The Human Organic Anion Transport Protein SLC21A6 Is Not Sufficient for Bilirubin Transport*

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A recent study (Cui, Y., Konig, J., Leier, I., Buchholz, U., and Kepper, D. (2001) J. Biol. Chem. 276, 9626–9630) suggests that human OATP2 (SLC21A6), also known as OATP-C and LST1, mediates hepatic bilirubin transport. Because of methodologic concerns, this study was designed to examine this issue using a bilirubin transport assay that was validated in overnight cultured rat hepatocytes. These studies showed that cultured rat hepatocytes transported bilirubin with kinetics virtually identical to the transport of sulfobromophthalein. This assay was then used to quantify bilirubin transport by HeLa cells that had been stably transfected with OATP2 under regulation of a metallothionein promoter. Immunoblot analysis revealed abundant expression of OATP2 after incubation of cells for 48 h in zinc, whereas uninduced controls did not express OATP2. In OATP2-expressing (zinc-induced) HeLa cells at 37 °C, the uptake of \[^{35}S\]sulfobromophthalein was substantial (51.6 ± 16.5 pmol/15 min/mg protein, n = 5) with little cell-associated ligand in non-expressing (uninduced) cells (0.54 ± 0.16 pmol/15 min/mg protein, n = 5, p < 0.002). In contrast, there was no difference (p > 0.2) in cell-associated \[^{3}H\]bilirubin in induced (OATP2-expressing) as compared with uninduced cells (11.25 ± 3.02 pmol/15 min/mg protein versus 9.15 ± 1.68 pmol/min/mg protein, respectively, n = 5). We obtained similar results in OATP2-transfected HEK293 cells that were used in the original report. The existence of a bilirubin transporter has been an important field of investigation for many years. Although the current study indicates that a role for OATP2 in hepatocyte bilirubin transport is unlikely, it provides new and sensitive tools that can be adapted to examine the function of putative bilirubin transporters in the future.

Bilirubin is a yellow pigment derived from the degradation of heme. Because of intramolecular hydrogen bonding, it is water insoluble (1). Following formation in various cells throughout the body, it is released into the circulation where it binds avidly to albumin (2, 3). Under normal circumstances, it is extracted from albumin and taken up rapidly by hepatocytes (4, 5). Although the kinetics of uptake suggests carriermediation, the nature of this carrier has remained elusive (6, 7). The fact that bilirubin can pass rapidly through lipid bilayers has led some investigators to question the necessity of postulating the existence of a carrier (8, 9). A recent report (10) indicates that the human organic anion transport protein SLC21A6 (also known as OATP2,‡ LST-1, and OATP-C) mediates high affinity transport of bilirubin. That study was performed using HEK293 cells that had been stably transfected with OATP2. Cells were harvested from poly-I-lysine-coated plastic dishes, and bilirubin uptake studies were performed on cells in suspension. Nonspecific binding was not reported although it was used to correct uptake data, and we were concerned that the harvesting procedure could result in cell permeabilization. To validate the results in that report, we devised a system in which we could test the ability of OATP2-transfected cells to transport bilirubin without the necessity of detaching the cells from the culture dishes. Initially, we established an assay to quantify bilirubin transport in overnight cultured rat hepatocytes. This assay was then used to examine bilirubin transport by HeLa cells that had been stably transfected with OATP2 under the regulation of a metallothionein promoter. OATP2 expression was induced by incubation of these cells in zinc for 48 h. Uninduced cells did not express OATP2 and were used as controls. These studies showed that cultured rat hepatocytes transported bilirubin with kinetics virtually identical to transport of sulfobromophthalein (BSP). In contrast, OATP2-expressing HeLa cells transported BSP but not bilirubin. Further studies, OATP2-transfected HEK293 cells that were obtained from Cui et al. (10) were also studied using the methodology that they described. We were unable to reproduce their results with respect to bilirubin transport, although these cells transported BSP as they described. The existence of a bilirubin transporter has been an important field of study for many years. The current studies using a newly established assay for bilirubin transport indicate that a role for OATP2 in hepatocyte bilirubin transport is unlikely.

**EXPERIMENTAL PROCEDURES**

Preparation of Radiolabeled Ligands

\[^{3}H\]Bilirubin—\[^{3}H\]Bilirubin (20 mCi/mmol) was purified from rat bile following intravenous administration of \[^{3,5-3}H\]aminolevulinic acid (3 Ci/mmol, PerkinElmer Life Sciences) as described previously (11). Radiochemical purity of \[^{3}H\]bilirubin was assessed by high pressure liquid chromatography and was greater than 95%.

\[^{35}S\]BSP—\[^{35}S\]BSP (5000 mCi/mmol) was prepared by sulfonation of phenoletetrabromophthalein with \(H_2\[^{35}S\]O_4\) (1000 Ci/mmol, PerkinElmer Life Sciences) as described previously (12). Radiochemical purity of \[^{35}S\]BSP was assessed by thin-layer chromatography (12) and was greater than 95%.

1 The abbreviations used are: OATP2, organic anion-transporting polypeptide 2; HEK, human embryonic kidney; BSP, sulfobromophthalein; SFM, serum-free medium; BSA, bovine serum albumin; HSA, human serum albumin.

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Procedures are shown. The Kpn instructions. For induction of OATP2, according to the manufacturer, stably transfected with pMEP4-OATP2 were prepared and hygromycin-resistant gene as a selectable marker (15). HeLa cells were cultured directly on plastic dishes as described under Experimental Procedure.

**Experimental Procedure**

Overnight cultured rat hepatocytes was determined as described under 2-3 studies.

Influence of isoosmotic substitution of NaCl in SFM on [3H]bilirubin uptake by cultured rat hepatocytes

Uptake of [3H]bilirubin over 5 min in the presence of 0.1% BSA by overnight cultured rat hepatocytes was determined as described under “Experimental Procedure” in SFM with or without isoosmotic substitution of NaCl by other compounds as indicated. Data are the average of 2-3 studies.

| SFM constituent | [3H]Bilirubin uptake |
|-----------------|---------------------|
| NaCl            | 100                 |
| KCl             | 86                  |
| Sodium glutonate| 41                  |
| Sucrose         | 27                  |

**OATP2-expressing Cell Lines**

HeLa Cells—Wild type OATP2 was cloned into the pEFHis,-Topo plasmid (Invitrogen) as described previously (13). The derived amino acid sequence was confirmed as identical to that originally published by König et al. (14). The OATP2 cDNA was excised from the plasmid using KpnI (3%) and NotI (3%) and was inserted into the corresponding restriction sites of pMEP4 (Invitrogen), a mammalian expression vector that uses the inducible metallothionein IIa promoter and also carries the hygromycin-resistant gene as a selectable marker (15). HeLa cells (ATCC) stably transfected with pMEP4-OATP2 were prepared and grown in selective medium as described previously (15) with the exception that they were transfected using LipofectAMINE plus (Invitrogen) according to the manufacturer’s instructions. For induction of OATP2, cells were cultured for 24 h in medium containing 100 μM ZnSO4 and then 24 h at 150 μM ZnSO4.

HEK293 Cells—HeK293 cells, stably transfected with OATP2 or vector (pCDA3.1) alone (10, 14), were a kind gift of Dr. Dietrich Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Before transport studies were performed, cells were cultured in the presence of 10 mM sodium butyrate for 24 h to maximize OATP2 expression (10, 14).

Isolated Rat Hepatocytes

Preparation—Hepatocytes were isolated from 200–250-g male Sprague–Dawley rats (Tacconi Farms, Farmington, NY) after perfusion of the liver with Collagenase Type I (Worthington Biochemical Corporation, Freehold, NJ) (16). All of the animals used in this study received humane care in compliance with the institution’s guidelines. Viability of isolated hepatocytes was >90% as judged by trypan blue exclusion.

Culture—Hepatocytes were cultured overnight as described previously (16, 17). Freshly isolated hepatocytes were suspended in Waymouth’s 552/1 medium (Invitrogen) containing 5% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, CA), 1.7 mM additional CaCl2, 5 μg/ml bovine insulin (Sigma), 100 units/ml penicillin (Invitrogen), 0.1 mg/ml streptomycin (Invitrogen), and 25 mM HEPES, pH 7.2. Approximately, 1.5 × 106 cells in 3 ml of medium were placed in 60-mm Primaria culture plates (BD Biosciences) and cultured in a 5% CO2 atmosphere at 37 °C. After 2 h, the medium was changed and cells were cultured overnight for ~18 h.

Assays of Transport of [3H]Bilirubin and [35S]BSP

**Cultured Hepatocytes and HeLa Cells**—Transport of [3H]bilirubin was quantified using methods described previously to quantify transport of [35S]BSP by cultured cells (16, 17). Cells were washed three times with 1.5 ml of serum-free medium (SFM) consisting of 135 mM NaCl, 1.2 mM MgCl2, 0.81 mM MgSO4, 27.8 mM glucose, 2.5 mM CaCl2, and 25 mM HEPES, pH 7.2. They were then incubated for 15 min at 37 °C in 1 ml of SFM containing 0.1% BSA. Following this period, cells were incubated for varied periods of time at 4 or 37 °C in 1 ml of SFM containing 0.1% BSA and either 1 μM [3H]bilirubin or 1 μM [35S]BSP. Following this incubation, the solution was rapidly aspirated and cells were washed seven times at 4 °C with 1.5 ml of SFM. The third wash contained 5% BSA and was allowed to stand at 4 °C for 5 min. Cells were harvested, and radioactivity was determined. In some studies, uptake was determined in SFM in which NaCl was replaced isoosmotically with KCl, sucrose, or sodium gluconate as we have described previously (4). In other studies, the concentrations of ligand or albumin (BSA or HSA) were varied as indicated. Cell protein was determined in replicate plates by the BCA assay (Pierce) according to the manufacturer’s instructions using BSA as standard.

**HEK293 Cells**—Because these cells do not attach firmly to plastic
dishes, they were detached from culture flasks and the transport of \(^{[3H]}\)bilirubin and \(^{[35S]}\)BSP was determined in suspension using methods identical to those described by Cui et al. (10).

Preparation of Antibody to OATP2—A cysteine-terminating peptide (DEANLESLNKNKHFC) corresponding to amino acids 666–679 of OATP2 was synthesized, cross-linked to keyhole limpet hemocyanin, and used to immunize rabbits (Covance Research Products, Inc. Denver, PA). This amino acid sequence is unique to OATP2 as determined by a BLAST search of available databases.

Immunoblot Analysis—Immunoblot analysis was performed on carbonate-extracted cell pellets as we have described previously (18). Cells were washed five times with ice-cold phosphate-buffered saline, pH 7.4, harvested, and centrifuged at 3000 \(g\) at 4 °C for 10 min, and pellets were kept on dry ice for 1 h. Pellets were resuspended in 30 ml of ice-cold 0.1 M Na\(_2\)CO\(_3\)-containing protease inhibitors (P8340, Sigma), rotated gently at 4 °C for 15 min, and centrifuged at 100,000 \(g\) at 4 °C for 1 h. Pellets were resuspended in ice-cold phosphate-buffered saline. 10% SDS-PAGE was performed on 14–26% g of protein in the presence or absence of 10 mM dithiothreitol, and proteins were transferred onto a polyvinylidene difluoride membrane and processed for immunoblot using an ECL detection system (PerkinElmer Life Sciences).

RESULTS

Comparison of Transport of \(^{[3H]}\)Bilirubin and \(^{[35S]}\)BSP by Overnight Cultured Rat Hepatocytes

As seen in Fig. 1, the uptake of \(^{[3H]}\)bilirubin and \(^{[35S]}\)BSP by cultured rat hepatocytes was highly temperature-dependent and linear over 15 min. The uptake assay included a 5-min incubation with 5% BSA at 4 °C with the aim of minimizing

### Table II

| OATP2 | \[^{[3H]}\)bilirubin | \[^{[35S]}\)BSP |
|-------|----------------------|------------------|
| Control | 0.41 pmol/min/mg protein | 0.06 pmol/min/mg protein |
| OATP2 | 0.30 | 1.95 |
| Experiment 1 | | |
| Control | 1.07 | 0.08 |
| OATP2 | 0.87 | 4.34 |
| Experiment 2 | | |
| Control | 1.63 | ND |
| OATP2 | 0.97 | ND |

Fig. 3. Uptake of \(^{[35S]}\)BSP and \(^{[3H]}\)bilirubin by OATP2-transfected HeLa cells. HeLa cells stably transfected with OATP2 under regulation of a zinc-inducible promoter were cultured for 48 h in the presence (Induced) or absence (Uninduced) of zinc as described under “Experimental Procedures.” A, representative studies of uptake over time by these cells of 1 \(\mu\)M \(^{[35S]}\)BSP or 1 \(\mu\)M \(^{[3H]}\)bilirubin that were determined in the presence of 0.1% BSA. The bars in B represent mean values for uptake over 15 min of \(^{[35S]}\)BSP (n = 5) or \(^{[3H]}\)bilirubin (n = 5). The error bars represent mean ± S.E.

Fig. 4. Uptake of \(^{[35S]}\)BSP and \(^{[3H]}\)bilirubin by OATP2-transfected HeLa cells in the presence of varied concentrations of BSA or HSA. HeLa cells stably transfected with OATP2 under regulation of a zinc-inducible promoter were cultured for 48 h in the presence (Induced) or absence (Uninduced) of zinc as described under “Experimental Procedures.” Uptake over time of 1 \(\mu\)M \(^{[35S]}\)BSP or 1 \(\mu\)M \(^{[3H]}\)bilirubin by these cells was determined in the presence of varied concentrations (2–16 \(\mu\)M) of BSA (A) or HSA (B). Studies of \(^{[35S]}\)BSP were also performed in the absence of albumin. Albumin-free studies were not performed with \(^{[3H]}\)bilirubin because of its water insolubility.

Transport of 1 \(\mu\)M \(^{[3H]}\)bilirubin or \(^{[35S]}\)BSP uptake by plasmid- and OATP2-transfected HEK293 cells in the presence of 0.1% BSA

Uptake of \(^{[3H]}\)bilirubin or \(^{[35S]}\)BSP in the presence of 0.1% BSA was determined in HEK293 cells in suspension as described under “Experimental Procedures.” Control cells were stably transfected with OATP2 under regulation of a zinc-inducible promoter to culture for 48 h in the presence (Induced) or absence (Uninduced) of zinc as described under “Experimental Procedures.” Uptake over time of 1 \(\mu\)M \(^{[35S]}\)BSP or 1 \(\mu\)M \(^{[3H]}\)bilirubin by these cells was determined in the presence of varied concentrations (2–16 \(\mu\)M) of BSA.
surface-associated ligand, which can be especially problematic with a highly water-insoluble compound such as bilirubin. That this strategy was effective can be seen from the low level of cell-associated bilirubin at 4°C as compared with 37°C (Fig. 1). It is noteworthy that cultured hepatocytes transported each of these ligands equally well (Fig. 1C). These results validate the use of this assay to quantify cellular transport of bilirubin. In previous studies, we have found that uptake of BSP by overnight cultured rat hepatocytes requires the presence of extracellular chloride (4, 17). A similar phenomenon was found when uptake of bilirubin by isolated and perfused rat liver was quantified (4). As seen in Table I, we have now found similar results for bilirubin uptake by overnight cultured rat hepatocytes in which there was approximately a 75% reduction in uptake following isosmotic substitution of NaCl by sucrose. These results provide a further level of validation for the bilirubin transport assay in cells.

**Preparation of HeLa Cells Stably Transfected with OATP2**

A HeLa cell line that was stably transfected with OATP2 under the regulation of a zinc-inducible promoter was prepared. Expression of the protein was examined by immunoblot (Fig. 2). In the absence of zinc induction, there was no expression of OATP2 (Fig. 2). Following 48 h of incubation in zinc, OATP2 expression was readily detectable. As distinct from results with rat Oatp1 (18), the apparent molecular mass of OATP2 changes upon reduction (Fig. 2). In the absence of reduction with dithiothreitol, OATP2 migrated as a major band of 188 ± 11.3 (mean ± S.D., n = 3), whereas after reduction, it migrated at 75.1 ± 0.17 kDa. These data suggest that the native protein is an oligomer of subunits that is held together by disulfide bonds.

**Comparison of Transport of [3H]Bilirubin and [35S]BSP by HeLa Cells Stably Transfected with OATP2**

Without zinc induction, there was little uptake of [35S]BSP by OATP2 stably transfected HeLa cells (Fig. 3). In those cells that had been preincubated with zinc to induce OATP2 expression, the uptake of [35S]BSP was substantial (Fig. 3) and of similar extent as that by overnight cultured rat hepatocytes. In contrast, the uptake of [3H]bilirubin was low and was unaffected by the expression of OATP2 (Fig. 3). To examine whether this absence of OATP2-mediated bilirubin transport was the result of the use of bovine rather than human albumin, we performed additional experiments as depicted in Fig. 4. There was no difference in the uptake of [3H]bilirubin between OATP2-expressing and non-expressing cells over a range (2–16 μM) of HSA or BSA. These results are in contrast to the transport of [35S]BSP that was highly dependent upon OATP2 expression (Fig. 4).

**Comparison of Transport of [3H]Bilirubin and [35S]BSP by HEK293 Cells Stably Transfected with OATP2**

In the presence of 0.1% BSA, OATP2-transfected cells but not control HEK293 cells avidly took up [35S]BSP (Table II). In contrast, cell association of [3H]bilirubin was relatively low in both cell lines, and uptake by OATP2-transfected cells was no higher than that of control cells (Table II). In these studies, BSA (14.7 μg) was in molar excess to bilirubin and BSP (1 μg). The uptake of 3 μM bilirubin was also examined in the presence of 3 μM BSA. As seen in Table III, there was much more association of bilirubin with cells under this condition. As in the preceding studies, there was no significant difference in bilirubin uptake in OATP-expressing cells as compared with control cells.

**DISCUSSION**

Bilirubin is a water-insoluble degradation product of heme that circulates bound tightly to albumin from which it is extracted and taken up rapidly by hepatocytes (4, 5, 19). Although the kinetics of uptake suggests carrier mediation (6, 7), the nature of this carrier has remained elusive and there may be carrier-mediated and passive diffusional components to this process (8, 20). In a recent publication (10), the human organic anion transport protein SLC21A6 (also known as OATP2, LST-1, and OATP-C) was reported to mediate high affinity transport of bilirubin. However, the transport assay that was used in that study had the potential for high background values and consequent unreliability. In this study, we established and validated an assay to quantify bilirubin transport by cells in culture that is based upon an assay that we used in previous studies of BSP transport (4, 17). BSP is a synthetic organic anionic dye that has been used in the past as a test of liver function based upon rapid disappearance from the circulation after intravenous injection. Like bilirubin, it circulates bound tightly to albumin from which it is extracted and taken up by hepatocytes (21, 22). Although hepatic uptake of bilirubin and BSP is saturable (6, 23) and uptake of these ligands is mutually exclusive, their uptake mechanisms were found to be partially independent in a previous study performed in isolated perfused rat livers following treatment of rats with nafenopin (21).

In this study, we found that the transport characteristics of bilirubin and BSP were essentially identical in overnight cultured rat hepatocytes. In particular, the uptake of these ligands was highly temperature- and time-dependent and required the presence of extracellular Cl⁻. Previous studies in overnight cultured rat hepatocytes demonstrated that upon isosmotic substitution of NaCl in the medium by sucrose, the uptake of BSP was reduced by ~75% (4, 17). This was not attributed to Na⁺ dependence of transport, because isosmotic substitution of NaCl in medium by KCl had little effect on transport (4). However, the substitution of NaCl by sodium gluconate reduced the uptake of these ligands by ~60% (4). Demonstration that BSP uptake requires external Cl⁻ and is not stimulated by unidirectional Cl⁻ gradients indicated that BSP uptake is not directly coupled to Cl⁻ transport (17). Rather, an ~10-fold increase in affinity of cells for BSP in the presence as compared with the absence of Cl⁻ was demonstrated (17). This study demonstrated similar Cl⁻ dependence...
of bilirubin uptake by cultured rat hepatocytes in accord with previous studies in the isolated perfused rat liver (4). CI dependence of BSP uptake was also seen in rat Oatp1-transfected HeLa cells in the presence but not absence of albumin (25). The physiologic significance of this phenomenon remains to be elucidated.

This assay was then used to quantify bilirubin and BSP transport by a HeLa cell line that we prepared in which OATP2 cDNA was stably transfected and in which OATP2 protein expression was under the regulation of a metallothionein promoter. Similar to our previous studies of a HeLa cell line that had been stably transfected with an analogous rat Oatp1 cDNA construct (15), in the absence of zinc induction, there was no immunologically detectable expression of OATP2. Following 48 h of incubation of cells in zinc-containing medium, the expression of OATP2 was readily seen (Fig. 2). This permitted uninduced cells to be used as a control when determining the influence of OATP2 on uptake function. As compared with the results following incubation of OATP2-HeLa cells in zinc, there was a substantial expression of BSP with uninduced cells. In contrast, the association of bilirubin with these cells was low, increased little over time, and was unaffected by OATP2 expression. To rule out a requirement for albumin in bilirubin uptake, similar studies were performed at varied concentrations of BSA and HSA. Under all of these conditions as compared with the uptake of BSP, cell association of bilirubin was low and was unaffected by OATP2 expression (Fig. 4). These studies confirm the previous report (10) that OATP2 can mediate cellular BSP transport. However, using an assay for bilirubin transport that clearly works in cultured rat hepatocytes, we were unable to find any evidence in support of a role of OATP2 in bilirubin transport in OATP2-expressing HeLa cells.

As it is possible that there could be cell specificity for OATP2 function, studies of BSP and bilirubin transport were performed in HEK293 cells that were stably transfected with OATP2. These cells were used in the initial report of OATP2-mediated bilirubin transport (10) and were kindly provided to us by the authors of that study. As in the initial report (10), controls for these studies were cells stably transfected with empty plasmid (i.e. not containing OATP2 cDNA). Because these cells do not adhere well to plastic culture dishes, we performed the transport assays on cells in suspension using the procedures described in the original report (10). We confirmed that OATP2-transfected cells but not control cells expressed OATP2 immunologically and that they avidly transported BSP (Table II). However, we found no influence of OATP2 expression on association of bilirubin with these cells even at a 1:1 ratio of albumin to bilirubin (Tables II and III). Recent studies performed in Xenopus laevis oocytes with 10 μM bilirubin in the presence of equimolar HSA were interpreted as showing that bilirubin uptake could be mediated by human OATP-A (SLC21A3), OATP2, and OATP-8 (SLC21A8) (26). Transport of bilirubin by OATP-A (27) and OATP8 (10) has not been seen by others, and elucidation of the reasons for these differences will require further study.

In summary, although we confirmed that OATP2 can mediate the transport of bilirubin, we were unable to confirm the previous report that it can mediate transport of bilirubin. The results that we obtained with OATP2-transfected HeLa cells and OATP2-transfected HEK293 cells lead us to conclude that this transporter does not have a substantial role in bilirubin transport. We have no explanation for the disparate results between the present study and that reported previously other than the possibility that bilirubin in the previous report had isomerized to derivatives that could be more readily transported by OATP2. We have ruled out this possibility in this study by high pressure liquid chromatography analysis of the [3H]bilirubin that was used. The existence and identity of a hepatocyte transporter for bilirubin has been a longstanding open question. Although the current study indicates that a significant role for OATP2 in hepatocyte bilirubin transport is unlikely, it provides new and sensitive tools that can be adapted to examine the function of putative bilirubin transporters in the future.

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