) (Cui et al., 2001), suggesting that bilirubin glucuronides pumped out by Mrp3 could be readily taken back into hepatocytes by Oatp1b2. Thus, we speculate that gender differences in hepatic expression of Ugt1a1, Mrp3, and Oatp1a1 may result in a gender difference in hepatic production, efflux, and reuptake of bilirubin glucuronides, leading to a conjugated hyperbilirubinemia selectively in Oatp1b2-null female mice.

In summary, the present study demonstrates a gender-selective development of conjugated hyperbilirubinemia in female Oatp1b2-null mice. Oatp1b2 plays key roles in hepatic uptake and toxicity of phalloidin and microcystin-LR, and is important in determining systemic disposition of DBSP, a model chemical used for liver function assay. Reduction in OATP1B1 function in populations with functional polymorphisms and/or in pathophysiological conditions is common in humans. Oatp1b2-null mice will be very useful to elucidate how a loss/decrease of Oatp1b2 and OATP1B1/1B3 function disturbs the homeostasis of endogenous chemicals and influences the hepatic uptake and systemic exposure to environmental chemicals and therapeutic drugs.

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