Increased renal expression of bilirubin glucuronide transporters in a rat model of obstructive jaundice

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Regulation of bilirubin glucuronide transporters in hepatic and extrahepatic tissues is not completely clear. In the present study, we evaluated the regulation of the bilirubin glucuronide transporters, multidrug resistance-associated proteins (MRP2 and 3, in rats with obstructive jaundice). Bile duct ligation (BDL) or sham operation was performed in Wistar rats. Liver and kidneys were removed 1, 3, and 5 days after BDL (n = 4, in each group). Serum and urine were collected to measure bilirubin levels just before animal killing. MRP2 and MRP3 mRNA expressions were determined by real-time RT-PCR. Protein expression of MRP2 and MRP3 was determined by Western blotting. Renal MRP2 function was evaluated by para-aminohippurate (PAH) clearance. The effect of conjugated bilirubin, unconjugated bilirubin, human bile, and sulfate-conjugated bile acid on MRP2 gene expression was also evaluated in the liver and kidney cell lines. Serum bilirubin and urinary bilirubin excretion increased significantly after BDL. In the liver, the mRNA expression of MRP2 decreased 59, 86, and 82%, and its protein expression decreased 25, 74, and 93% compared with sham-operated animals after 24, 72, and 120 h of BDL, respectively. In contrast, the liver expression of MRP3 mRNA increased 138, 2,137, and 3,295%, and its protein expression increased 560, 634, and 612% compared with sham-operated animals after 24, 72, and 120 h of BDL, respectively. PAH clearance was significantly increased after BDL. The mRNA expression of MRP2 increased in renal proximal tubular epithelial cells after treatment with conjugated bilirubin, sulfate-conjugated bile acid or human bile. Upregulation of MRP2 in the kidneys and MRP3 in the liver may be a compensatory mechanism to improve bilirubin clearance during obstructive jaundice.

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of this transporter in the liver during obstructive jaundice. To demonstrate this hypothesis we measured the renal expression of MRP2 in an experimental model of obstructive jaundice caused by bile duct ligation (BDL) in rats.

MATERIALS AND METHODS

Reagents. Bilirubin ditaurate disodium salt was purchased from Porphyrin Products (Logan, Utah). Bilirubin was purchased from Nacalai Tesque (Kyoto, Japan). Taurolithocholic acid 3-sulfate disodium salt (TLCA sulfate) and taurine were purchased from Sigma (St. Louis, MO).

Bile. Bile was sampled through a bile drainage tube from an 86-yr-old woman with obstructive jaundice caused by common bile duct carcinoma. The bile sample was sterilized through a 0.22-μm filter (Millipore, Bedford, MA) before use. The concentration of conjugated bilirubin in bile was 238 μM. Informed consent was obtained from the patient before sampling.

Animal model. Male Wistar rats weighing 200–250 g were subjected to BDL under general anesthesia (pentobarbital sodium 50 mg/kg body wt ip). All rats received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, recommended by the National Academy of Science and published by the National Institutes of Health (NIH publication 86–23, revised 1985). Liver and kidneys were removed after 24, 72, and 120 h of BDL (n = 4 each). The organs were perfused with phosphate-buffered saline, cut into small pieces, and snap-frozen in liquid nitrogen until use. Control animals underwent sham surgery that consisted in exposure of bile duct but without ligating it. Liver and kidneys were removed 24, 72, and 120 h after sham surgery (n = 4 each).

Blood samples were collected during organ removal. For urine sampling, the rats were placed in metabolic cages and urine was collected for 24 h. The concentrations of bilirubin and bilirubin glucuronide were determined separately using a test kit (Wako Pure Chemical, Tokyo, Japan) by the alkaline azobilirubin method.

Cell culture. The human hepatoma cell line HepG2 (Riken Cell Bank, Tsukuba, Japan) was grown in DMEM supplemented with 10% (vol/vol) fetal bovine serum, 50 μg/ml penicillin, and 50 μg/ml streptomycin. Human renal proximal tubular epithelial cells (RPTEC), obtained from Bio-Whittaker (Walkersville, MD), were cultured in renal epithelial cell basalmedium supplemented with 10% (vol/vol) fetal bovine serum and 0.5 mg/ml hydrocortisone, 10 μg/ml human epidermal growth factor, 0.5 mg/ml epinephrine, 10 mg/ml transferrin, 5 mg/ml insulin, 6.5 μg/ml triiodothyronine, gentamicin sulfate, and amphotericin-B. Cells were passaged using standard trypsinization procedures. All cell lines were incubated at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂.

DNA isolation from cultured cells and PCR. Total RNA was isolated from HepG2 and RPTEC cells using TRIzol reagent (n = 3, each; Invitrogen, Carlsbad, CA) after treatment for 24 h with 100 μM bilirubin ditaurate, 10 μM unconjugated bilirubin, 100 μM TLCA sulfate, or 20% (vol/vol) human bile. Reverse transcription and PCR were carried out as described previously with minor modifications (36, 43). The primers used for amplification of human MRP2 were 5′-TCTGCTTCCACCACCACACATCTT-3′ (nucleotides 3400–3420) and 5′-TCTGCTTCCACCACACATCTT-3′ (nucleotides 4023–4043). The primers for GAPDH amplification were 5′-GGACGAGCAAGTACGG-3′ (nucleotides 150–169) and 5′-TCTAGACGGGACAGTGTCAGG-3′ (nucleotides 720–743). PCR was performed under the following conditions: 94°C for 60 s, annealing for 60 s, and elongation at 72°C for 60 s, for a total of 30 cycles for MRP2 and 25 cycles for GAPDH. A final elongation at 72°C for 10 min was performed. Annealing temperature for both MRP2 and GAPDH was 55°C. The PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining. The bands were scanned and semiquantified by densitometric analysis using the public domain NIH Image program (Wayne Rasband, Research Service Branch of the National Institute of Mental Health, Bethesda, MD).

Real-time PCR. Total RNA for real-time PCR was extracted from liver and kidney specimens using the Total SV RNA Isolation System Kit (Promega, Madison, WI) according to the manufacturer’s instructions. Isolated RNA was purified by ethanol precipitation and stored at −80°C until use. Primers and probes for MRP2 and MRP3 were prepared using Primer Express version 1 (PE Biosystems, Foster city, CA). For the rat MRP2, the sense primer was 5′-TTCTGT-TTGGCCTTGTCT-3′ (nucleotides 3658–3674), the antisense primer was 5′-CTTCTGCGCTCATCTTAC-3′ (nucleotides 3784–3766), and the Taqman probe was 5′-TGTCACAGCC-CCTCAATATCACA-3′ (nucleotides 3725–3747) (29). For rat MRP3, the sense primer was 5′-GAAGACACACTCGAC-CACC-3′ (nucleotides 2694–2712), the antisense primer was 5′-CTCTGGCGACCTGTAT-3′ (nucleotides 2760–2743), and the Taqman probe was 5′-ACACAGACCTGACAGACC-3′ (nucleotides 2714–2736). The rat GAPDH was used as a control. For amplification of the rat GAPDH, the sense primer was 5′-TCTCCACACTACCGAGA-3′ (nucleotides 1758–1777), and the antisense primer was 5′-TTGCGACCTTGATAGT-3′ (nucleotides 1833–1814) was used. For the rat GAPDH, the Taqman probe 3′-TCCGTTTGG- GCAGAGAGATGCA-3′ (nucleotides 1782–1805) was used.

Real-time PCR was carried out as previously described using the ABI PRISM 7700 Sequence Detection System (PE Biosystem) (2, 6, 18, 24). First, 0.4 μg of liver RNA or 1 μg of kidney RNA were reverse transcribed using TaqMan Gold reverse transcription reagent (PE Biosystems). Then, the cDNA were amplified using 200 nM of primer of the respective genes and 100 nM Taqman probe in a total volume of 50 μl containing RT-PCR buffer [50 mM KCl, 0.1 mM EDTA, 100 mM Tris·HCl (pH 8.3), and 60 mM passive reference], 5.5 mM MgCl₂, 200 μM of each of dATP, dCTP, dGTP, and 400 μM of dUTP, 0.025 U/μl AmpliTaq Gold DNA polymerase, and 0.01 U/μl of AmpErase uracil-N-glycosidase. Before cDNA amplification, the PCR mixture was first incubated at 50°C for 10 min and then at 95°C for 10 min. Thereafter, PCR was performed under the following conditions: 95°C for 15 s and annealing at 55°C for 20 s and at 60°C for 40 s for a total of 45 cycles.

Each sample was measured in duplicate, and the amount of RNA was calculated from a standard curve drawn using serial dilutions of total RNA extracted from normal Wistar rat liver. Standard samples were run in parallel during each analysis. The coefficient of correlation between threshold cycle and starting MRP2 or GAPDH amount was r ≥ 0.95, and the slope was constant in each experiment. The amount of MRP2 in each sample was normalized by the GAPDH content.

Western blotting analysis. The rabbit polyclonal antibody against rat MRP2, EAG15 (30), was kindly provided by Professor D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The rabbit polyclonal antibody against rat MRP3 (17) was a kind gift from Professor Y. Sugiyama (Graduate School of Pharmaceutical Science, University of Tokyo, Tokyo, Japan). Crude plasma membrane
(CPM) was prepared from rat liver and kidney homogenates as described previously (33, 41). Fifty micrograms of liver CPM or 80 μg of kidney membrane proteins were separated on a 7.5% polyacrylamide gel. After the membrane was transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), it was blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membrane was then incubated with anti-MRP2 (1:5,000) or anti-MRP3 (1:1,000) antibody, and after it was washed appropriately, it was incubated for 1 h with a peroxidase-conjugated goat anti-rabbit IgG antibody (1:3,000). After the membrane was washed four times with TBST buffer, chemiluminescence development was performed using an Immune-Star chemiluminescent protein-detection system (Bio-Rad). The immunoreactive bands on the autoradiography films were scanned and semiquantified by densitometric analysis using the public domain NIH Image program. Control blot was prepared in parallel following the same protocol but using nonimmune rabbit serum.

Evaluation of renal function by PAH clearance. PAH clearance was determined after 24, 72, and 120 h of BDL (n = 4 each) after previously reported methods with minor modifications (38, 44). In brief, the right jugular vein and the left carotid artery were cannulated with heparinized polyethylene tubes. Rats were given an intravenous bolus infusion (0.4 ml/100 g body wt) of 1% PAH in saline solution via the jugular vein catheter followed by infusion (50 μl/min) of PAH solution through a pump for 1 h. Bladder catheter was placed to collect urine for 30 min after the infusion. Arterial blood samples (300 μl) were drawn 15 min after the infusion. Serum and urine PAH concentration were determined by an automated chemistry analyzer. PAH clearance (ml·min⁻¹·100 g⁻¹) was calculated using the following formula: [urine volume (ml/min)/body wt (100 g)]×[urine PAH (mg/dl)/plasma PAH (mg/dl)].

RESULTS

Serum and urinary bilirubin levels after BDL. BDL was associated with an increase in the serum bilirubin level and in the daily urinary conjugated bilirubin excretion. Total serum bilirubin increased at 24 and 72 h after BDL and slightly recovered after 120 h of operation (Fig. 1A). Most of elevated serum bilirubin was of direct-reacting type. Daily urinary conjugated bilirubin excretion markedly increased at 24 and 72 h after BDL and recovered slightly 120 h after operation (Fig. 1B).

Effect of BDL on MRP2 and MRP3 mRNA expression. MRP2, MRP3 and GAPDH mRNA expressions were determined by real-time RT-PCR (Fig. 2). The standard curve performed with serial dilutions of the
samples showed a constant slope between experiments, indicating the constant efficiency of the PCR. The liver MRP2 mRNA significantly decreased 59, 86, and 82% compared with sham-operated animals after 24, 72, and 120 h of BDL, respectively.

In contrast to the liver, the renal mRNA expression of MRP2 significantly increased in 162, 73, and 21% of sham-operated animals after 24, 72, and 120 h of BDL, respectively. On the other hand, liver MRP3 mRNA expression significantly increased in 138, 2,137, and 3,295% of sham-operated animals after 24, 72, and 120 h of BDL, respectively.

**Effect of BDL on MRP2 and MRP3 protein levels.** MRP2 protein levels on the apical membrane were determined in liver and kidneys after 24, 72, and 120 h of BDL (Fig. 3, A and B). Rat liver MRP2 protein expression significantly decreased in 25, 74, and 93% of sham-operated animals after 24, 72, and 120 h of BDL, respectively. On the other hand, liver MRP3 protein level significantly increased in 560, 634, and 612% of sham-operated animals after 24, 72, and 120 h of BDL, respectively. In contrast to liver MRP2, renal MRP2 protein expression increased significantly in 387, 558, and 472% of sham-operated animals after 24, 72, and 120 h of BDL, respectively.

**Fig. 3.** Change of MRP2 and MRP3 protein expressions in the liver and MRP2 protein expression in the kidneys during BDL. MRP2 and MRP3 expressions were examined by Western blotting using EAG15 antibody and anti-rat MRP3 antibody. Immunoreactive bands were semiquantitated by a densitometric analysis. Data were expressed as means ± SD (n = 4, each). *P < 0.05, **P < 0.01, compared with sham-operated rats.

**Fig. 4.** Para-amine aminohippurate (PAH) clearance during BDL. PAH clearance (ml-min⁻¹-100 g⁻¹) was calculated as described in MATERIALS AND METHODS. Marked increase of PAH clearance was observed 72 h after BDL. Data were expressed as means ± SD (n = 4, each). **P < 0.01, compared with sham-operated rats.

**Fig. 5.** Effect of bilirubin conjugate, diurate, disodium salt, tauro-lithocholic acid (TLCA) sulfate, human bile, unconjugated bilirubin, and taurine on MRP2 expression in renal proximal tubular epithelial cells (RPTEC). MRP2 expression was evaluated by RT-PCR, and it was normalized by GAPDH expression. Bilirubin diurate, TLCA sulfate, and human bile increased the mRNA expression of MRP2 in RPTEC cells. Data were expressed as means ± SD (n = 3, each). *P < 0.05, **P < 0.01, compared with control. Control indicates without any stimulant.
Effect of BDL on PAH clearance. Renal function was evaluated by PAH clearance. BDL induced a remarkable increment of PAH clearance of \(0.42 \pm 0.16, 0.76 \pm 0.23\), and \(0.53 \pm 0.27 \text{ml/min}^{-1} \cdot \text{100 g body wt}^{-1}\) compared with sham-operated animals after 24 and 72 h of BDL, respectively (Fig. 4). PAH clearance changed in parallel with daily urinary bilirubin excretion.

Effect of bilirubin ditaurate, TLCA sulfate, unconjugated bilirubin, and human bile on the mRNA expression of MRP2 in HepG2 and RPTEC cells. The mRNA expression of MRP2 was evaluated by RT-PCR, and it was normalized by the GAPDH content. The mRNA expression of MRP2 in HepG2 cells did not change significantly compared with controls in the presence of bilirubin ditaurate, TLCA sulfate, and so on. (data not shown). However, bilirubin ditaurate, TLCA sulfate, or human bile increased the mRNA expression of MRP2 in RPTEC cells (Fig. 5).

DISCUSSION

In this study, we evaluated the expression of MRP2 and MRP3 in liver and kidneys from BDL rats. The serum levels of the conjugated bilirubin, which mainly increased in our experimental model, decreased after 120 h, suggesting the occurrence of extrahepatic excretion of bilirubin during BDL. In the present study, we hypothesized that the kidneys excrete the excess circulating levels of bilirubin during obstructive jaundice.

It has been reported that liver MRP2 protein is downregulated after BDL; however, the precise mechanism of this MRP2 downregulation in the liver is still controversial (20, 31). In the present study, we carried out real-time RT-PCR to quantify precisely the level of mRNA expression of MRP2 during BDL-associated obstructive jaundice. The results showed that liver MRP2 mRNA expression is decreased after BDL in rats. This decrease in mRNA expression was also associated with a concomitant decrease in protein expression of MRP2, as demonstrated by Western blotting analysis. This result agrees with findings of previous studies (20).

Under normal physiological conditions, the expression of MRP2 is high and that of MRP3 is low in hepatocytes; therefore, in the normal liver, most of conjugated organic anions are transported to bile canaliculi via MRP2. Low expression of MRP2 and concomitant increased expression of MRP3, which is located on basolateral membrane of hepatocytes (16, 21), may explain the elevation of serum bilirubin during BDL. MRP3 in the liver is upregulated by bilirubin and bilirubin glucuronides (11, 12, 19). This compensatory effect of MRP3 may play an important role in reducing injury to hepatocytes from cytotoxic materials that increase during obstructive jaundice.

In addition to liver excretion, conjugated bilirubin has been reported to be excreted also through the kidneys. Previous studies suggested that urinary excretion occurs mainly by glomerular filtration (13, 25). However, in conditions associated with cholestasis, renal tubular excretion of sulfate-conjugated bile acids may also take place (9). In our present experimental BDL model, in contrast to the liver, the protein and mRNA expressions of MRP2 were significantly increased in the kidneys after 24 h. This upregulation of MRP2 was associated with a concomitant elevation of renal PAH clearance up to 72 h after BDL. These results suggest that increased renal MRP2 is functionally active during obstructive jaundice and that bilirubin is transported into urine, at least in part, via renal MRP2. The increased MRP2 expression in the kidneys may provide an alternative pathway for accelerating excretion of bilirubin conjugates during obstructive jaundice. However, to what degree this tubular secretion contributes to renal excretion of conjugated bilirubin needs to be clarified.

An important finding that needs clarification is the mechanism by which BDL affects the expression of MRP2 in the liver and kidneys. To gain some insights into this mechanism, in the present study, we evaluated the effects of synthetic conjugated bilirubin, sulfate-conjugated bile acid, human bile, and unconjugated bilirubin on human MRP2 expression in an in vitro system using hepatocyte and renal cell lines. In our in vitro study, we found that bilirubin ditaurate, TLCA sulfate, and human bile upregulate the expression of MRP2 in renal tubular cells but not in hepatocytes. These findings are consistent with changes in renal MRP2 expression observed in our animal BDL model. These results suggest that conjugated bilirubin, sulfate-conjugated bile acid, and some components of bile may, by themselves, regulate the expression of renal MRP2 and that this regulation varies according to the type of cells. Both conjugated bilirubin and sulfate-conjugated bile acid are well-characterized substrates of MRP2. The fact that conjugated bilirubin (bilirubin ditaurate), TLCA sulfate, and human bile but not unconjugated bilirubin upregulated the expression of MRP2 in renal tubular cells suggests that stimulation of MRP2 expression in these cells is substrate specific (14, 15, 40). Overall, findings in our in vitro experiments carried out using liver and renal tubular cells may explain the contrasting effect of BDL in our animal model. Further studies should be carried out to identify the cellular mechanisms involved in the opposing effects of glucuronide conjugates in different cell types.

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