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Regulation of transporter expression in mouse liver, kidney, and intestine during extrahepatic cholestasis

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Abstract

It is hypothesized that during cholestasis, the liver, kidney, and intestine alter gene expression to prevent BA accumulation; enhance urinary excretion of BA; and decrease BA absorption, respectively. To test this hypothesis, mice were subjected to either sham or bile-duct ligation (BDL) surgery and liver, kidney, duodenum, ileum, and serum samples were collected at 1, 3, 7, and 14 days after surgery. Serum total BA concentrations were 1–5 μmol/l in sham-operated mice and were elevated at 1, 3, 7, and 14 days after BDL, respectively. BDL decreased liver Ntcp, Oatp1a1, 1a5, and 1b2 mRNA expression and increased Bsep, Oatp1a4, and Mrp1–5 mRNA levels. In kidney, BDL decreased Oatp1a1 and increased Mrp1–5 mRNA levels. In intestine, BDL increased Mrp3 and Ibat mRNA levels in ileum. BDL increased Mrp1, 3, 4, and 5 protein expression in mouse liver. These data indicate that the compensatory regulation of transporters in liver, kidney, and intestine is unable to fully compensate for the loss of hepatic BA excretion because serum BA concentration remained elevated after 14 days of BDL. Additionally, hepatic and renal Oatp and Mrp genes are regulated similarly during extrahepatic cholestasis, and may suggest that transporter expression is regulated not to remove bile constituents from the body, but instead to remove bile constituents from tissues.

Keywords: Cholestasis; Bile-duct ligation; Multidrug resistance-associated protein; Organic anion transporting polypeptide; Transporter; Bile salt export pump

1. Introduction

Bile acids (BA) are synthesized in liver and excreted via the common bile duct into the small intestine. Once in the intestine, they aid in the emulsification and absorption of dietary fats. After reabsorption into enterocytes, BAs enter the circulation and most are transported into the liver via sodium-dependent and sodium-independent transport processes, with a small amount being excreted into urine.

Several transporters are known to transport BAs from blood into liver and from liver into bile. BAs are transported into the liver by the Sodium taurocholate-cotransporting polypeptide (Ntcp) and some members of the organic anion polypeptide transporter superfamily (Oatps), such as Oatp1a1, 1a4, and 1b2 [1]. Ntcp mediates sodium-dependent BA transport, whereas Oatps mediate sodium-independent BA and organic anion transport. In liver, Ntcp, Oatp1a1, Oatp1a4, and Oatp1b2 are localized to the sinusoidal membrane [2–5].

Conversely, the bile salt export pump (Bsep) and some members of the multidrug resistance-associated protein (Mrp) and Organic solute transporter family aid in transport of BAs out of the liver into either bile (i.e. Mrp2 and Bsep), or blood (i.e. Mrp3, Mrp4, Ostα/β) [1,6–8]. In humans, Progressive Familial Intrahepatic Cholestasis 2 is associated with mutations

Abbreviations: Ntcp, Slc10a1, Sodium taurocholate cotransporting polypeptide; Oatp, Slc21a, Organic anion transporting polypeptide; Ibat, Slc10a1, Ileal bile acid transporter; Bsep, abcb11, Bile salt-export pump; Mrp, Abcc, Multidrug resistance-associated protein; BDL, Bile duct ligation; CDCA, Chenodeoxycholic acid

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in the coding sequence of BSEP [9]. In mice, deletion of Bsep causes a milder progressive intrahepatic cholestasis and does not impede bile flow, although it decreases biliary excretion of hydrophobic BAs, such as taurocholate [6]. This observation suggests that other transporters aid in the export of BAs out of hepatocytes. Mutations in rat Mrp2 result in hyperbilirubinemia and moderate elevation of serum BAs [10]. In humans, similar mutations in MRP2 cause Dubin–Johnson syndrome, which is also associated with the accumulation of conjugated bilirubin and bile acids in blood [11,12]. In liver, Bsep and Mrp2 are localized to the canalicular membrane to transport chemicals from hepatocytes into bile, whereas Mrp1, 3, 4, and 5 are localized to the basolateral membrane, mediating transport of chemicals from liver to blood [13–18].

The mechanisms responsible for BA transport in kidney are not nearly as well-defined or characterized as those in liver. In rats and mice, Oatp1a1, 1a4 and Oatp1a6 mRNAs are expressed in kidney [19–22]. Oatp1 is localized to the apical membrane of renal proximal tubules where it is thought to aid in tubular reabsorption of organic compounds, and is responsible for gender differences in taurocholate excretion [23]. Mrp1, 2, 3, and 4 mRNAs are also detected in rat and mouse kidney [24–26]. Mrp2 and Mrp4 are localized to the apical membrane of proximal tubules, and are thought to aid in excretion of organic anions and BAs into urine [26].

BAs in serum are mainly recovered by enterohepatic circulation, rather than produced via *de novo* synthesis in liver [1,27]. Intestinal absorption of BAs occurs mainly in the ileum by the ileal BA transporter (Ibat) [1]. Recently, the heterodimeric organic solute transporter alpha-beta (Ostαβ) has been localized to the basolateral membrane of enterocytes in ileum and is believed to mediate bile acid efflux into blood circulation [28]. Mrp3 is also localized to the basolateral membrane of enterocytes, and plays a less important role for efflux of BAs from enterocytes into blood [29]. In a human colon cancer cell line (Caco-2), Mrp3 expression is induced by BAs via Liver receptor homologue-1 (Lrh-1) [30].

Cholestasis is defined as decreased or impaired bile flow. Various models exist to study intrahepatic and extrahepatic cholestasis. In this study, common bile-duct ligation (BDL) was employed to model extrahepatic cholestasis, which is associated with obstruction of the bile duct and diseases such as primary sclerosing cholangitis. After BDL, the liver accumulates BAs, and other compounds such as bilirubin, bilirubin–glucuronide, and eventually BA levels increase in liver, serum, and urine. During BDL, adaptive changes in transporter expression may occur in liver, kidney, and intestine to decrease levels of potentially toxic BAs and other waste in blood, and decrease absorption of BAs. For example, expression of the bile acid uptake transporter, Ntcp, in liver is downregulated after BDL, which is hypothesized to be a protective response to prevent further accumulation of bile acids in liver [31–33]. Similarly, expression of the basolateral bile acid export transporters, Mrp3 and 4, markedly increase after BDL, a response which lowers hepatic bile acid levels by increasing efflux of bile acids out of liver into the blood [34–36]. Studies using Mrp3- and Mrp4-null mice have demonstrated that these transporters contribute to liver accumulation of BAs during obstructive cholestasis. During obstructive cholestasis, liver BA levels are higher in Mrp3-null mice and serum BA levels are lower in serum of Mrp4-null than wild-type controls [8,35]. In addition, cholestasis increases Ostβ mRNA and protein expression in liver in rats, mice, and humans, which is another potential compensatory mechanism that is induced to decrease BA concentrations in liver [7]. Ost alpha and beta mRNA and protein is increased in patients with PBC, as well as after bile-duct ligation in the mouse [7].

To date, there is no single published studies that thoroughly characterize gene expression changes in the mouse BDL model. Therefore, the purpose of this study was to provide a comprehensive characterization of changes in the expression of several transporters/transporter families, namely the Oatps, Mrps, Ntcp, and Bsep in mouse liver, kidney and small intestine after BDL.

2. Materials and methods

2.1. Animal model

Adult male C57BL/6 mice were purchased from Jackson Laboratories (Bar Habor, ME). The mice were housed in a temperature-, light-, and humidity-controlled environment in cages with hardwood chips. The mice were fed Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI) *ad libitum*. Under pentobarbital-induced anesthesia (50 mg/kg, ip), the abdominal cavity was opened and the common bile-duct was ligated with 4-0 surgical silk, with the gall bladder intact. The abdominal muscle was sutured with Ethicon 4-0 dissolvable suture material and the wound was closed with surgical staples. Sham surgeries were performed by the same method, but without BDL. Serum, liver, kidneys, duodenum, and ileum were collected 1, 3, 7, and 14 days after BDL. Tissues were snap frozen in liquid nitrogen and stored at −70 °C until analysis. All animal studies were conducted according to Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

2.2. Serum BA concentration

Trunk blood was collected, allowed to coagulate, and then centrifuged at 5000 rpm for 5 min. The resulting supernatant (serum) was collected for analysis. Serum BA concentrations were quantified according to the manufacturer's protocol by measuring the production of formazan by the action of 3α-hydroxysteroid dehydrogenase and diaphorase, which is directly proportional to the BA concentration (Trinity Biotech, Wicklow, Ireland).

2.3. RNA extraction

Total RNA from liver, kidney, duodenum, and ileum tissue was extracted using RNA Bee Reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. RNA integrity was confirmed by formaldehyde-agarose gel electrophoresis.

2.4. Branched DNA signal amplification (bDNA) assay

Mouse Ntcp, Bsep, Oatp1a1, 1a4, 1b2, 4a1, Mrp1–5, Heme Oxygenase-1 (Ho-1) mRNA were quantified using the branched signal amplification assay (QuantiGene® High Volume bDNA Signal Amplification Kit, Genospectra, Freemont, CA) with modifications [24,37]. Probes to detect mouse Ntcp, Oatp1a1, 1a4, 1a5, 1b2, Mrp1–5, and Ho-1 have been described previously [38–42]. Multiple oligonucleotide probe sets (containing capture, label, and blocker probes) specific to a single mRNA transcript were designed using Probe Designer

software v1.0 (Bayer Corp., Emeryville, CA). Probes were designed with a Tm of approximately 63 °C, which enabled the hybridization conditions to be held constant (i.e. 53 °C) during each hybridization step and for each probe set. Each probe developed using ProbeDesigner was submitted to the National Center for Biotechnology Information for nucleotide comparison by the basic local alignment search tool (BLASTn), to ensure minimal cross-reactivity with other known mouse sequences and ESTs. Oligonucleotides with a high degree of similarity (≥80%) to other mouse gene transcripts were excluded from the design.

Total RNA (1 μg/μl; 10 μl/well) was added to each well of a 96-well plate containing 50 μl capture hybridization buffer and 50 μl of each diluted probe set. For each gene, total RNA was allowed to hybridize to the probe set overnight at 53 °C. Subsequent hybridization steps were carried out per the manufacturer's protocol, and luminescence was measured with a Quantiplex® 320 bDNA Luminometer interfaced with Quantiplex® (Bayer Corp.-Diagnostics, Div., Emeryville, CA). Data Management Software Version 5.02 for analysis of luminescence from 96-well plates. The luminescence for each well was reported as relative light units (RLUs) per 10 μg total RNA.

2.5. Preparation of crude membrane fractions

Livers were homogenized in ST buffer (0.25 M sucrose, 10 mM Tris–HCl, pH 7.4) and centrifuged at 100,000×*g* for 60 min at 4 °C. The resulting pellet containing the crude membrane fraction was resuspended in ST buffer. Protein concentration was determined using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA).

2.6. Western blot analysis of transport proteins

Membrane proteins (50 μg protein/lane) were electrophoretically resolved without boiling using polyacrylamide gels (8% resolving, 4% stacking) and transblotted overnight at 4 °C onto PVDF-Plus membrane (Micron Separations Inc., Westborough, MA). Immunochemical detection of Ho-1 was performed as previously described [43]. Immunochemical detection of Mrp1 (~190 kDa), Mrp2 (~190–200 kDa), Mrp3 (~180–190 kDa), Mrp4 (~160–170 kDa), Mrp5 (~160–180 kDa), and Mrp6 (~165 kDa) protein was performed using MRPr1, M2III-5, M3II-2, M4I-10, M5I-60, and M6II-68 antibodies, respectively [43]. Anti-Mrp antibodies were provided by George Scheffer, VU Medical Center, Amsterdam, the Netherlands. Membranes were blocked with 1% NFDM in PBS-Tween for 1 h and incubated for 1 h with primary antibody diluted in blocking buffer (1:2000 for MRPr1, M3II-2, M4I-10, M6II-68, 1:600 for M2III-5, and 1:50 for M5I-60). A species-appropriate peroxidase-labeled secondary antibody (Sigma Chemical Co., St. Louis, MO) was diluted (1:2000) in blocking buffer and incubated with blots for 1 h Protein–antibody complexes were detected using an ECL chemiluminescent kit (Amersham Life Sciences, Arlington Heights, IL) and exposed to Fuji Medical X-ray film (Fisher Scientific, Pittsburgh, PA). The intensity of protein bands was quantified using the Discovery Series Quantity One 1-D Analysis software (Biorad, Hercules, CA).

2.7. Statistics

Statistical differences between groups at each time point were determined by a Student's t-Test. Asterisks (*) represent a statistical difference from control ($p<0.05$).

3. Results

3.1. Serum BA concentration after BDL

Total BA concentrations in serum of mice that underwent sham and BDL surgeries are shown in Fig. 1. Serum total BA concentrations were 1–5 μmol/l in sham-operated mice and were elevated to 1166, 1222, 2279, 2957 μmol/l at 1, 3, 7, and 14 days after BDL, respectively. One and 3 days after BDL, serum BA levels increased approximately 200–250 fold above those detected in serum from sham mice. At 7 and 14 days after BDL, serum BA concentrations were approximately 1800 fold higher than that detected in sham mice.

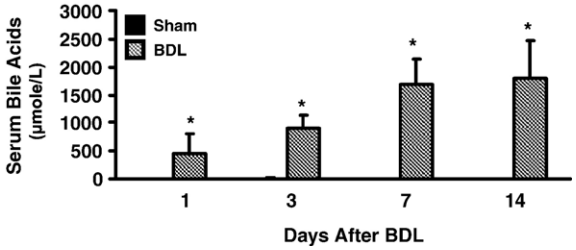

Fig. 1. Bile acid concentration in serum from male C57BL/6 mice 1, 3, 7, and 14 days after sham or bile-duct ligation (BDL) surgery. The data are presented as mean+S.E.M. ($n=4$–7 mice). Asterisks (*) represent a statistical difference ($p<0.05$) between sham and BDL groups.

3.2. Ntcp and Bsep mRNA expression in liver after BDL

Ntcp and Bsep are considered to be the primary BA transporters in liver, and are responsible for hepatic uptake and efflux of BAs, respectively. The effect of BDL on Ntcp and Bsep mRNA expression in mouse liver is shown in Fig. 2. At 1 and 7 days after BDL, Ntcp mRNA expression was reduced approximately 50%. Bsep mRNA levels in liver increased 40–50% 1 and 7 days after BDL.

3.3. Oatp mRNA expression in liver and kidney after BDL

In liver, Oatps are thought to mediate sodium-independent uptake of a wide variety of compounds, including bile acids. The effect of BDL on Oatp mRNA expression in mouse liver is shown in Fig. 3. BDL decreased Oatp1a1 and 1b2 mRNA expression in liver. One day after BDL, Oatp1a1 mRNA expression in liver was decreased by 80%, and by more than 90% thereafter. BDL doubled Oatp1a4 mRNA expression in liver by day 3 and increased mRNA expression by 5 fold at 2 weeks. The mRNA expression of Oatp1b2, also known as Liver-Specific Transporter (Lst), was decreased about 20% by BDL. Basal mRNA expression of Oatp1a5 is relatively low in rat liver [44], and BDL did not alter Oatp1a5 levels (data not shown).

The effect of BDL on Oatp mRNA expression in mouse kidney is shown in Fig. 4. BDL decreased renal Oatp1a1 expression by 50% on day 1, and about 90%, thereafter. Oatp1a4 expression in kidney varied over time after BDL. After BDL, Oatp1a4 mRNA expression in kidney increased at day 1, did not differ from sham expression on day 3, and was decreased at days 7 and 14. BDL doubled Oatp4a1 RNA expression in kidney.

3.4. Mrp mRNA expression in liver and kidney after BDL

In liver, some multidrug resistance-associated proteins are thought to transport BA and bilirubin–glucuronide out of hepatocytes into bile or blood, namely Mrp3 [8]. The effect of BDL on Mrp mRNA expression in mouse liver is shown in Fig.

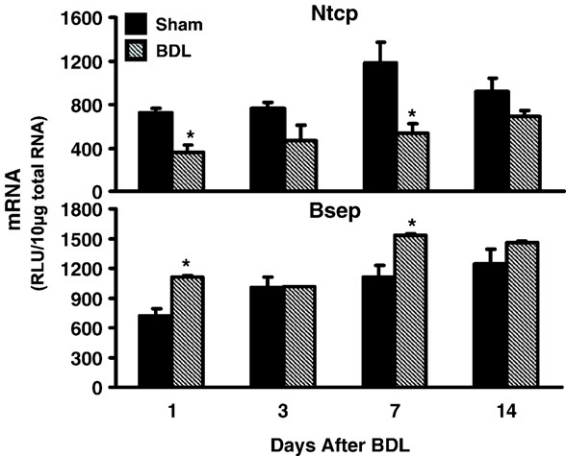

Fig. 2. Time course of Sodium taurocholate-cotransporting polypeptide (Ntcp) and Bile salt export pump (Bsep) mRNA expression in mouse liver after bile-duct ligation (BDL). Total RNA was isolated from mouse livers collected 1, 3, 7, and 14 days after sham or BDL surgery and analyzed by the bDNA assay for Ntcp and Bsep expression. The data are presented as mean relative light units (RLU)+S.E.M. (n=4–7 animals). Asterisks (*) represent a statistical difference (p<0.05) between sham and BDL groups.

5. In general, Mrp1–5 mRNA expression was elevated after BDL in liver. At 1 day after BDL, Mrp3 and Mrp5 mRNA levels were elevated 1.4 and 3 fold, respectively. At 3 days after BDL, Mrp1, 3, 4, and 5 mRNA expression was increased by at least 40% in livers from mice that underwent BDL surgery. At 7 and 14 days after BDL, mRNA expression of Mrps1–5 in liver was significantly higher than that detected in livers from mice that underwent sham surgery. All of the Mrps were increased 2 to 10 fold 7 and 14 days after BDL.

The effect of BDL on Mrp mRNA expression in mouse kidney is shown in Fig. 6. BDL also increased Mrp mRNA expression in kidney. Mrp2 and 4 mRNA expression was significantly increased 1 day after BDL (30 and 50%, respectively). BDL increased Mrp3 and 4 mRNA expression 3, 7, and 14 days after BDL. Mrp5 expression in kidney was also doubled 7 days after BDL.

3.5. Transporter mRNA expression in intestine after BDL

The function of Ibat is to aid in absorption of bile acids from intestinal lumen into enterocytes, and Mrp3 may participate in export of BA from enterocytes into blood. The effect of BDL on the expression of the bile acid transporters, Ibat in ileum and Mrp3 in duodenum and ileum, is shown in Fig. 7. Ibat mRNA expression in duodenum was very low (data not shown), but was high in ileum. Ibat mRNA expression in ileum approximately doubled 7 and 14 days after BDL. BDL did not affect Mrp3 expression in duodenum, but did increase Mrp3 expression in ileum. Mrp3 mRNA expression increased by 50% in ileum 14 days after BDL.

3.6. Mrp1–6 protein expression in liver after BDL

Mrp1–6 protein expression after BDL was measured in liver membrane fractions (Fig. 8). BDL increased Mrp1, 3, 4, and 5 protein expression in mouse liver. Mrp1 expression in liver was increased by 3.5 and 2.8 fold over sham controls at 3 and 7 days after BDL, respectively. Mrp2 protein levels were slightly increased 1 day after BDL, but unchanged at 3 and 7 days after BDL. Mrp3 protein expression was increased 6.6 and 12.4 fold over sham controls at 3 and 7 days after BDL, respectively. Mrp4 protein expression was increased 2.5 and 3.1 fold over sham controls at 3 and 7 days after BDL, respectively. Mrp5 protein expression was increased 5.5 and 6.6 fold over sham controls at 3 and 7 days after BDL, respectively. BDL did not affect Mrp6 protein levels.

3.7. Induction of Heme Oxygenase-1 (Ho-1) mRNA and protein expression in liver after BDL

Heme Oxygenase-1 is a microsomal enzyme that catalyzes the conversion of heme to biliverdin, the precursor to bilirubin. The Ho-1 gene is inducible and is known to be induced through activation of the nuclear receptor, NF-E2-related factor-2 (Nrf2) [45]. Fig. 9 illustrates the time course of Ho-1 expression in liver after BDL. BDL induced Ho-1 mRNA expression in mouse liver by about 2–5 fold at 1, 3, 7, and 14 days. Similarly,

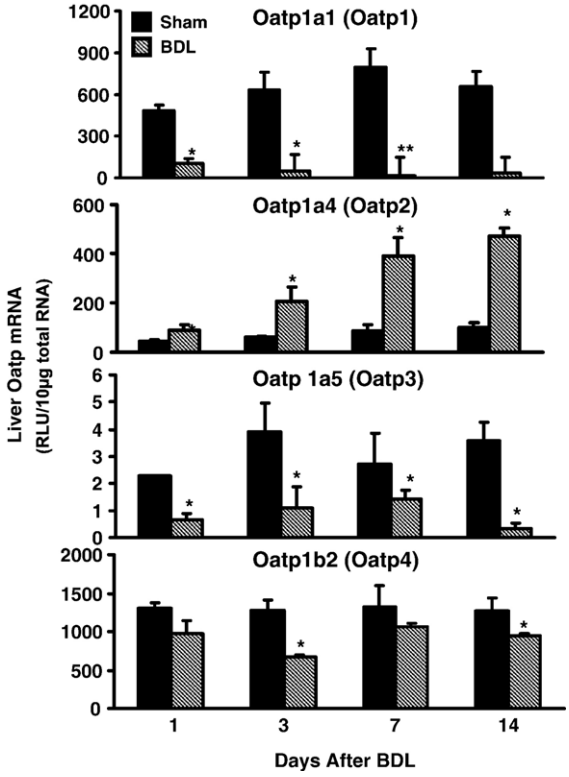

Fig. 3. Time course of organic anion transporting polypeptide (Oatp) 1a1, 1a4, 1a5, and 1b2 mRNA expression in mouse liver after bile-duct ligation (BDL). Total RNA was isolated from mouse livers collected 1, 3, 7, and 14 days after sham or bile-duct ligation (BDL) surgery and analyzed by the bDNA assay for organic anion transporting polypeptide (Oatp) 1a1, 1a4, 1a5, and 1b2 expression. The data are presented as mean relative light units (RLU)+S.E.M. (n=4–7 animals). Asterisks (*) represent a statistical difference (p<0.05) between sham and BDL groups.

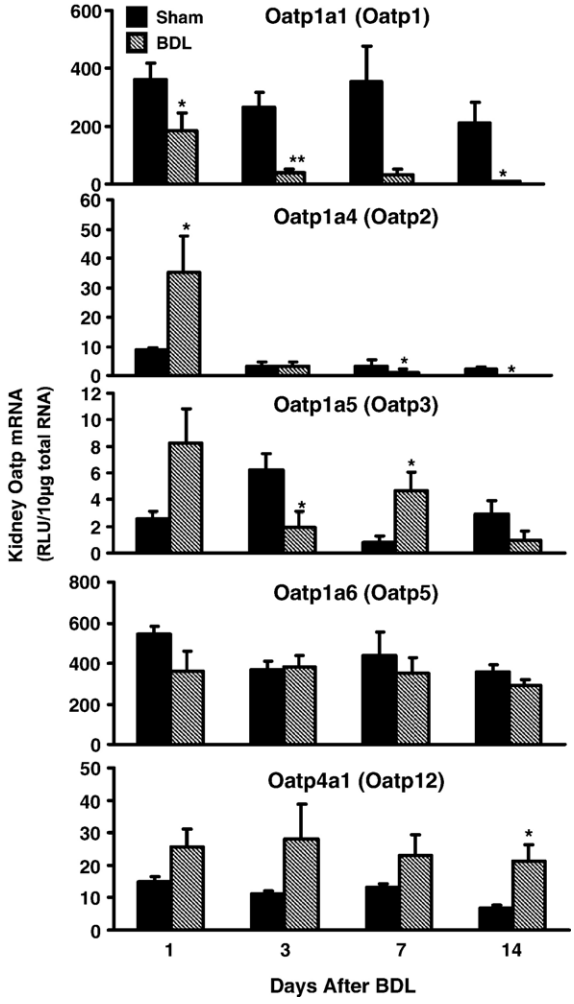

Fig. 4. Time course of organic anion transporting polypeptide (Oatp) 1a1, 1a4, 1a5, 1a6n and 4a1 mRNA expression in mouse kidney after bile-duct ligation (BDL). Total RNA was isolated from mouse kidneys collected 1, 3, 7, and 14 days after sham or BDL surgery and was analyzed by the bDNA assay for Oatp 1a1, 1a4, 1a5, 1a6, and 4a1 expression. The data are presented as mean relative light units (RLU)+S.E.M. (n=4–7 animals). Asterisks (*) represent a statistical difference ($p<0.05$) between sham and BDL groups.

BDL increased Ho-1 protein expression liver by about 3 fold at 3 and 7 days.

4. Discussion

It is important to understand changes that occur during cholestasis at the molecular level. Understanding of how the liver and kidney adapt in order to decrease tissue bile acid levels is particularly important for understanding the pathophysiology of cholestasis, and how drug distribution may be changed during cholestasis. The goal of this study was to provide a thorough characterization of the effects of BDL on hepatic, renal, and intestinal transporter expression in mice. Given the growing availability of knock-out mice and completion of sequencing for the mouse genome, the mouse is becoming an even more important model for studying gene expression and function, as well as certain disease states. Therefore, identifying changes in mRNA expression of genes in mouse tissues important for transporting endogenous and exogenous compounds by the liver, kidney, and intestine in mice during BDL, is important for gaining insight into how tissues adapt to high levels of bile acids, bilirubin, and other chemicals, as well as how drug distribution could be changed during obstructive cholestasis.

In this study, serum BA levels increased 1, 3, 7, and 14 days after BDL. The level of BA in serum was markedly elevated 14 days after BDL. The continued elevation of serum BA levels after BDL over 14 days suggests that BA production/transfer to blood is occurring faster than renal excretion of BAs from the blood and excretion into urine. Upregulation of Ibat, Mrp3, and Ostα/β in ileum, and thus increased BA absorption from gut, could also add to the observation of elevated serum BA levels in mice throughout 14 days of BDL. These data also imply that adaptive changes in liver, kidney, and intestine after BDL are not sufficient to compensate for the decreased excretion of BA into

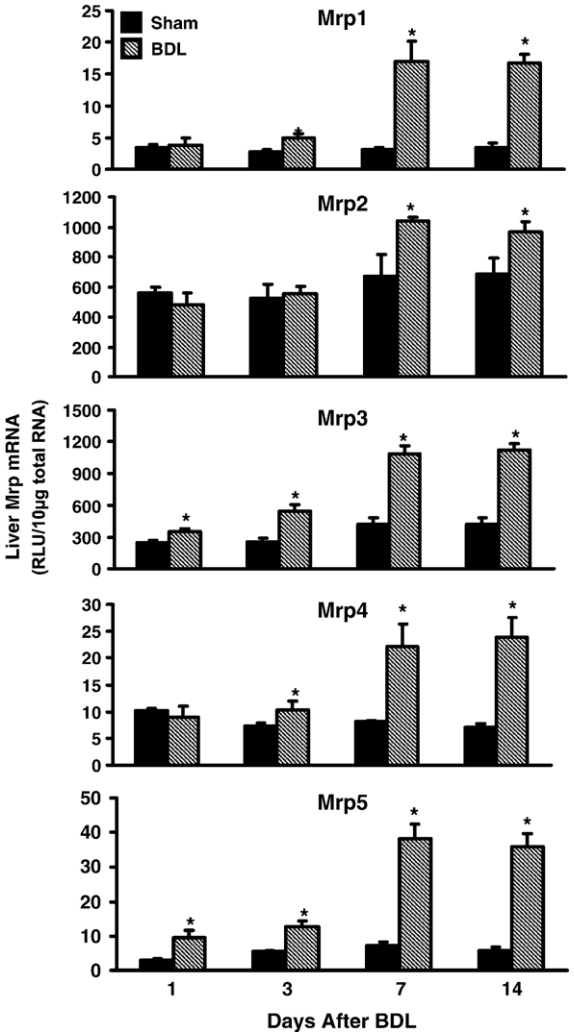

Fig. 5. Time course of Multidrug resistance-associated protein (Mrp) 1–5 mRNA expression in mouse liver after bile-duct ligation (BDL). Total RNA was isolated from mouse livers collected 1, 3, 7, and 14 days after sham or BDL surgery and was analyzed by the bDNA assay for Mrp expression. The data are presented as mean relative light units (RLU)+S.E.M. (n=4–7 animals). Asterisks (*) represent a statistical difference ($p<0.05$) between sham and BDL groups.

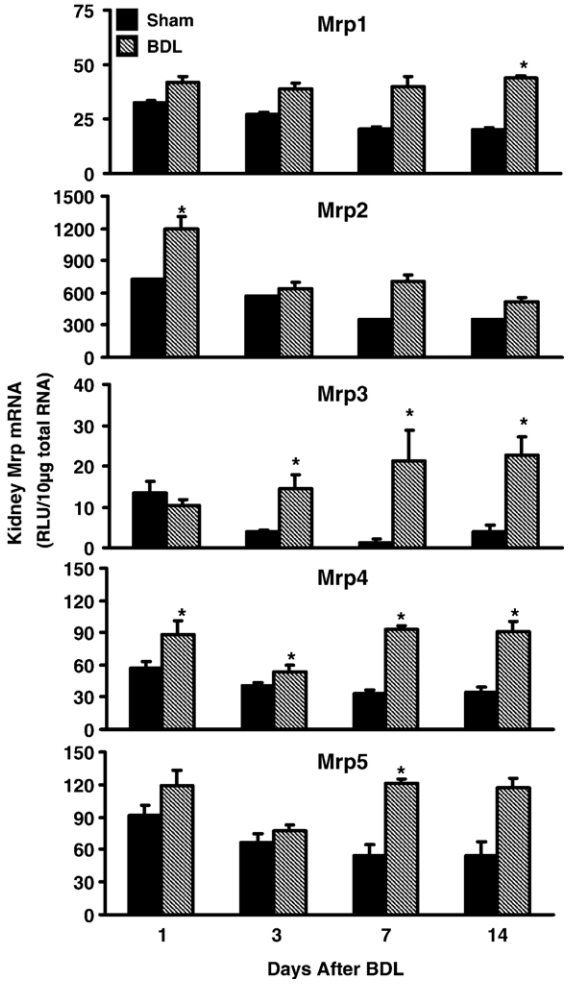

Fig. 6. Time course of Multidrug resistance-associated protein (Mrp) 1–5 mRNA expression in mouse kidney after bile-duct ligation (BDL). Total RNA was isolated from mouse kidney at 1, 3, 7, and 14 days after sham or BDL surgery and was analyzed by the bDNA assay for Mrp 1–5 expression. The data are presented as mean relative light units (RLU)+S.E.M. (n=4–7 animals). Asterisks (*) represent a statistical difference (p<0.05) between sham and BDL groups.

bile after BDL, because serum BA levels increase throughout the 14-day interval. The observation that BDL increases serum BA levels is consistent with a previous report that demonstrated elevations in serum total BA levels 3 and 7 days after BDL in mice [18]. Interestingly, the observed increase in serum BA levels after BDL in mice differs from results after BDL in rats in which serum BA peaked 3 days after BDL, and declined thereafter [46]. There are several possible reasons for this observed species difference. First, the presence of a gallbladder in mice may affect the concentration and amount of BA that the liver is exposed to over time. Initially, the gallbladder in mice, compared to rats, may act as a reservoir for BA, decreasing the exposure of liver to high concentrations of BA. Bile flow rates do not differ significantly between rats and mice [47]. Because rats lack gallbladders, it is likely that after BDL, rat liver is exposed to higher concentrations of BA and other bile constituents at an earlier time than mouse liver. Second, the composition of bile acids for rats and mice may be different, such that rat BA are better signaling molecules for factors (such as nuclear hormone receptors) that can affect Cyp7a (bile acid formation) and transporter expression. Third, differences in response elements in the upstream regulatory regions of genes for the various transporters may exist between rats and mice, such that the rat response elements are more markedly activated or repressed. An example of this is Mrp3 induction in liver after BDL in rats and mice. In rats, Mrp3 mRNA expression is induced approximately 6 fold, but in mice Mrp3 mRNA is only induced about 2–3 fold, 7 days after BDL [18,36]. The mRNA and protein data in this study supports this finding. Thus, changes in liver, kidney, and intestinal expression of transporters that can transport BA after BDL observed in this study, in part, explain how BDL increases serum bile acids in mice.

BAs are extracted from blood into liver via several known transporters, including Ntcp and several Oatps. The role of Oatps in renal absorption of bile acids is not defined. In the present study, BDL altered the expression of several mouse Oatps in liver and kidney. Mouse Oatp1a1 can transport taurocholate and rat Oatp1a1, 1b2, and 4a1 can transport bile acids. The data in this manuscript show that Ntcp, Oatp1a1, and Oatp1b2 mRNA expression in liver are all markedly decreased after BDL. In particular, BDL decreased Oatp1a1 mRNA expression in liver by 93%. If the decrease in hepatic mRNA expression of Ntcp, Oatp1a1, and Oatp1b2 causes a corresponding decrease in hepatic protein expression, this could, in part, explain the increased serum BA levels because the transporters would extract less BA from blood into liver.

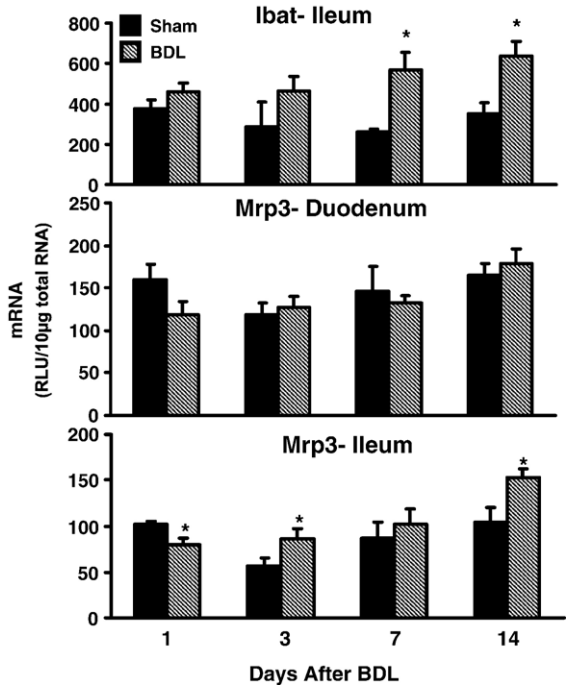

Fig. 7. Time course of Ileal bile acid transporter (Ibat) and Multidrug resistance protein 3 (Mrp3) mRNA expression in mouse duodenum and ileum after bile-duct ligation (BDL). Total RNA from duodenum and ileum was isolated from mice 1, 3, 7, and 14 days after sham or BDL surgery and analyzed by the bDNA assay for Ibat and Mrp3 expression. The data are presented as mean relative light units (RLU)+S.E.M. (n=4–7 animals). Asterisks (*) represent a statistical difference (p<0.05) between sham and BDL groups.

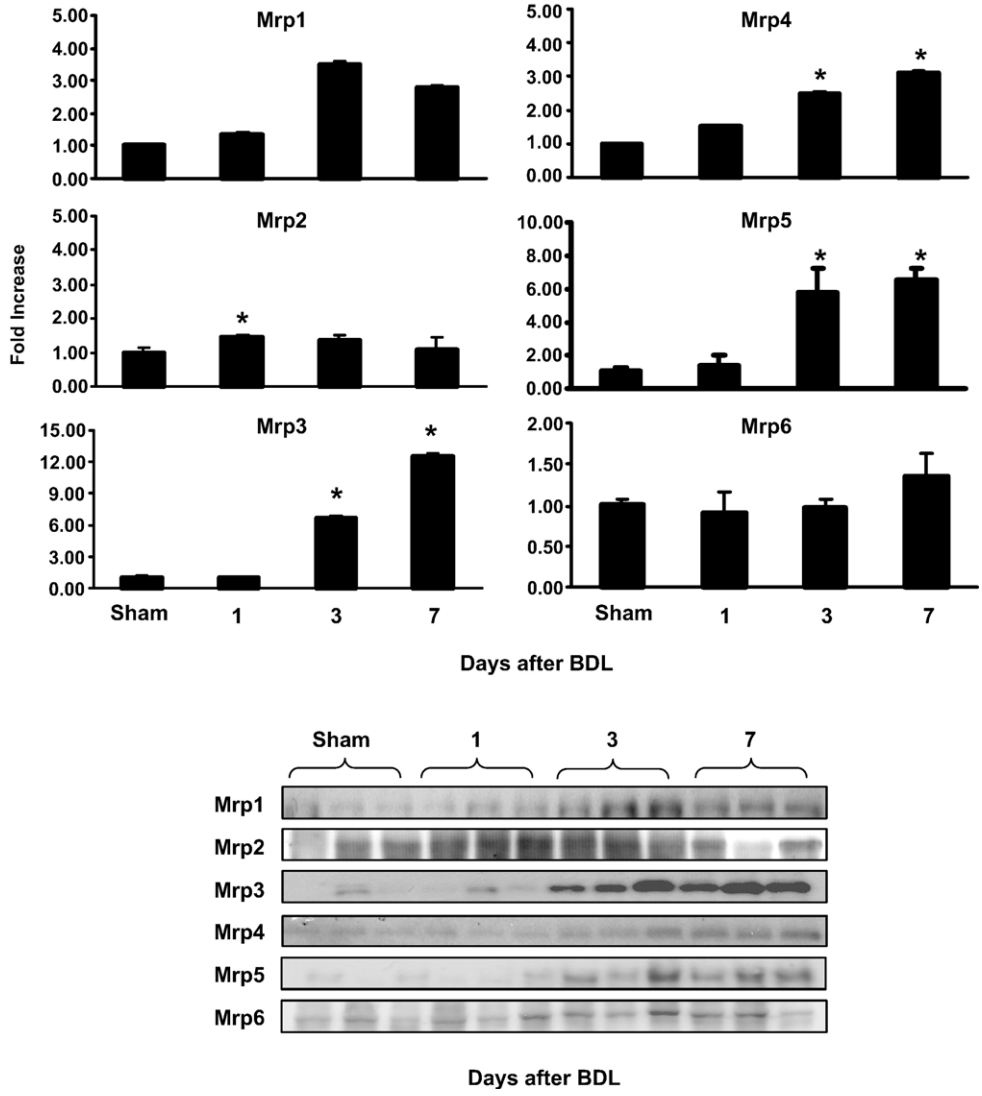

Fig. 8. Time course of Mrp1–6 protein expression in liver crude membrane fractions after bile-duct ligation (BDL). Upper panel: crude membrane fractions were isolated from livers of mice 1, 3, 7, and 14 days after sham or BDL surgery and analyzed by Western blot analysis for Mrp1–6 expression. Lower panel: quantification of Western blots. The data are presented as Fold Increase+S.E.M. ($n=4$–7 animals). Asterisks (*) represent a statistical difference ($p<0.05$) between sham and BDL groups.

In contrast to Oatp1a1 and 1b2, liver Oatp1a4 mRNA and protein expression was increased after BDL. It is unclear how induction of these transporters may affect BA levels in serum and liver after BDL. It is not known whether Oatp1a4 can mediate BA uptake into liver or efflux out of liver. Whereas it is accepted that Oatp1a4 transports some xenobiotics (i.e. cardiac glycosides) into liver, it is unclear whether it also transports BAs into liver. A recent publication has suggested that human OATP8/1B3 acts as an efflux pump that symports with glutathione to extrude organic anions from liver [48]. Thus, during cholestasis, Oatp1a4 may serve a function similar to OATP8/1B3. However it has been shown that Oatp1a4 has a relatively low affinity for taurocholate so it is possible that induction of Oatp1a4 will have little or no effect on BA levels in serum or liver after BDL. An alternate explanation is that induction of Oatp1a4 is hepatoprotective because induction of Oatp1a4 may increase BA transport into liver, which in turn, may increase BA hydroxylation by Cytochrome P450 3a11 (which is also upregulated after BDL), and subsequently detoxifies the BA.

In the present study, BDL altered the mRNA and protein expression of several mouse Mrps in liver, kidney, and intestine. Mrps are efflux pumps in liver, transporting compounds such as bile acids as well as glucuronide, glutathione, and sulfate-conjugated compounds from liver into bile, and from liver into blood. Bile acids are predominantly eliminated from hepatocytes into bile via Bsep and Mrp2, or out of hepatocytes into blood via Mrp3, Mrp4, or Ostα/β, which are localized to the basolateral membrane of hepatocytes and transport bile acids out of hepatocytes into blood. Mrp3- and 4-null mice have been used to study the contribution of these transporters during obstructive cholestasis. Data regarding the role of Mrp3 in BA disposition during cholestasis is somewhat unclear due to conflicting studies—one reported that serum BA levels do not differ in Mrp3-null mice [49], whereas another study reported

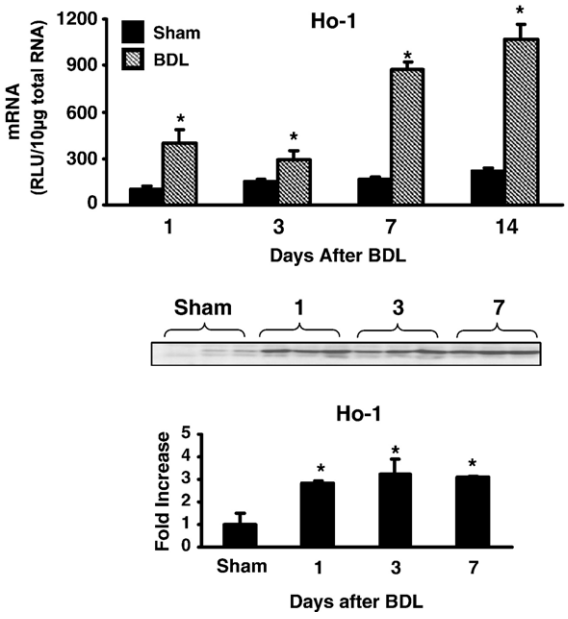

Fig. 9. Time course of Heme Oxygenase (Ho-1) mRNA and protein expression in mouse liver after bile-duct ligation (BDL). Upper panel: total RNA from liver was isolated from mice 1, 3, 7, and 14 days after sham or BDL surgery and analyzed by the bDNA assay Ho-1 expression. The data are presented as mean relative light units (RLU)+S.E.M. (n=4–7 animals). Asterisks (*) represent a statistical difference ($p<0.05$) between sham and BDL groups. Lower panel: crude membrane fractions were isolated from livers of mice 1, 3, 7, and 14 days after sham or BDL surgery and analyzed by Western blot analysis for Ho-1 expression. The data are presented as fold increase+S.E.M. (n=4–7 animals). Asterisks (*) represent a statistical difference ($p<0.05$) between sham and BDL groups.

increased liver BA levels in Mrp3-null after BDL [8]. BDL-induced liver injury was more severe and serum BA levels were lower in Mrp4-null mice [35]. The data in this study show that Mrps1, 3, 4, and 5 in mouse liver and kidney are induced after BDL. BDL induced Mrp1 and 5 mRNA expression approximately 5 fold, and Mrp 3 and 4 mRNA expression approximately 3 fold in liver. In kidney, among the Mrps, Mrp3 was the most highly induced (approximately 3 fold). BDL also increased Mrp1 and 3–5 protein levels in liver, whereas Mrp2 and Mrp6 protein levels were not significantly changed. These findings in mice are consistent with a previous report that documents elevation of Mrp3 and Mrp4 mRNA and protein levels in liver after BDL [18]. The data in this study demonstrate a slight induction of Mrp2 in mouse kidney 1 day after BDL, which was similar to a study that showed BDL increased Mrp2 expression in rat kidney 1 day after BDL [46,50]. In this study, Mrp2 mRNA levels in liver were unchanged after BDL and slightly increased 7 and 14 days after BDL. Mrp2 protein levels were not markedly changed at 1, 3, or 7 days after BDL. A previous report demonstrated that in mice, Mrp2 mRNA levels in liver were slightly decreased 1 and 3 days after BDL and similar to that detected in livers from sham-operated mice 7 days after BDL [18].

There are several mechanisms by which BA could regulate gene expression. Bile acids can activate the nuclear hormone receptors Farnesoid-X-receptor (FXR) and Pregnane-X-receptor (PXR) [51,52]. FXR is a “bile acid-sensor” and has a high affinity for chenodeoxycholic acid (CDCA) [53], however, CDCA levels in liver decrease after BDL [18]. However, FXR is also activated in mice by cholic acid feeding [51], and BDL increases cholic acid levels in livers of mice [18]. BA treatment decreases Ntcp expression in vivo in an FXR-dependent manner [51], and corresponds with suppression of protein binding to RXR:RAR response element in the Ntcp promoter in vitro. Lithocholic acid induces Oatp1a4 mRNA expression in mouse liver in a PXR-dependent manner [52], however BDL decreases lithocholic acid concentrations in mouse liver [18]. Thus, it is unclear whether changes in transporter expression after BDL are mediated via BA activation of FXR and PXR. Recently, Wagner et al. reported that FXR expression was important for basal Bsep and Mrp4 expression, but not for Mrp3 and 4 induction in mouse liver [18]. To date, there are no published studies that address the role of FXR and PXR in transporter regulation after BDL in kidney. It is possible that transporters in kidney and intestine are regulated via activation of FXR and PXR, because both are present in mouse kidney and intestine [54,55]. In addition to FXR and PXR, the orphan nuclear receptor, Liver receptor homolog-1 (Lrh-1, also named alpha fetoprotein transcription factor) may also be important for induction of transporters in liver and intestine after BDL, because Lrh-1 is activated in a human intestinal cell line (Caco-2) by bile acids, and is associated with induction of human Mrp3 [30]. In mice, Lrh-1 is expressed in liver, intestine, and colon, with relatively low expression in kidney [56].

Data from the present study also demonstrates that BDL increases the mRNA and protein expression of Ho-1, a microsomal enzyme that catalyzes the conversion of heme to biliverdin. The constitutive and inducible expression of Ho-1 is dependent upon Nrf2 [45,57]. Thus, induction of Ho-1 mRNA expression may indicate activation of Nrf2, which indicates a novel pathway for regulation of transporter expression during obstructive cholestasis. A recent study has described induction of other Nrf2 target genes after BDL, namely NAD(P)H: quinone oxidoreductase 1 [58]. In the present study, Mrp2 expression in liver was elevated at 7 and 14 days after BDL. Recent publications demonstrate that mouse the mouse Mrp2 gene contains Nrf2-binding consensus sequences (antioxidant response elements, AREs) that maintain basal Mrp2 expression and mediate Mrp2 induction [59]. Thus, elevation of Mrp2 in this model may be mediated through activation of Nrf2, and increased binding of Nrf2 to AREs present in the mouse Mrp2 gene.

Bile duct ligation is also associated with inflammation. BDL elevates serum levels of Interleukin-1 (IL-1), Interleukin-6 (IL-6), and Tumor Necrosis Factor alpha (TNFα) in both rats and mice [13,60]. TNFα has been implicated in the regulation of Mrp3 expression in liver during cholestasis [13]. In TNFα receptor-null mice, Mrp3 is not significantly induced after BDL, and this lack of induction corresponds with Lrh-1 binding to CPF/FTF/Lrh-1-response element in the mouse Mrp3 promoter [13]. Elevated serum cytokines may also be involved in mediating the alterations in kidney transporter expression after BDL. A recent study also implicates a role for cytokines in

affecting renal function after BDL. In mice, chronic BDL (7 days) exacerbated acute renal failure and was associated with elevation in serum IL-1 levels [61].

BDL decreased the mRNA expression of Ntcp, Oatp1a1, 1a5, and 1b2 in liver and Oatp1a1 in kidney. It has been proposed that down-regulation of Ntcp and Oatps during cholestasis is due to downregulation of the transcription factors, hepatocyte nuclear factor 1 alpha (Hnf1α) [1]. Ntcp expression in liver is down-regulated after lipopolysaccharide treatment and obstructive cholestasis [31,62]. Downregulation of Ntcp during obstructive cholestasis appears to be cytokine-independent and occurs through decreased protein expression of the Hnf1α and 4, transcription factors that maintain basal Ntcp transcription [33,56]. Ablation of Hnf1α expression affects the expression of a large number of genes in liver, and markedly decreases expression of Oatp1a1 and Oatp1b2 [63,64]. In human cultured hepatoma cells, CDCA treatment decreased Hnf1α expression [65]. Because Hnf1α is also expressed in kidney, it is possible that the observed decrease in renal Oatp1a1 mRNA expression after BDL is due to decreased Hnf1α expression and/or binding to the Oatp1a1 promoter [66].

It could be speculated that during obstructive cholestasis, the liver, kidney, and intestine will alter gene and protein expression to lower serum levels of BA and enhance BA excretion into urine to ultimately protect the organism. However, the data presented in this study suggest that this compensation is not adequate. For the most part, gene expression changes in liver and kidney are in a manner consistent with decreasing the cellular BA concentrations in those organs. Changes in transporter expression in liver, kidney, and intestine during extrahepatic cholestasis may not only be important for handling excess bile acid levels in tissues, but also for the disposition of drugs that are substrates for the Oatp and Mrp families of drug transporters. In conclusion, BDL altered the gene expression for Ntcp, Bsep, and members of the Mrp and Oatp family in mouse liver and/or kidney.

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