Hepatic Uptake of Bilirubin and Its Conjugates by the Human Organic Anion Transporter SLC21A6

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Bilirubin, the end product of heme catabolism, is taken up from the blood circulation into the liver. This work identifies a high-affinity transport protein mediating the uptake of bilirubin and its conjugates into human hepatocytes. Human embryonic kidney cells (HEK293) permanently expressing the recombinant organic anion-transporting polypeptide 2 (human OATP2, also known as LST-1 or OATP-C; symbol SLC21A6) showed uptake of [3H]monoglucuronosyl bilirubin, [3H]bisglucuronosyl bilirubin, and [3H]sulfobromophthalein and its glucuronate conjugates into human hepatocytes. Human embryonic kidney cells (HEK293) permanently expressing human OATP2 and OATP8 enabled us to answer the question of whether these two major human hepatic OATP transport proteins localized them to the basolateral membrane of hepatocytes (16, 19). Northern blot analyses demonstrated an apparently exclusive hepatic expression of both transport proteins in human liver, we and other groups have recently cloned a new member of this transporter family, human OATP2 (also known as LST1 or OATP-C, gene symbol SLC21A6) (16–18). Most recently, we cloned an additional human liver OATP isoform termed OATP8 (gene symbol SLC21A8), which shares 80% identical amino acids with human OATP2 (19). Antibodies raised against both transport proteins localized them to the basolateral membrane of human hepatocytes (16, 19). Northern blot analyses demonstrated an apparently exclusive hepatic expression of both transporters (16, 19). The availability of cell lines stably expressing human OATP2 and OATP8 enabled us to answer the question of whether these two major human hepatic OATP family members are capable of transporting bilirubin and its conjugates from blood across the basolateral membrane into hepatocytes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cell Lines—**HEK293 cells were cultured in minimum essential medium (Sigma) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 95% humidity, and 5% CO₂, as described recently (16, 19). HEK-OATP2 cells (16) and HEK-OATP8 cells (19) permanently expressed high levels of human recombinant OATP2 and OATP8, respectively. The GenBank™/European Molecular Biology Laboratory accession numbers for the sequences of OATP2 and OATP8 are AJ132573 and AJ251506, respectively.

**Biosynthesis of [3H]Bilirubin—**[3H]Bilirubin was obtained biosynthetically in rats in a procedure similar to the one described by Crawford et al. (20). Two Harlan Sprague-Dawley rats were given an intravenous or intraportal injection of 500 μCi of [3,5,6-3H]deltaaminolevulinic acid (25.9 GBq/mmol; NEN Life Sciences, Boston, MA) at a dose of 83 and 42 MBq/kg body weight, respectively. [3H]Bilirubin was isolated from bile by hydrolysis of its glucuronides and extraction

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1 The abbreviations used are: BSP, sulfobromophthalein; E217G, 17β-glucurononosyl estradiol; HSA, human serum albumin; ICG, indocyanine green; OATP, organic anion-transporting polypeptide; HPLC, high pressure liquid chromatography.
with chloroform (20). The purity of the [3H]bilirubin was confirmed by HPLC, and the specific radioactivity was 120,000–140,000 dpm/nmol (2.0–2.3 GBq/mmol) for the material obtained from both rats. In all experiments, [3H]bilirubin was protected from exposure to light.

**Synthesis of [3H]Monoglucuronosyl Bilirubin and [3H]Bisglucuronosyl Bilirubin** —Bilirubin glucuronides were prepared using recombinant UDP-glucuronosyl transferase 1A1 and UDP-1[3H]glucuronic acid (0.56 TBq/mmol; Biotechnology, Köln, Germany) together with unlabeled bilirubin as described previously (6, 7) and purified using radio-HPLC.

**Uptake Studies** —For uptake assays, cells were seeded in 6-well plates (coated with 0.1 mg/ml poly-lysin) at a density of 1.5–2 × 10⁶ cells/well and cultured with 10 mm sodium butyrate for 24 h. Before the uptake experiments, cells were washed with uptake buffer (142 mm NaCl, 5 mm KCl, 1.2 mm KH₂PO₄, 1.5 mm CaCl₂, 5 mm glucose, and 12.5 mm HEPES, pH 7.3). The transport assay was started by the addition of 1 ml of uptake buffer containing [3H]-labeled substrate (18.5–37 MBq/ml) to the cells. [H]-labeled substrates were obtained from NEN Life Sciences.

[3H]BSP (0.5 TBq/mmol) was obtained by custom synthesis (Hartmann Analytik, Köln, Germany); its purity (>98%) was confirmed by reverse-phase HPLC analysis on a C₁₈ Hypersil column (5-μm particles; Shandon, Runcorn, United Kingdom) using two different systems. The isocratic elution was performed with 45% methanol/55% water containing 50 m NaH₂PO₄ and 5 m Na₂SO₄ at pH 2.8. The linear gradient elution was performed from 100% buffer A (45% methanol/55% water containing 2 mm tetrabutylammonium hydroxide at pH 6.0) to 100% buffer B (90% methanol/10% water containing 2 mm tetrabutylammonium hydroxide at pH 6.0). The specific radioactivity of [3H]BSP did not change during repeated HPLC analyses, indicating that [3H] exchange was below detectability. [3H]BSP strictly co-chromatographed with unlabeled BSP during HPLC. Moreover, the unlabeled BSP and the [H]-labeled BSP were analyzed by nondenaturing mass spectrometry as described by Lehmann and Kaspersen (21). The isotopic pattern of molecular ions of unlabeled BSP and [3H]BSP was very similar, and quantitative evaluation indicated the following relative amounts: unlabeled BSP, 63.1%; singly labeled [3H]BSP 27.4%; and doubly labeled [3H][3H]BSP, 9.5%. These data correspond to a specific radioactivity of 0.5 TBq/mmol.

For inhibition studies, inhibitors were included in the uptake buffer. After incubation at 37 °C, transport was stopped at different time points by the addition of 1 ml of cold uptake buffer. Cells were subsequently washed three times with uptake buffer and lysed with 1 ml of 0.2% SDS in water. Aliquota (250 μl) of the lysate were counted for radioactivity. Protein content was determined by the Lowry method using 100 μl of lysate.

**Uptake Studies with [3H]Bilirubin** —Due to high background binding of [3H]bilirubin to the poly-L-lysine-coated plastic dishes, uptake of [3H]bilirubin into transfected cells was measured in cell suspension. Cells were cultured with 10 mm butyrate for 24 h as described previously (16). For uptake assays, cells were detached from culture flasks by knocking, washed twice with uptake buffer, and resuspended in uptake buffer at a density of 3 × 10⁶ cells/ml. [3H]Bilirubin was diluted with human serum albumin (HSA; Sigma; fatty acid-free) in uptake buffer (75,000–100,000 dpm/ml). Unlabeled bilirubin was added to give the desired final concentrations. Uptake was started by mixing 1 ml of cell suspension with 1 ml of bilirubin/albumin solution to give a final radioactivity of 37,500–50,000 dpm/ml and stopped at different time points by centrifugation of the mixture at 13,000 rpm for 10 s. Cell pellets were washed twice with 1 ml of uptake buffer containing HSA and lysed in 2 ml of 0.2% SDS in water. Aliquota (300 μl) of the lysate were counted for radioactivity. To determine the nonspecific binding of [3H]bilirubin, cells were incubated with [3H]bilirubin in the presence of HSA for 1 min at 4 °C. Cell-associated radioactivity measured under these conditions was used as a blank and subtracted from all other values. No differences between this method and the method using adherent cells were observed for other substrates like BSP.

**Immunoblot Analysis** —Preparation of crude membrane fractions from transfected cells and immunoblot analysis were performed as described previously (16, 19). The polyclonal antibody ESL (16) was used to detect recombinant human OATP2.

**RESULTS**

Sulforhodamine B, Monoglucuronosyl Bilirubin, and Bisglucuronosyl Bilirubin Are High-affinity Substrates for Human OATP2—BSP, a widely used anionic model compound for studies on uptake into the liver, is a high-affinity substrate for OATP2 with a Kₘ value of 140 nM (Fig. 1). In comparison with OATP8, OATP2 showed a 24-fold higher affinity for BSP (Table I). In addition, monoglucuronosyl bilirubin and bisglucuronosyl bilirubin were identified as high-affinity substrates for OATP2 with nanomolar Kₘ values. Monoglucuronosyl bilirubin also showed an equivalent concentration of 0.5 GBq/mmol, while OATP8 had a concentration of 0.5 GBq/mmol; Biotrend, Köln, Germany) together with unlabeled bilirubin as described previously (6, 7) and purified using radio-HPLC.

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TABLE I

Kinetic constants for substrate transport mediated by OATP2 and OATP8

<table>
<thead>
<tr>
<th>Substrate</th>
<th>OATP2</th>
<th>OATP8</th>
</tr>
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<tbody>
<tr>
<td>Monoglucuronosyl bilirubin</td>
<td>0.10 ± 0.03</td>
<td>0.50 ± 0.16</td>
</tr>
<tr>
<td>Bisglucuronosyl bilirubin</td>
<td>0.28 ± 0.13</td>
<td>0.62 ± 0.18</td>
</tr>
<tr>
<td>Sulfochromophthalein</td>
<td>0.14 ± 0.03</td>
<td>3.3 ± 0.9</td>
</tr>
<tr>
<td>17β-Glucuronosyl estradiol</td>
<td>8.2 ± 1.8</td>
<td>5.4 ± 3.1</td>
</tr>
<tr>
<td>Cholyl taurine</td>
<td>10.0 ± 5.5</td>
<td>6.0 ± 3.6</td>
</tr>
<tr>
<td>Cholate</td>
<td>11.4 ± 2.3</td>
<td>6.0 ± 3.6</td>
</tr>
<tr>
<td>Estrone 3-sulfate</td>
<td>12.5 ± 2.1</td>
<td>6.0 ± 3.6</td>
</tr>
<tr>
<td>Dehydroepiandrosterone 3-sulfate</td>
<td>21.5 ± 2.5</td>
<td>&gt;30'</td>
</tr>
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* No significant transport was detected at a concentration of 1 μM.
* No significant transport was detected at a concentration of 5 μM.
* No saturation up to a concentration of 30 μM.

Fig. 2. Effect of HSA on BSP uptake mediated by OATP2 and OATP8. A, uptake of [3H]BSP was measured at 50 nM for OATP2 and 1 μM for OATP8 in the presence of 1 μM (OATP2) or 20 μM (OATP8) HSA (BSP:HSA = 1:20 in both cases). No significant effect of albumin on [3H]BSP uptake was observed for OATP2, whereas OATP8-mediated [3H]BSP uptake was completely abolished by the addition of albumin. B, uptake of 1 μM [3H]BSP measured with OATP2 and OATP8 in the presence of increasing concentrations of HSA ranging from 0.2 to 5 μM. HSA had a much greater effect on the OATP8-mediated [3H]BSP uptake than on the OATP2-mediated uptake (p < 0.01). Data are the means ± S.D. (n = 6).

Inhibited OATP2-mediated uptake in a competitive manner with a Kᵢ value of 112 nm. Although BSP and ICG were thought to be taken up into hepatocytes by the same mechanism (3), patients with apparently normal hepatic BSP uptake but delayed ICG clearance have been reported (11). Our studies raise the possibility that such patients may have a deficiency in OATP2, leading to delayed ICG clearance, but normal OATP8, which may function in BSP uptake under this condition.

Unlike ICG, the drugs pravastatin, rifamycin SV, and rifampicin inhibited both OATP2- and OATP8-mediated uptake of [3H]E₂17βG (Fig. 3B). The HMG-CoA reductase inhibitor pravastatin was a competitive inhibitor of OATP2-mediated transport of E₂17βG with an inhibition constant of 53 μM. This is in line with a recent study showing that pravastatin is a substrate for human OATP2 with a Kᵢ value of about 30 μM (17).

Unconjugated Bilirubin Is Transported by OATP2 in the Presence of Albumin—The fact that BSP, monoglucuronosyl bilirubin, and bisglucuronosyl bilirubin are high-affinity substrates for human OATP2 and the fact that ICG inhibits OATP2-mediated uptake competitively at nanomolar concentrations suggested that OATP2 might be the long-sought uptake transporter for unconjugated bilirubin in the basolateral hepatocyte membrane. We therefore measured [3H]bilirubin uptake by OATP2-transfected HEK293 cells (HEK-OATP2) at a concentration of 1 μM in the presence of 2 μM HSA. The calculated free bilirubin concentration under this condition, using the dissociation constant for HSA and bilirubin (12), is about 25 nM. As shown in Fig. 4A, [3H]bilirubin was taken up by HEK-OATP2 cells time-dependently at 37 °C. The uptake rate into HEK-OATP2 cells (8.5 pmol·min⁻¹·mg protein⁻¹) differed from that into vector-transfected HEK293 cells (1.5 pmol·min⁻¹·mg protein⁻¹) by a factor of 5.7 (p < 0.01). When the uptake was determined at 4 °C, no significant difference in uptake rates was detected between OATP2-expressing cells and control cells. Uptake of bilirubin mediated by recombinant human OATP2 was concentration-dependent as shown in Fig. 4B. In these experiments, the HSA concentration was kept constant at 20 μM so that the bilirubin:HSA ratio did not exceed 0.5. Because of the uncertainty of calculated free bilirubin concentrations (12), the Kᵢ value for free unconjugated bilirubin could only be estimated and was about 160 nm. For comparison, we investigated whether OATP8 would also transport unconjugated bilirubin. At the same [3H]bilirubin and HSA concentrations and under the same conditions used for OATP2 (Fig. 4), no significant uptake of [3H]bilirubin was detected with HEK293 cells expressing human OATP8 (data not shown).

Bilirubin, which was examined as a complex with HSA, inhibited the uptake of E₂17βG and BSP by human OATP2, with 50% inhibition at 5 μM with E₂17βG as substrate and at 20 μM with BSP as substrate. Uptake of [3H]bilirubin into HEK-OATP2 cells was strongly inhibited by BSP (Fig. 4C), demonstrating mutual inhibition of BSP and bilirubin uptake.

DISCUSSION

We conclude that uptake of bilirubin into human hepatocytes, the first step of its detoxification, is mediated by OATP2, a major transport protein localized to the basolateral membrane of hepatocytes, but not by the isoform OATP8 localized to the same membrane domain. Our conclusion is based on the following experimental data: (a) the structurally and chemically related lipophilic anionic compounds BSP, monoglucuronosyl bilirubin, and bisglucuronosyl bilirubin were high-affinity substrates for OATP2, with nanomolar Kᵢ values, whereas OATP8 transported BSP and monoglucuronosyl bilirubin with markedly lower affinity (Fig. 1 and Table 1); (b) OATP2, but not OATP8, was able to extract substrates from albumin (Fig. 2) to which bilirubin binds with high affinity; (c) ICG inhibited OATP2 at nanomolar concentrations but exerted no inhibitory effect on OATP8 at concentrations up to 10 μM (Fig. 3); and (d) [3H]bilirubin uptake by OATP2 was directly demonstrated by uptake studies with OATP2-expressing HEK transfected (Fig. 4). Together with previous data, we propose the following scheme for the detoxification and elimination
pathway of bilirubin in human liver (Fig. 5): bilirubin (B) bound to albumin is taken up across the basolateral membrane by OATP2 and conjugated in the hepatocyte by the UDP-glucuronosyl transferase 1A (UGT1A1), resulting in monoglucuronosyl bilirubin (BGA) and bisglucuronosyl bilirubin (B(GA)2). The excretion of BGA and B(GA)2 is mediated by the apical ATP-dependent conjugate export pump, multidrug resistance protein 2 (symbol ABCC2) (6, 7).

Our results here establish a carrier-mediated uptake of bilirubin into hepatocytes. However, we do not exclude additional bilirubin uptake through passive diffusion. The differentiation between carrier-mediated and diffusional bilirubin uptake into the liver will be supported by the identification of mutations in the OATP2 (SLC21A6) gene leading to the loss or functional impairment of OATP2 in the basolateral membrane of hepatocytes. Moreover, in view of the fact that current knowledge of the human OATP family is not complete, additional transport proteins may further contribute to the selective uptake of bilirubin from the blood circulation into liver.

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