Adaptive responses of renal organic anion transporter 3 (OAT3) during cholestasis

Jiarong Chen, Tomohiro Terada, Ken Ogasawara, Toshiya Katsura, and Ken-ichi Inui

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto, Japan

Bile acids are amphiphilic molecules synthesized from cholesterol exclusively in the liver and are essential for effective absorption of dietary fat. They exist as anions at physiological pH and require transport across the membranes of enterohepatic tissues. During cholestasis, expressions of hepatic transporter genes are changed to reduce the intracellular accumulation of toxic biliary constituents as hepatoprotective responses. For example, alternative bile acid excretion during cholestasis is accomplished by the induction of hepatic sinusoidal multidrug resistance-associated protein (MRP) 3 and MRP4, which contribute to the cytotoxic responses in obstructive cholestasis (11). Similarly, expressions of renal transporter genes are increased to facilitate the excretion of bile acids at the renal basolateral membrane. In the present study, therefore, we investigated whether OAT1 and/or OAT3 take part in adaptive responses in the kidney during cholestasis using the Eisai hyperbilirubinemic rats (EBHR), mutant Sprague-Dawley (SD) rats lacking MRP2, and MRP4, which is localized at the brush-border membranes of renal proximal tubular cells, is upregulated to assist urinary bile acid excretion (8, 18). In contrast to apical transporters, there is little information about the renal basolateral transporters that mediate the cellular uptake of bile acids.

At the renal basolateral membranes, organic anion transporters OAT1 and OAT3 have been demonstrated to play important roles in the membrane transport of a wide variety of anionic compounds, including endogenous metabolites, drugs, and xenobiotics (12, 24, 28). We previously found that the mRNA level of OAT3 was higher than that of any other members of the organic ion transporter family in the human kidney (21). OAT3 possessed greater activity to transport β-lactam antibiotics including cefazolin than OAT1 in an in vitro experiment (35). Furthermore, clinical pharmacokinetic and gene expression analyses showed that only the mRNA level of OAT3 among OAT1–4 significantly correlated with the apparent elimination rate constant of the free fraction of cefazolin in patients with mesangial proliferative glomerulonephritis (25, 26). Regarding to the regulatory aspects of OAT1 and OAT3, it has been reported that both transporters are regulated in various conditions (33). In acute obstructive cholestasis, the protein expression level of OAT1 was increased, but that of OAT3 was unchanged (5). On the other hand, at 3 days after bile duct ligation, the protein expression level of OAT1 was decreased, whereas that of OAT3 was increased (4). These findings suggest that OAT1 and OAT3 are enumerated as candidate transporters for bile acids at the renal basolateral membrane. However, convincing evidences for the expression of renal OAT1 and OAT3 during cholestasis have not been obtained. In addition, the transport activity of bile acids and their conjugates by OAT1 or OAT3 has not been fully elucidated.

In the present study, therefore, we investigated whether OAT1 and/or OAT3 take part in adaptive responses in the kidney during cholestasis using the Eisai hyperbilirubinemic rats (EBHR), mutant Sprague-Dawley (SD) rats lacking MRP2 expression. EHBFR shows hyperbilirubinemia and elevation of serum bile acid concentration and has been used as an intrahepatic cholestatic animal model.

METHODS

Materials. p-[14C]aminohippurate (PAH; 1.9 GBq/mmoll) and [3H]estrostone sulfate (ES) ammonium salt (2.1 TBq/mmoll) were purchased from PerkinElmer Life Analytical Sciences (Boston, MA). [carboxy-1-14C]cholic acid (1.85 MBq/mmoll), [glycine-1-14C]glycocholic acid (1.85 MBq/mmoll), and [3H]taurocholic acid (1.85 MBq/mmoll) were purchased from American Radiolabeled Chemicals (St. Louis, MO). Cefotiam was a gift from Takeda Chemical Industries (Osaka, Japan). Bile acid sodium salts and their conjugates were purchased from Wako (Osaka, Japan). Antibodies for MRP3 and MRP4 were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of the highest purity available.

Measurement of the serum and urinary total bile acid concentration. Male EHBFR and SD rats aged 6–7 wk were purchased from Japan SLC (Hamamatsu, Japan), and cared for according to the Guidelines for Animal Experiments of Kyoto University. Serum and urinary total bile acid concentrations of EHBFR and SD rats were
quantified by bile acid C test Wako (Wako) according to the manufacturer’s protocol. For the measurement of blood urea nitrogen (BUN) and serum creatinine levels, we used commercial kits (Wako).

Western blot analysis. Crude plasma membranes were prepared from the kidney cortex of SD rats and EHBR, and Western blot analysis was performed as described previously (13). Antibodies for OAT1 and OAT3 were prepared as described previously (13). Antibodies for OAT1 (1:1,000), OAT3 (1:1,000), MRP3 (1:500), and MRP4 (1:1,000) were used as the primary antibody. The bound antibodies were detected on X-ray film by enhanced chemiluminescence with horseradish peroxidase-conjugated secondary antibodies and cyclic diacylhydrazides (GE Healthcare Bio-Science, Piscataway, NJ). The density of bands was determined using ImageJ 1.38x (National Institutes of Health).

Real-time PCR. Total RNA was isolated from the rat kidney cortex using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and was reverse-transcribed to yield cDNA. For quantification of the amounts of MRP3, MRP4, OAT1, OAT3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), real-time PCR was carried out by using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) as described previously (29). The primer-probe sets were purchased from Applied Biotech (Belgium, WI). GAPDH mRNA was used as an internal control.

Uptake experiments. HEK293 cells (American Type Culture Collection CRL-1573) were cultured as described previously (36). Transfection and cellular uptake experiments were carried out as described previously (35, 36). The IC50 values were estimated by nonlinear regression analysis as described previously (35). Inhibition constant (K) values were calculated from IC50 values according to the method of Cheng and Prusoff (9).

In vivo experiments. In vivo experiments were performed as described previously (22) with some modifications. Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg), and the femoral artery was cannulated with a polyethylene tube (SP-31; Natsume, Tokyo, Japan) for blood sampling. To investigate the pharmacokinetics of cefotiam, the femoral vein of rats was also cannulated and cefotiam dissolved in saline solution was injected into the femoral vein instantaneously at a dose of 2 mg/kg. Blood samples were collected from the femoral artery at 1, 3, 5, 10, 15, 30, and 60 min after the end of the injection. The blood samples were centrifuged at 14,000 rpm for 3 min, and 100 µl of plasma was deproteinized by adding 200 µl of methanol. The mixtures were centrifuged at 14,000 rpm for 3 min, and the supernatants were filtered through a Millipore filter (SGJVL, 0.45 µm). The filtrates were analyzed by HPLC as described previously (35).

Pharmacokinetic analysis. The pharmacokinetic parameters observed clearance (CLobs), area under the plasma concentration vs. time curve (AUC), and the concentrations of serum bile acid and urinary bile acid in EHBR (solid bars) were ~4- and 6-fold-higher than those in control (open bars), respectively. Each column represents means ± SE of 4 rats. *P < 0.05, **P < 0.01 significantly different from control.

Fig. 1. Serum (A) and urinary (B) concentrations of total bile acid in control rats [Sprague-Dawley (SD) rats] and Eisai hyperbilirubinemic rats (EHBR). The concentrations of serum bile acid and urinary bile acid in EHBR (solid bars) were ~4- and 6-fold-higher than those in control (open bars), respectively. Each column represents means ± SE of 4 rats. *P < 0.05, **P < 0.01 significantly different from control.

Fig. 3. Real-time PCR for OAT1 (A), OAT3 (B), MRP3 (C), and MRP4 (D) in SD rats (open bars) and EHBR (solid bars). Total RNA isolated from kidney cortex of SD rats and EHBR, and Western blot analysis were performed as described previously (29). The primer-probe sets were purchased from Applied Biotech (Belgium, WI). GAPDH mRNA was used as an internal control. Each value represents means ± SE of 4 rats. *P < 0.05, **P < 0.01 significantly different from control.
after one-way ANOVA. Probability values of <0.05 were considered statistically significant.

RESULTS

Serum and urinary bile acid concentrations in EHBR. At first, we examined the urinary secretion of bile acids in EHBR. As shown in Fig. 1, urinary concentration of total bile acid in EHBR was significantly higher than that in SD rats, as well as serum concentrations of total bile acid. In addition, BUN and serum creatinine levels were compared between SD rats and EHBR. No significant changes were observed in BUN and serum creatinine levels between both rats [BUN: 14.16 ± 0.66 (SD rats) vs. 12.42 ± 0.44 (EHBR); serum creatinine: 20.99 ± 1.67 (SD rats) vs. 24.89 ± 0.49 (EHBR)]. These findings suggested that the renal function of EHBR is not affected by cholestatic conditions.

Expression of renal MRP3, MRP4, OAT1, and OAT3 in EHBR. To elucidate the involvement of OATs in the renal handling of bile acids, we examined the protein expression levels of OAT1 and OAT3 together with MRP3 and MRP4 as positive controls. Consistent with previous reports (8, 18), renal MRP3 and MRP4 expression levels were significantly increased in EHBR compared with SD rats (Fig. 2). The protein expression level of OAT3 in EHBR was also upregulated, whereas that of OAT1 was not different between SD rats and EHBR (Fig. 2).

To assess whether expression changes occurred in the transcriptional process, mRNA expression levels of each transporter were determined by real-time PCR analysis. mRNA expression level of OAT3 was not different between SD rats and EHBR (Fig. 3B), suggesting that posttranscriptional mechanisms are involved in the upregulation of OAT3 protein in EHBR. OAT1 mRNA level was slightly decreased in EHBR compared with SD rats (Fig. 3A).

mRNA expression level of OAT3 was not different between SD rats and EHBR (Fig. 3B), suggesting that posttranscriptional mechanisms are involved in the upregulation of OAT3 protein in EHBR. OAT1 mRNA level was slightly decreased in EHBR compared with SD rats (Fig. 3A).

Functional interaction of OAT3 with bile acids. Expression analyses suggested that OAT3 is involved in bile acid transport. Next, we examined the effects of various bile acids on the transport activity of rat (r) OAT1 and rOAT3. As shown in Fig. 4A, PAH uptake by rOAT1 was not inhibited by bile acids except for deoxycholic acid (DCA). On the other hand, [3H]ES uptake by rOAT3 was markedly inhibited by various bile acids, especially DCA, chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA) and glycochenodeoxycholic acid (Fig. 4B). The same experiments were performed using human (h) OAT1- and hOAT3-expressing cells, and similar results were obtained (data not shown).

We then estimated $K_i$ values of various bile acids from the dose/inhibition curves. Typical curves using cholic acid (CA), taurocholic acid (TCA), and UDCA are shown in Fig. 5, and $K_i$ values are summarized in Table 1. Among bile acids tested, UDCA showed the highest affinity for rOAT3 and hOAT3 with $K_i$ values of 62.3 and 50.0 μM, respectively, whereas TCA showed the lowest affinity for rOAT3 and hOAT3 with $K_i$ values of 882 and 2,360 μM, respectively.

To obtain direct evidence of bile acid transport via OAT3, uptake experiments of [14C]CA and its conjugates [14C]glycochenodeoxycholic acid (GCA) and [3H]TCA by OAT1 and OAT3 were carried out. As shown in Fig. 6, CA and its conjugates were significantly transported by rat and human OAT3, except for GCA by hOAT3. This may be due to the low transport activity of OAT3 in EHBR.
Table 1. $K_i$ values of various bile acids for $[3H]$ES uptake by rOAT3 and hOAT3

<table>
<thead>
<tr>
<th>Bile Acids</th>
<th>rOAT3, µM</th>
<th>hOAT3, µM</th>
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<tr>
<td>CA</td>
<td>230±15</td>
<td>554±86</td>
</tr>
<tr>
<td>DCA</td>
<td>72.7±4.8</td>
<td>188±20</td>
</tr>
<tr>
<td>CDCA</td>
<td>33.5±3.1</td>
<td>137±33</td>
</tr>
<tr>
<td>UDCA</td>
<td>62.3±8.6</td>
<td>50.0±3.5</td>
</tr>
<tr>
<td>GCA</td>
<td>203±34</td>
<td>350±11</td>
</tr>
<tr>
<td>GCDCA</td>
<td>75.7±3.7</td>
<td>54.1±3.7</td>
</tr>
<tr>
<td>TCA</td>
<td>882±45</td>
<td>2360±300</td>
</tr>
<tr>
<td>TCDA</td>
<td>207±45</td>
<td>77.0±8.3</td>
</tr>
</tbody>
</table>

Data represent means ± SE of 3 independent experiments. $K_i$, inhibition constant; ES, estrone sulfate; rOAT3 and hOAT3, rat and human organic anion transporter 3, respectively; CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; UDCA, ursodeoxycholic acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; TCA, taurocholic acid; TCDA, taurochenodeoxycholic acid.

In obstructive cholestasis, the main route to excrete bile acids is urinary excretion. However, molecular mechanisms for renal tubular secretion of bile acids, especially for bile acid transport across the basolateral membranes of renal proximal tubular cells, remain unclear. To elucidate the pathophysiological and pharmacokinetic roles of the kidney in cholestasis, it is important to understand how the kidney adapts to decreased serum bile acid levels. In the present study, we clearly demonstrated that OAT3 can interact with various bile acids and that the $K_i$ values of bile acids (~100 µM) are comparable to those of hOAT3 compared with rOAT3, as demonstrated by ES transport (Fig. 6D). Rat and human OAT1 did not transport any of the bile acids used.

Pharmacokinetic profile of OAT3-specific substrate in EHBR. Finally, we investigated the effects of pathophysiological states of EHBR on the pharmacokinetics of OAT3-specific substrate in vivo. Previously, we reported that β-lactam antibiotics such as cefotiam are selectively transported by hOAT3, but not by hOAT1 (35). As shown in Fig. 6A, cefotiam was also demonstrated to be a specific substrate for rOAT3. Because cefotiam is not metabolized and is mainly excreted in the urine as an unchanged form (10), we carried out pharmacokinetic analyses using cefotiam. Figure 8 shows the mean plasma concentration-time profiles for cefotiam in SD rats and EHBR. The plasma concentration of cefotiam in EHBR was higher during the distribution and elimination phases than in SD rats. Table 2 shows the pharmacokinetic parameters calculated from the data in Fig. 8. AUC from 0–60 min in EHBR was significantly higher than that in SD rats, whereas $CL_{obs}$ was decreased in EHBR compared with SD rats.

**DISCUSSION**

In obstructive cholestasis, the main route to excrete bile acids is urinary excretion. However, molecular mechanisms for renal tubular secretion of bile acids, especially for bile acid transport across the basolateral membranes of renal proximal tubular cells, remain unclear. To elucidate the pathophysiological and pharmacokinetic roles of the kidney in cholestasis, it is important to understand how the kidney adapts to decreased serum bile acid levels. In the present study, we found that 1) OAT3 protein expression level is increased in EHBR, 2) OAT3 interacts with various bile acids, and 3) OAT3 can transport various bile acids and its conjugates such as CA, a major component of serum bile acids. These findings were not observed in OAT1, another major renal basolateral organic anion transporter. Furthermore, we demonstrated that the pharmacokinetic profile of cefotiam, an OAT3-specific substrate, is altered in EHBR. These results suggested that renal OAT3, but not OAT1, plays critical roles in the adaptive responses to the renal handling of bile acids in cholestasis.

From in vitro transport studies, endogenous compounds such as cortisol, conjugated hormones (ES, dehydroepiandrosterone sulfate, and estradiol-17β-glucuronide) and prostaglandins have been demonstrated to be transported by OAT3, indicating that the physiological function of renal OAT3 is the secretion of steroid hormones, their conjugates, and prostaglandins (24). In addition to these substrates, TCA was also reported to be transported by OAT3 (7, 32). However, so far, there have been no systematic analyses of the interaction of bile acids with OAT3. In the present study, we clearly demonstrated that OAT3 can interact with various bile acids and that the $K_i$ values of bile acids (~100 µM) are comparable to those of
other substrates of OAT3, although TCA showed relatively low affinity (~1 mM). Furthermore, we showed that CA and its conjugates, major serum bile acids in cholestasis, were transported by OAT3. These findings strongly suggest that the pathophysiological role of renal OAT3 is the secretion of bile acids to reduce the serum bile acid concentration in the choles-
tophysiological role of renal OAT3 is the secretion of bile acids transported by OAT3. These findings strongly suggest that the

In addition to endogenous substrates, OAT3 transports various types of drugs such as β-lactam antibiotics, H2 blockers, diuretics, and nonsteroidal anti-inflammatory drugs (24). Previously, we demonstrated that the β-lactam antibiotic cefotiam is a specific substrate for hOAT3, but not for hOAT1 (35). Demonstrating that OAT3 also selectively transported cefotiam (Fig. 7), we then performed in vivo pharmacokinetic analysis using this drug to elucidate the pharmacokinetic role of OAT3 in cholestasis. Before experiments, we speculated that cefotiam would be more rapidly cleared in EHBR than in SD rats because of upregulation of the OAT3 protein in EHBR. However, contrary to expectations, cefotiam clearance was significantly reduced in EHBR. From a dose-inhibition curve, we determined the $K_i$ value of cefotiam for rOAT3 (760 ± 110 μM from three independent experiments). This $K_i$ value was about eightfold higher than those of most bile acids, suggesting that cefotiam transport via rOAT3 in EHBR is inhibited by elevated serum bile acids. Under cholestatic conditions, serum bile acid levels markedly increased, ranging from 14 to 252 μM in humans (6), which is comparable to $K_i$ values of bile acids for hOAT3. The balance between $K_i$ values of bile acids and those of other drugs for hOAT3 should be considered to predict the drug interaction in the renal secretion of anionic drugs under cholestatic conditions. Furthermore, bile acids have been utilized for therapeutic applications. For example, CDCA and UDCA have been widely used for cholesterol gallstone dissolution (16), and UDCA was recently introduced for cholestatic liver disease therapy (23). CDCA and UDCA showed a very high affinity for hOAT3, indicating the possibility of interaction between UDCA/CDCA and other drugs transported via hOAT3.

The protein expression level of OAT3 was clearly increased in EHBR, but no significant change was observed in the mRNA expression level of OAT3 between SD rats and EHBR. We confirmed that mRNA expression levels of MRP3 and MRP4 were significantly increased in EHBR as described previously (8, 18). It is therefore suggested that the upregulation of OAT3 protein in EHBR was caused by the posttranscriptional modification and/or stabilization of OAT3 protein. There are considerable evidences that several bile acid transporters in hepatocytes are posttranscriptionally regulated by bile acids and that their cell surface expression is increased by recruitment from intracellular pools (1, 14, 34). For example, the recruitment of bile salt export pump (BSEP) to the canalicular membrane by vesicular targeting from the intracellular pool is induced by hypoosmolarity in response to cAMP and bile acids (15, 27). The vesicular trafficking of BSEP is dependent on microtubule cytoskeleton (2), and several cellular kinases and signal transduction molecules such as protein kinase C, phosphatidylinositol 3-kinases (PI3Ks), and p38 mitogen-activated protein kinase (MAPK) are involved in BSEP regulation by membrane trafficking (3, 17, 19, 20). OAT3 transport activity is also regulated by protein kinases such as PI3Ks (31), MAPK, and protein kinase A (30), which may function as signal transducers in intracellular membrane trafficking. Although further studies are needed, augmentation of renal OAT3 protein expression in cholestasis may be associated with signaling pathways mentioned above.

In conclusion, we demonstrated that renal OAT3 protein expression level is increased in EHBR, cholestatic animal model, and that OAT3 can interact with various bile acids. These findings indicate that OAT3 plays an important pathophysiological role in protecting tissues from cholestatic injury.
by stimulating the renal secretion of bile acids. From a pharmacokinetic standpoint, it is possible that increased serum bile acids and/or administration of bile acids such as UDCA for therapy could influence the tubular secretion of anionic drugs via OAT3, and therefore, in these cases, much more attention should be paid to prevent the occurrence of drug interaction or drug-induced toxicity.

REFERENCES