

Changes in Transporters and Metabolizing Enzymes in the Livers of Rats with Bile Duct Ligation

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Received, July 11, 2019; Revised, September 8, 2019; Accepted, September 9, 2019; Published, September 16, 2019.

ABSTRACT - Purpose: Bile duct ligation (BDL) in experimental animals is widely used as an animal model of liver cholestasis and fibrosis. The transcriptional process and plasma membrane localization of transporters are regulated by nuclear receptors and scaffold proteins, respectively. However, the detailed changes of these factors in the livers of BDL rats remain unclear. To clarify the effects of BDL on the levels of transporters and metabolizing enzymes, nuclear receptors, and scaffold proteins, we investigated changes in mRNA and protein levels of livers from BDL rats. **Methods:** Membrane proteins and microsomes were prepared from rats with BDL. The mRNA levels of transporters and nuclear receptors in livers of control and BDL rats were examined by real-time reverse transcription polymerase chain reaction. The protein levels of transporters, metabolizing enzymes and scaffold proteins in membrane proteins and microsomes were determined by liquid chromatography-tandem mass spectrometry-based targeted proteomics. **Results:** Mdr1a mRNA was significantly decreased at 1 and 2 weeks of BDL. The mRNA levels of MRP2 were significantly decreased. The mRNA levels of nuclear receptors were significantly decreased in livers of 1-week BDL rats. The protein levels of P-gp were significantly increased by BDL. Regarding scaffold proteins, the protein levels of ezrin, moesin and EBP50 were significantly decreased at 2 weeks of BDL. The protein levels of radixin were significantly increased at 1 week of BDL. In 1-week BDL rats, the protein levels of metabolizing enzymes such as CYP and UGT were significantly decreased. **Conclusions:** This study reports the comprehensive changes of transporters, metabolizing enzymes, nuclear receptors, and ezrin/radixin/moesin proteins in the livers of BDL rats. The expression levels of nuclear receptors and radixin that regulate the transcription and localization of CYP and/or transporters were decreased by BDL.

INTRODUCTION

The inhibition of the biliary excretion of bile acid leads to an increased accumulation of bile acid in hepatocytes. Occasionally, the intracellular accumulation of bile acid triggers cholestasis and fibrogenesis following liver injury (1,2). Bile duct ligation (BDL) in experimental animals is widely used as an animal model of liver cholestasis and fibrosis (3–5). BDL rats exhibit a high level of cirrhosis with morphological changes similar to human biliary cirrhosis (4).

The hepatic concentrations of drugs and drug metabolites are regulated by the interplay between transporters and metabolizing enzymes in hepatocytes. For example, drug metabolites formed by metabolizing enzymes such as cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) underwent biliary excretion via efflux transporters such as P-glycoprotein (P-gp/ABCB1), multidrug resistance-associated protein 2 (MRP2/ABCC2) and breast cancer resistance protein (BCRP/ABCG2) on a canalicular membrane. Metabolizing enzymes and transporters are transcriptionally regulated by

nuclear receptors such as pregnane X receptor (PXR, NR1I2), constitutive androstane receptor (CAR, NR1I3), retinoid X receptor (RXR) and cytoplasmic CAR retention protein (CCRP) (6–8). The plasma membrane localization of efflux transporters on the canalicular membrane is important for this transport activity. Ezrin/radixin/moesin (ERM) proteins participate as a scaffold protein in the plasma membrane localization of efflux transporters (9–11). Previous report shows that radixin knockout mice loses the localization of MRP2 on the canalicular membrane (12).

The hepatic expression of some metabolizing enzymes in BDL rats is decreased (13,14). The expression of efflux transporters on the canalicular membrane might have been elevated because of an adaptation to the liver injury (15–17).

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However, a comprehensive understanding of the changes in transporters, metabolizing enzymes, nuclear receptors, and ERM proteins in the livers of BDL rats is poorly understood, although these changes might affect drug disposition, efficiency and adverse reaction of various drugs. This background prompted us to clarify the effects of BDL on changes in metabolizing enzymes and transporters in the liver.

METHODS

Animals and treatment

The study protocol was approved by the Committee for the Care and Use of Laboratory Animals of the School of Pharmacy of Kindai University (Osaka, Japan). Male Wistar/ST rats (7-weeks-old, body weight 200–220 g) were purchased from Japan SLC (Shizuoka, Japan). The animals were housed in an air-conditioned room at $22 \pm 0.5^\circ\text{C}$ and relative air humidity of $55 \pm 10\%$ with a 12-h lighting schedule (7:00 AM–7:00 PM) and were given free access to standard laboratory food (MF, Oriental Yeast Co., Tokyo, Japan) and water on the light/dark schedule for 1 week before they were divided into groups. Rats were anesthetized with pentobarbital (50 mg/kg, *i.p.*, this treatment did not affect the CYP expression in 1, 2, and 3 weeks control and BDL rats). The bile duct was exposed and double ligated with 5-0 silk after making an approximately 2-cm incision in the abdomen with a microdissecting scissor. The bile duct of sham-operated animals was exposed but not ligated and these were used as control rats. After suturing the skin, rats were maintained under the above conditions. The livers of rats anesthetized with isoflurane were perfused with ice-cold physiologic (0.9%) saline and removed at 1, 2 or 3 weeks after ligation of the bile duct. After flash

freezing in liquid nitrogen, each sample was preserved at -80°C until used for RNA extraction, and microsome and membrane protein preparation.

Chemicals and reagents

Sepasol RNA I Super G was from Nacalai Tesque (Kyoto, Japan). ReverTra Ace was from Toyobo Life Science (Osaka, Japan). Fast SYBR Green Master Mix, Mem-PER eukaryotic membrane protein extraction reagent kit, and BCA protein assay kit were from Thermo Fisher Scientific (Waltham, MA, USA). Oligonucleotide primers were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mass spectrometry (MS) grade porcine pancreatic trypsin and OASIS HLB were obtained from Wako Pure Chemicals (Osaka, Japan) and Waters (Milford, MA, USA), respectively. All other chemicals and solvents were of MS grade or the highest commercially available purity.

Determination of mRNA levels by real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from livers of control and BDL rats using Sepasol RNA I Super G. The mRNA expression levels were measured by RT-PCR as described previously (18). The oligonucleotide sequences for each mRNA target are shown in Table 1. Data were analyzed using StepOne Software (Thermo Fisher Scientific) using the multiplex comparative method.

Preparation of membrane proteins and liver microsomes

Membrane proteins were extracted from livers using a Mem-PER eukaryotic membrane protein extraction reagent kit.

Table 1. Primer sequences used in PCR assays

Gene	Forward (5'–3')	Reverse (5'–3')
<i>Mdr1a</i>	GTGAAAGGGCTACAGGGTC	AGTGTCAATTGCCAGCCGTA
<i>Mdr1b</i>	GGCCTTAACGGAACAGCAGA	CTGCATAGCGAAACATCCCG
<i>MRP2</i>	CTCGGTCTTATGCGGCGTATT	TCTGGAAACCGTAGGAGACGAA
<i>MRP3</i>	TGAAGTTCAAAGACTCCCGCA	TCTACCTGCTCCAAGAAGGTGG
<i>BCRP</i>	TGTGTAGGTCGGTGTGCGAG	ATCTATGCCTTTCTAGCTGTCCC
<i>BSEP</i>	AAGCTGGCAAAGGGTTGTTG	AGCCAACCTCGAATGTCTCCC
<i>OCT1</i>	CCAATAGCGGCCTCGAATCT	TGCAGCTCATGCGGGATAAA
<i>OCTN1</i>	AAGCCGCAAAGATGAATGGC	TTGTGGCAATGTTCCGAGTC
<i>OCTN2</i>	ACTACGTGGCAGCATTGTC	TATGCAAACAGAGGCAGCAC
<i>PXR</i>	GACGGCAGCATCTGGAACACTAC	TGATGACGCCCTTGAACATG
<i>CAR</i>	CCACGGGCTATCATTTCAT	CCCAGCAAACGGACAGATG
<i>RXR</i>	CCGCTCCATAGCTGTGAAAGA	TCCGTTAGCACCCCTGTCAAAG
<i>CCRP</i>	TGCAATGGCAGCATGTCGTA	ATACTCCATGACCGCATTGGC
<i>GAPDH</i>	CAACGACCCCTTCATTGA	CAGTGATGGCATGGACTG

Liver microsomes from control and BDL rats were prepared according to previously described methods (19). The protein concentrations of membrane proteins and liver microsomes were measured using a BCA protein assay kit.

Measuring protein levels of transporters, metabolizing enzymes and scaffold proteins by targeted proteomics

The protein expression of liver microsomes and plasma membrane proteins of the liver were determined by liquid chromatography-tandem mass spectrometry-based targeted proteomics, as described previously (20,21). Liver microsomes were used to determine metabolizing enzyme expressions and plasma membrane proteins were used to determine transporter and scaffold protein levels.

STATISTICAL ANALYSIS

Data are shown as the mean \pm standard error of the mean ($n = 3-5$). Relationships between the mRNA levels of nuclear receptors and transporters were evaluated by Pearson's correlation and regression analysis using GraphPad Prism software version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Significance of differences between mean values was determined by Dunnett's test after analysis of variance using GraphPad Prism software. Values of $p < 0.05$ were considered to indicate statistical significance.

RESULTS

The effects of BDL on the mRNA levels of transporters and nuclear receptors in livers are shown in Figures 1 and 2. mRNA levels of transporters such as Mdr1a, Mdr1b, MRP2, MRP3, BCRP, bile salt export pump (BSEP), organic cation transporter (OCT)1, carnitine/organic cation transporter (OCTN)1 and OCTN2 in livers from control and BDL rats relative to controls are shown in Figure 1. The mRNA levels of Mdr1a were significantly increased and whereas mRNA levels of Mdr1b were reduced in BDL rats. Mdr1a mRNA was significantly decreased at 1 and 2 weeks of BDL and Mdr1b mRNA was significantly increased at 1 week of BDL. Regarding the other efflux transporters on the canalicular membrane, the mRNA levels of MRP2 were significantly decreased and the mRNA levels of BCRP tended to decrease similar to Mdr1a mRNA. The mRNA levels of the other examined transporters in BDL rats also tended

to decrease compared with control rats. Relative mRNA levels for PXR, CAR, RXR and CCRP in livers from control and BDL rats are shown in Figure 2. The mRNA levels of PXR, CAR, RXR and CCRP were significantly decreased in livers of 1-week BDL rats compared with control rats. The decreased mRNA levels of CAR and RXR were maintained up to 3 weeks.

Expression levels did not necessarily exhibit corresponding changes between mRNA and protein levels. The protein levels for transporters, scaffold proteins and metabolizing enzymes in the livers of control and BDL rats are shown in Figures 3 and 4. Protein levels for P-gp, MRP2, BCRP, OAT2, ezrin, radixin, moesin and ezrin-radixin-moesin-binding phosphoprotein of 50 kDa (EBP50) in membrane proteins of livers from control and BDL rats relative to controls are shown in Figure 3. The protein levels of P-gp were significantly increased by BDL at 1, 2 and 3 weeks. The other efflux transporters such as MRP2 and BCRP on the canalicular membrane were significantly increased at 1 week but not 2 and 3 weeks of BDL. Protein levels of OAT2 tended to be decreased. The protein levels of the scaffold proteins ezrin, moesin and EBP50 were significantly decreased at 2 weeks of BDL. Ezrin and EBP50 were significantly decreased at 3 weeks of BDL. The protein levels of radixin were significantly increased at 1 week but not 2 and 3 weeks of BDL. Relative protein levels of CYP1A1, CYP1A2, CYP1B1, CYP2A1, CYP2C6, CYP2C11, CYP2D1, CYP2D3, CYP2E1, CYP3A1, CYP3A2, CYP3A9, UGT1A1, UGT1A3 and UGT2B1 in microsomes of livers from control and BDL rats are shown in Figure 4. In 1-week BDL rats, the protein levels of CYP1A1, CYP1B1, CYP2A1, CYP2C6, CYP2C11, CYP2D1, CYP2D3, CYP2E1, CYP3A1, CYP3A9, UGT1A1 and UGT2B1 were significantly decreased. In 2-week BDL rats, the protein levels of CYP1A1, CYP2A1, CYP2D1, CYP3A1 were significantly decreased. In 3-week BDL rats, the protein levels of CYP1A1, CYP2C11, CYP2D3, CYP3A1 and UGT2B1 were significantly decreased.

DISCUSSION

The profiling of metabolizing enzymes and transporters in livers of 1-, 2- and 3-week BDL rats, an animal model of liver cholestasis and fibrosis, were examined. Regarding mRNA expressions, correlations of transporters with nuclear receptors were evaluated. To determine changes in the protein levels of efflux transporters, the protein levels of scaffold proteins were measured.

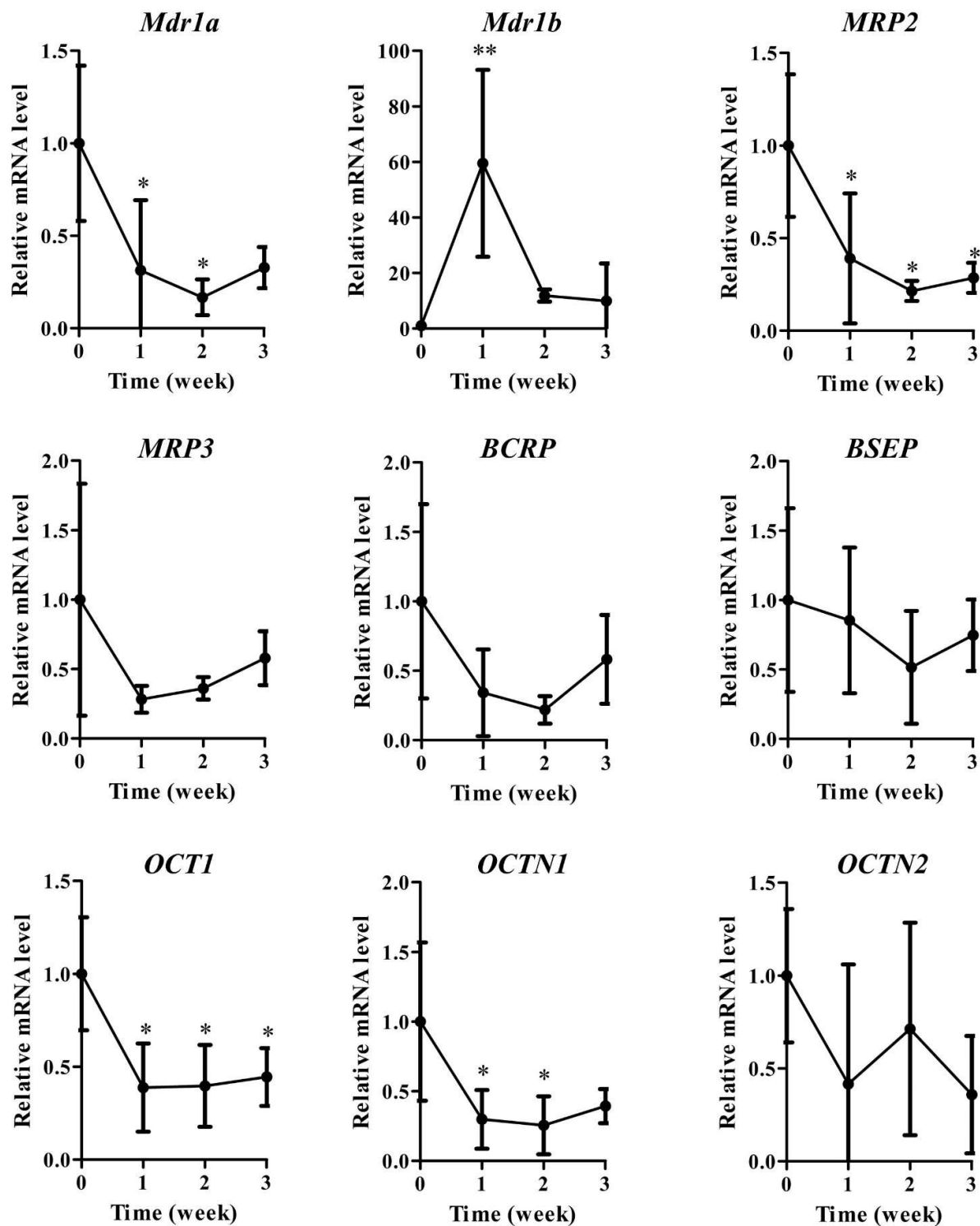


Figure 1. mRNA levels of *Mdr1a*, *Mdr1b*, *MRP2*, *MRP3*, *BCRP*, *BSEP*, *OCT1*, *OCTN1* and *OCTN2* in livers from control and BDL rats relative to controls. *Mdr1a* and *Mdr1b* are mRNA for P-gp. Significant differences (* $p < 0.05$ and ** $p < 0.01$) vs controls.

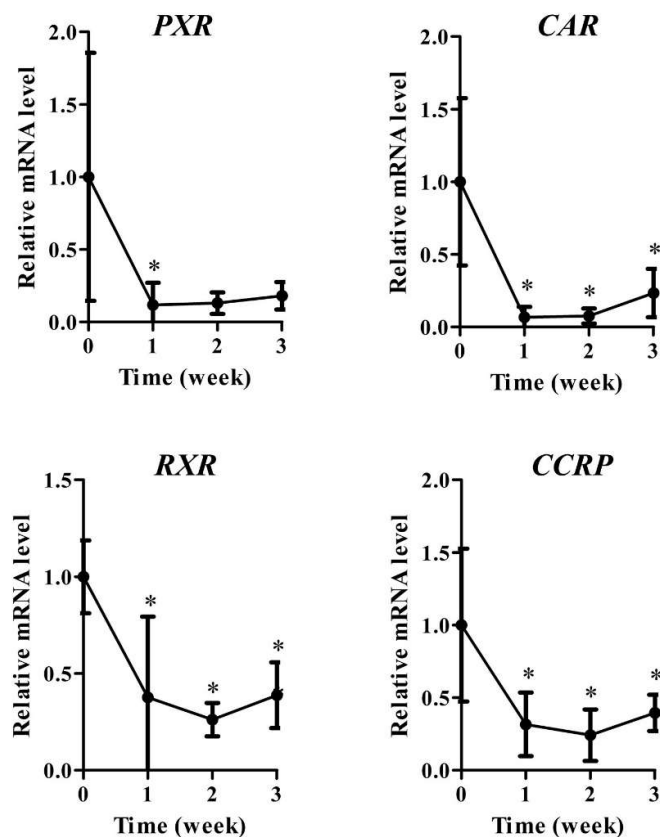


Figure 2. mRNA levels of *PXR*, *CAR*, *RXR* and *CCRP* in livers from control and BDL rats relative to controls. Significant differences (* $p < 0.05$) vs controls.

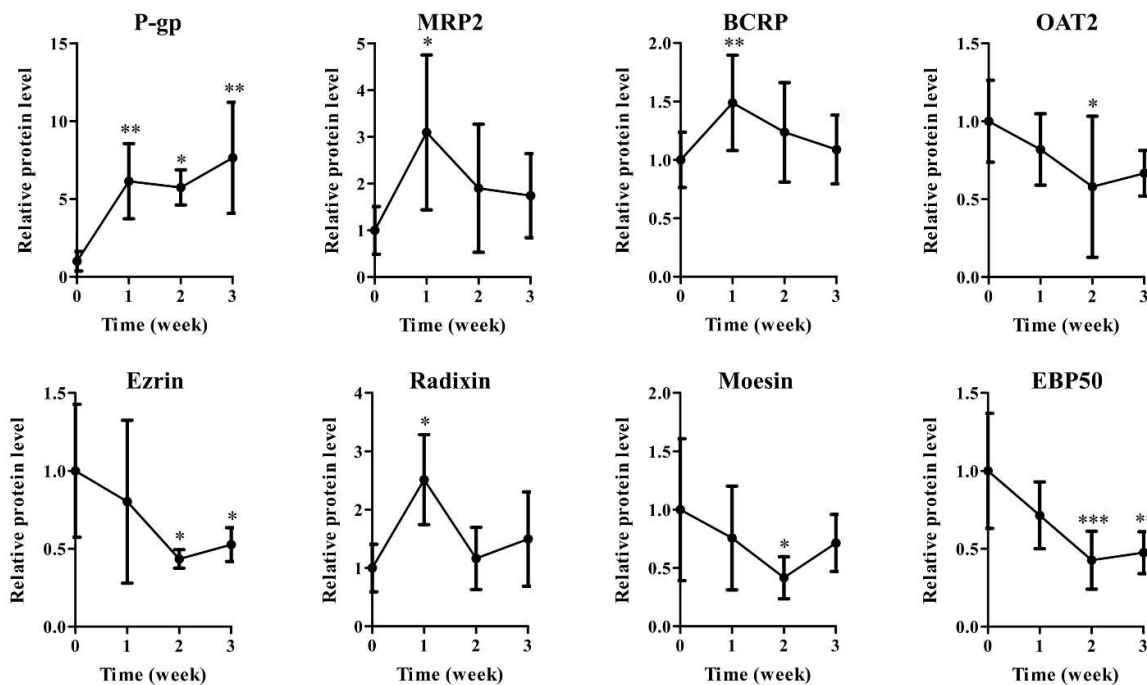


Figure 3. Protein levels of P-gp, MRP2, BCRP, OAT2, ezrin, radixin, moesin and EBP50 in membrane proteins of livers from control and BDL rats relative to controls. Significant differences (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$) vs controls.

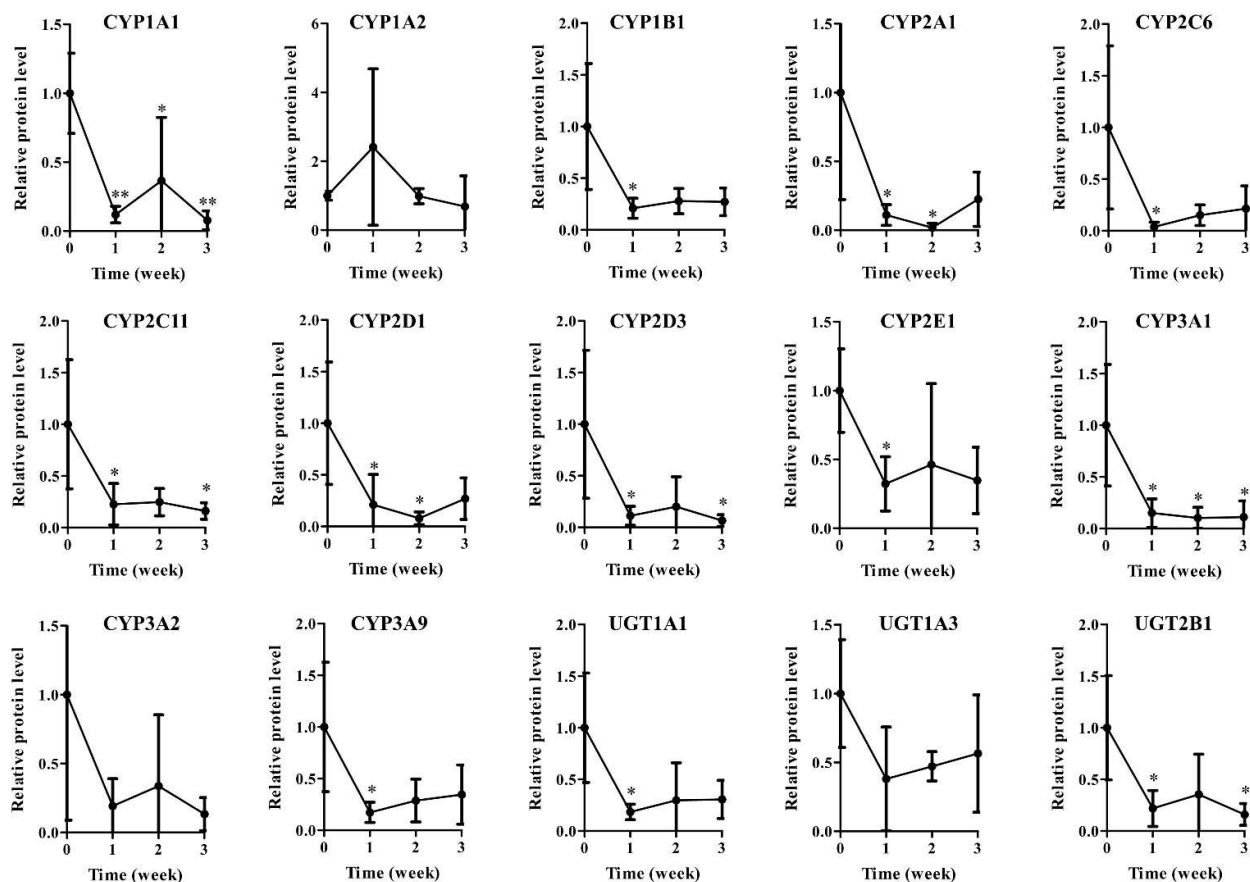


Figure 4. Protein levels of CYP1A1, CYP1A2, CYP1B1, CYP2A1, CYP2C6, CYP2C11, CYP2D1, CYP2D3, CYP2E1, CYP3A1, CYP3A2, CYP3A9, UGT1A1, UGT1A3 and UGT2B1 in microsomes of livers from control and BDL rats relative to controls. Significant differences (* $p < 0.05$ and ** $p < 0.01$) vs controls.

The mRNA levels of transporters except Mdr1b in the livers of BDL rats were significantly decreased or tended to decrease. These results are consistent with the results of previous reports (22,23). The mRNA levels of Mdr1a and Mdr1b changed in the opposite direction. Other report also shows that the hepatic mRNA levels of Mdr1a and Mdr1b are oppositely altered by inflammation (24). The mRNA levels of nuclear receptors in BDL rats were examined, because the consideration should be given to the intracellular localization (cytoplasm or nucleus) and phosphorylation state of nuclear receptors in the determination of protein levels of nuclear receptors. The mRNA levels of nuclear receptors in BDL rats were decreased similar to transporter mRNAs. In particular, the mRNA levels of nuclear receptors in 1-week BDL rats were decreased. PXR and CAR, which binds as a heterodimer with RXR in the nucleus, regulate the transcriptional process of CYP and transporters. CCRP forms complexes with CAR and heat shock protein 70 (25,26). The mRNA levels of Mdr1a, MRP2, MRP3, BCRP, OCT1, OCTN1 strongly

correlated with the mRNA levels of nuclear receptors, suggesting that a reduction of transcriptional regulation by nuclear receptors might decrease transporter mRNAs in BDL rats. There was no correlation between BSEP and nuclear receptors. Therefore, the transcription of BSEP is probably regulated by other nuclear receptors such as farnesoid X receptor and small heterodimer partner (27,28).

Previous studies report that the mRNA and protein levels of uptake transporters such as organic anion transporting polypeptide and OCT1 are reduced by BDL (22,29,30). In contrast, efflux transporters such as MRP2 are increased by BDL (31), although the mRNA levels of MRP2 are decreased by an intraperitoneal injection of inflammatory cytokines such as interleukin-1 α , interleukin-6 and tumor necrosis factor- α to mice (32). P-gp protein is also increased in the livers of BDL rats up to 3 weeks similar to previous reports (15–17). These results support the view that an increase in efflux transporters on the canalicular membrane reflect an adaptation to liver injury. The

amount of bile acid in the liver is altered by BDL. For example, the concentration of bile acid in the livers of BDL rats was increased approximately 10-fold and the concentrations of deoxycholic acid and hyodeoxycholic acid are decreased (33). However, the involvement of bile acid in the regulation of transporters is likely to be minor (32). To date, changes in hepatic efflux transporter expression in BDL rats are controversial (17,34–36). Further studies are needed to clarify the changes of MRP2 in BDL rats.

The localization of transporters such as MRP2 and P-gp on the canalicular membrane is regulated by scaffold proteins such as ERM proteins. Radixin has a linker activity for efflux transporters (12,37). The protein levels of radixin in the membrane proteins of 1-week BDL rats corresponded to those of MRP2 (Figure 3). The protein levels of ezrin and EBP50 in 2- and 3-week BDL rats were significantly decreased compared with control rats. These results suggested that radixin is dominantly involved in the plasma membrane localization of efflux transporters compared with ezrin, moesin and EBP50. The changed expression of nuclear receptors and radixin in BDL rats might participate in the regulation of the expression of transporter proteins.

The protein levels of transporters did not exhibit corresponding changes with their mRNA levels, suggesting the post-transcriptional process and/or plasma membrane localization of transporters as well as the transcriptional process might be modulated by BDL. Therefore, changes in the mRNA levels of transporters in BDL rats might not reflect the activity of transporters.

The expressions of CYP in liver microsomes and of transporters is transcriptionally regulated by nuclear receptors (38–40). Previous reports demonstrate that the hepatic levels of CYP are reduced by BDL (13,41). The current study also shows that the protein levels of the CAR target gene CYP2C and PXR target gene CYP3A in liver microsomes of BDL rats were significantly decreased (Figure 4). The decreased levels of nuclear receptors such as CAR and PXR in the liver microsomes of BDL rats might inhibit the transcriptional process of CYP isoforms. Drug pharmacokinetics are affected by decreased levels of CYP isoforms in BDL rats (42,43). Thus, the elimination of other drugs from the body might be delayed by BDL. Further studies on the effects of BDL on altered drug pharmacokinetics are required. BDL rats show the states of liver injury or toxicity accompanying cholestasis and fibrosis (3–5). The decreases of CYP activities in these states might

result in the reduction of metabolism of drugs and induction of adverse effects of drugs.

In conclusion, this study reports on the comprehensive changes of transporters, metabolizing enzymes, nuclear receptors, and ERM proteins in the livers of BDL rats. The decrease in nuclear receptors might participate in downregulating the expression of CYP in the liver microsomes of BDL rats. In addition to nuclear receptors, reduced radixin in BDL rats might inhibit the plasma membrane localization of transporters.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by JSPS KAKENHI Grant Number 18K06806, the Japanese Ministry of Education, Culture, Sports, Science, and Technology (MEXT). We thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

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