Vectorial transport of unconjugated and conjugated bile salts by monolayers of LLC-PK1 cells doubly transfected with human NTCP and BSEP or with rat Ntcp and Bsep

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Bile formation is an essential function of the liver. Vectorial transport of bile salts across the hepatocyte is the major driving force for bile flow, which is mediated by several transporters. Bile salts are taken up from the portal vein by Na+-dependent Na+-taurocholate-cotransporting polypeptide (rat Ntcp/Slc10a1 and human NTCP/SLC10A1) (6, 7) as well as by the Na+-independent organic anion transporting polypeptides (rat Oatps/Slc21a and human OATPs/SLC21A). Much evidence indicates that NTCP plays a major role in this uptake process (25). On the bile canalicular membrane, bile salts are secreted into bile by the ATP-dependent transporter bile salt export pump (rat Bsep/Abcb11 and human BSEP/ABCB11) (1, 3, 5, 18), whereas 3-sulfated dipolar bile salts are excreted by multidrug resistance protein 2 (rat Mrp2/Abcc2 and human MRP2/ABCC2) (1, 10, 12, 24). Thus it appears to be well established that NTCP and BSEP play key roles in the vectorial transport of bile salts across hepatocytes.

It is reported that these transporters accept various kinds of bile salts and share substrate specificity: taurochenodeoxycholate (TCDC), tauroursodeoxycholate (UDC), taurocholate (TC), cholate (CA), and glycocholate (GC) have been reported to be transported by human NTCP-expressing oocytes (16), rat Ntcp-expressing oocytes, and Chinese hamster ovary (CHO) cells (16, 21). Efficient transport was observed for TCDC, glycochenodeoxycholate (GCDC), TUDC, TC, and GC by rat Bsep-expressing Sf9 vesicles and TUDC, TCDC, TC, and GC by human BSEP-expressing Sf9 vesicles (3, 18).

In a previous study, we established the coexpression system of rat Ntcp and rat Bsep as an in vitro model that can reproduce the vectorial transport of bile salts across hepatocytes. This expression system is useful for detecting the transport of bile salts mediated by Ntcp and Bsep and allows the identification of new substrates. Until this study, it had been difficult to study Bsep function in intact mammalian cells, because most Bsep substrates cannot penetrate the cell membrane without the aid of uptake transporters. Therefore, Bsep has been studied using inside-out membrane vesicles prepared from Bsep-expressing cells, and this expression system should be useful for screening drugs that inhibit or enhance the function of Bsep. However, a human NTCP- and human BSEP-expressing experimental system is essential for such analysis to connect the experimental data and clinical observations because there may be species differences in transport properties and/or drug sensitivity between rats and humans.

The aim of this study was to construct a coexpression system of human NTCP and human BSEP that transports bile salts vectorially. We report the construction of such a coexpression system in LLC-PK1 cells, a cell line devoid of endogeneous bile salts transport activity. We then used these cells grown in polarized monolayers to characterize the transcellular transport of a series of bile salts and compared the results with those obtained in a study in which rat transporters were transfected.

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Materials and Methods

Chemicals. [3H]cholic acid (24.5 Ci/mmol), [3H]taurocholic acid (2 Ci/mmol), and [14C]chenodeoxycholic acid (48.6 mCi/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). [14C]glycochenodeoxycholic acid (57.3 mCi/mmol), and [14C]lithocholic acid (LCA) (57.3 mCi/mmol) were purchased from American Radiolabeled Chemicals, (St. Louis, MO). [3H]taurochenodeoxycholic acid (10 Ci/mmol), [3H]glycochenodeoxycholic acid (11 Ci/mmol), [3H]ursodeoxycholic acid (12 Ci/mmol), [3H]taursodeoxycholic acid (10 Ci/mmol), [3H]glycodeoxycholic acid (11 Ci/mmol), [3H]gallchenodeoxycholic acid (11 Ci/mmol), and [3H]galldeoxycholic acid (30 Ci/mmol) were kindly provided by Dr. Benjamin Shneider (Mount Sinai Medical Center)(22). Antibodies for human BSEP (N-16, goat IgG) were kindly provided by Mitsubishi Pharma (Osaka, Japan). All other chemicals were used commercially available and of reagent grade.

Antibodies. Antibodies raised in rabbits for human NTCP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Both antibodies were used at a dilution of 1:1,000 for immunoblotting and 1:100 for immunostaining.

Cell culture and transfection. Parental LLC-PK1 cells were grown in medium 199 (M199) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (GIBCO-BRL; 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B) at 37°C under 5% CO2. Full-length human NTCP cDNA subcloned into pcDNA3.1 (Invitrogen) was transfected into LLC-PK1 cells with FuGENE 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. Transfectants expressing NTCP were selected with G418 (800 μg/ml). The clone with the highest NTCP activity was screened by uptake activity for TC.

Immunoblotting. NTCP or vector transfected LLC-PK1 cells were harvested for 24 h after infection, and expression of NTCP was induced by 10 mM sodium butyrate. After 24 h of induction, cells were then fixed with ice-cold methanol for 10 min, permeabilized with 1% Triton X-100 in PBS for 10 min, and incubated for 1 h with primary antibodies at room temperature. After this, cells were washed three times with PBS, incubated with secondary antibody conjugates, Alexa Fluor, and cyanine monomer TO-PRO-3 iodide (642/661) (Molecular Probes) diluted 250-fold in PBS for 1 h at room temperature, and mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). The coverslips were examined using an LSM 510 microscope (Zeiss; Oberkochen, Germany).

Transport assays. NTCP- or vector-transfected LLC-PK1 cells were seeded on transwell membrane inserts (pore size of 3 μm; Falcon, Bedford, MA) in 24-well plates at a density of 1.4 × 105 cells per insert. After a 2-day culture, confluent cells were infected by recombinant adenovirus containing cDNAs for BSEP or GFP (100 MOI). Cells were harvested 48 h after infection, and expression of NTCP was induced with 10 mM sodium butyrate (4). Then, 24 h after induction, cells were washed with transport buffer (in mM: 118 NaCl, 23.8 NaHCO3, 4.83 KCl, 0.96 KH2PO4, 1.20 MgSO4, 12.5 HEPES, 5 glucose, and 1.53 CaCl2 adjusted to pH 7.4). Subsequently, 3H- or 14C-labeled substrates were added to the transport buffer in either the apical (250 μl) or basal compartment (950 μl). After the times indicated, the radioactivity in the opposite compartment was measured. The intracellular accumulation of radioactivity was determined at the end of the experiments by lysing the cells with 500 μl 0.2 N NaOH in distilled water and measuring the radioactivity in the cell lysates. Aliquots (50 μl) of cell lysate were used to determine protein concentrations by the method of Lowry (14) with bovine serum albumin as a standard. The apparent intracellular concentration of substrates was determined by assuming that the cellular volume per milligram of cellular protein was 4 μl.

Data analysis. The transcellular transport of [3H]TC was determined based on the transcellular transport of bile salts during the 2-h incubation. The transcellular transport at 30 and 60 min was linearly increased in a time-dependent manner for 2 h (data not shown).

Fig. 1. Western blot analysis of Na+-taurocholate-cotransporting protein (NTCP) and bile salt export protein (BSEP). The expression level of NTCP and BSEP was determined by Western blot analysis. Crude membrane fractions (30 μg) from the control, LLC-NTCP, LLC-BSEP, and LLC-NTCP/BSEP cells were separated by 12.5% and 8.5% SDS-PAGE for NTCP and BSEP, respectively. *Nonspecific bands.
the transcellular transport of substrates (pmol·min⁻¹·mg protein⁻¹) and S, Kₘ, Vₘₐₓ, and PS diff represent substrate concentration in medium (µM), Michaelis-Menten constant (µM), maximum uptake rate (pmol·min⁻¹·mg of protein⁻¹), and nonsaturable permeability-surface area (PS) product expressed as a clearance (µl·min⁻¹·mg protein⁻¹), respectively. The concentration-dependent transport of bile salts was fitted to this equation by the nonlinear least-squares method with a MULTI program (26) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values.

Kinetic analysis of the transcellular transport was performed according to a previous report (17). PSnet is given as a hybrid parameter consisting of PSbasal, PSapical, and PSbasal,eff:

\[
PS_{\text{net}} = PS_{\text{basal}} \times \frac{PS_{\text{apical}}}{PS_{\text{apical}} + PS_{\text{basal,eff}}}
\]

where PSnet (µl·min⁻¹·mg protein⁻¹) is the PS product in the apical compartment defined for the ligand concentration in the medium [Cmed (pmol/µl)]. PSbasal (µl·min⁻¹·mg of protein⁻¹) is the clearance for the influx of ligand across the basal membrane, which is defined for Cmed. PSapical (µl·min⁻¹·mg protein⁻¹) is the clearance for the efflux of ligand across the apical membrane, which is defined for the ligand apparent concentration in the cells [Ccell (pmol/µl)]. PSbasal,eff (µl·min⁻¹·mg protein⁻¹) is the clearance for the efflux of ligand across the basal membrane from the cell to the basal compartment, which is defined for Ccell.

Under conditions where PSapical > PSbasal,eff, PSnet can be approximated

\[
PS_{\text{net}} \approx PS_{\text{basal}}
\]

In the present study, PSnet and PSapical were calculated by dividing the rate for the transcellular transport of ligands determined over 2 h by the medium concentration of ligands and by the apparent cellular concentration of ligands determined at the end of the experiments (2 h), respectively. When PSapical > PSbasal,eff in NTCP- and BSEP-coexpressing LLC-NTCP/BSEP and BSEP-expressing LLC-PK1 monolayers (LLC-BSEP), the difference between PSnet for LLC-NTCP/BSEP and PSnet for LLC-BSEP represents the clearance for the uptake mediated by NTCP (PSNTCP). Consequently, PSNTCP

Fig. 2. Subcellular localization of NTCP and BSEP in LLC-PK1 cells. The localization of NTCP and BSEP was confirmed in various types of LLC-PK1 cells. LLC-NTCP (A), LLC-BSEP (B), and LLC-NTCP/BSEP (C and D) cells were stained with rabbit antiserum against NTCP (red) or BSEP (green). Nuclei were stained with TO-PRO-3 iodide (blue). In each panel, the top shows the en face image and the bottom shows the Z-sectioning image with a horizontal line in the en face image. Bars = 20 µm (A–C) and 100 µm (D).

Fig. 3. Time profiles of the transcellular transport of [³H]taurocholate (TC) across various types of LLC-PK1 monolayers. Transcellular transport of [³H]TC (1 µM) across LLC-PK1 monolayers was examined as a function of time. A–D represent the data for the control, LLC-NTCP, LLC-BSEP, and LLC-NTCP/BSEP monolayers, respectively. Open and closed circles represent the transcellular transport in the apical to basal and basal to apical directions, respectively. Each point and vertical bar represents the mean ± SE of 3 determinations.

Where vertical bars are not shown, the SE was contained within the limits of the symbol.

AJP-Gastrointest Liver Physiol • VOL 290 • MARCH 2006 • www.ajpgi.org
was calculated according to the following equation in the present study

$$PS_{NTCP} = PS_{apical for LLC-NTCP/BSEP} - PS_{net for LLC-BSEP}$$  \( (5) \)

The efflux clearance mediated by BSEP \((PS_{BSEP})\) was calculated according to the following equation;

$$PS_{BSEP} = PS_{apical for LLC-NTCP/BSEP} - PS_{apical for LLC-NTCP}$$  \( (4) \)

**RESULTS**

**Expression and localization of NTCP and BSEP in LLC-PK1 cells.** The expression of NTCP and BSEP in the transfected LLC-PK1 cells was confirmed by immunoblotting (Fig. 1). As shown in Fig. 1, NTCP expression was detectable as a band of 50 kDa in LLC-NTCP and LLC-NTCP/BSEP. The expression of BSEP was also detected at 160 kDa in both BSEP-expressing LLC-PK1 (LLC-BSEP) and NTCP- and BSEP-expressing LLC-PK1 (LLC-NTCP/BSEP) (Fig. 1). In the control LLC-PK1 cells, no expression of NTCP or BSEP could be detected (Fig. 1).

Subcellular localization of the immunostained transporters in the transfectants was assessed using confocal laser scanning microscopy. In LLC-NTCP and LLC-NTCP/BSEP, NTCP was localized to the basolateral membrane (Fig. 2, A and C). In contrast, BSEP was observed at the apical membrane in LLC-BSEP and LLC-NTCP/BSEP (Fig. 2, B and C). The amount of NTCP- and BSEP-positive cells in LLC-NTCP/BSEP cells was ~100% and 50%, respectively (Fig. 2D).

**Vectorial transport of TC mediated by NTCP and BSEP.**

The function of NTCP and BSEP was studied by measuring the transcellular transport of \(^{3}H\)TC across cell monolayers (Fig. 3). In the case of the control cells and cells expressing only NTCP or BSEP, transport of TC was rarely observed. In contrast, vectorial transport of \(^{3}H\)TC was significantly increased in LLC-NTCP/BSEP monolayers. In addition, \(^{3}H\)TC remaining in LLC-NTCP/BSEP was significantly lower than that present in LLC-NTCP at the end of the experiment (2 h; 12 vs. 66 \(\mu\)M). The intracellular concentration in the control cells was 2 \(\mu\)M. Saturation of the basal-to-apical transport of TC across the LLC-NTCP/BSEP monolayers was observed (see Fig. 7A and Table 1).

**Vectorial transport of a series of bile salts across LLC-PK1 monolayers.** In addition to transport of TC, the transcellular transport of a variety of conjugated and unconjugated bile salts was characterized (Figs. 4–6). Basal to apical transcellular transport of GC, CA, TCDC, GCDC, chenodeoxycholate (CDCA), TUDC, glycodeoxycholate (GUDC), Ursodeoxycholate (UDCA), taurodeoxycholate (TDC), and glycodeoxycholate (GDC) across the LLC-NTCP/BSEP monolayers was compared with that by the control monolayers. For LCA, basal to apical transport was not significantly enhanced even in LLC-NTCP/BSEP (Fig. 6D).

The transport clearance of each of the examined bile salts was normalized by comparing its clearance with that of TC, which was determined in the same experiment. Basal to apical transport patterns were similar among GC, CA, TCDC, GCDC, TUDC, and GUDC. For unconjugated CDCA and UDCA, vectorial transport was observed not only in the LLC-NTCP/BSEP monolayers but also in LLC-NTCP or LLC-PK1-BSEP monolayers, probably because of the relatively high membrane permeability of both basolateral and apical membranes (Fig. 6, B and D). The transport of the secondary bile salts TDC and GDC was less efficient (Figs. 4D and 5D). For GUDC, the saturation of the basal to apical transport across LLC-NTCP/BSEP monolayers was examined as well as TC (Fig. 7B and Table 1). The \(K_m\) value and the nonsaturable flux \(PS_{diff}\) of basal to apical transport for GUDC were similar to that of TC. The \(V_{max}\) for GUDC was almost comparable with TC, although slightly smaller, showing that the transport by LLC-NTCP/BSEP was as efficient for GUDC as TC, as already suggested in the comparison of basolateral to apical transport clearance of the tracer amount of GUDC and TC (Fig. 5).

**Comparison of the PS\(_{NTCP}\) and PS\(_{BSEP}\) values of bile salts across LLC-PK1 monolayer-expressing human NTCP/BSEP and rat Ntcp/Bsep.** The species difference in substrate specificity between human NTCP and rat Ntcp and human BSEP and rat Bsep was examined by comparing the results in the present study using human transporters with those of a study using rat transporters. The uptake clearance mediated by human NTCP (PS\(_{NTCP}\)) and the efflux clearance mediated by human BSEP (PS\(_{BSEP}\)) were calculated using transcellular transport of \([^3H]TC\) remaining in LLC-NTCP/BSEP was significantly lower than that present in LLC-NTCP at the end of the experiment (2 h; 12 vs. 66 \(\mu\)M). The intracellular concentration in the control cells was 2 \(\mu\)M. Saturation of the basal-to-apical transport of TC across the LLC-NTCP/BSEP monolayers was observed (see Fig. 7A and Table 1).
For the PSBSEP values, rank order was exhibited as glycine-coursodeoxycholate (GUDC), and [3H]glycodeoxycholate (GDC) across the control (LLC), LLC-NTCP, LLC-BSEP, and LLC-NTCP/BSEP monolayers determined at the same time. Transcellular transport data for TC, GC, CA, TCDC, GCDC, CDCA, TUDC, GUDC, UDCA, TDC, and GDC (Fig. 8) as described in MATERIALS AND METHODS. The vectorial transport of these bile salts was also measured using in LLC-Ntcp/Bsep. PSNTCP and PSBSEP were calculated using these data. Both PSNTCP and PSBSEP well correlated between rats and humans (Fig. 8). PSNTCP values increased in rank order: taurine-conjugated bile salts > glycine-conjugated bile salts > unconjugated bile salts. For the PSBSEP values, rank order was exhibited as glycine-conjugated bile salts and unconjugated bile salts > taurine-conjugated bile salts.

**DISCUSSION**

In the present study, we established LLC-PK1 cells expressing both bile salt uptake transporter NTCP and bile salt efflux transporter BSEP as an in vitro model that can mimic the vectorial transport of bile salts across the hepatocyte. We previously demonstrated that MDCK cells expressing both rat Ntcp and rat Bsep exhibit vectorial transport of several kinds of bile salts (17). However, this model was not a suitable system for the characterization of Bsep because MDCK cells have an endogenous efflux transport system for taurine-conjugated bile salts at their apical membrane (17). For taurine-conjugated bile salts, such as TUDC and TCDC, basal to apical transport was not enhanced even in Bsep-expressing MDCK cells, presumably due to the high background efflux by this endogenous transport system. We found that LLC-PK1 cells do not have such an endogenous transport system. Therefore, this LLC-PK1 system is a more powerful tool than the previous one in that quantitative functional analysis of Bsep for various kinds of bile salts and other organic anions can be carried out.

The basolateral localization of NTCP and the apical localization of BSEP were confirmed by immunohistochemical analysis (Fig. 2), which is consistent with the fact that these transporters exhibited luminal and canalicular membranes in hepatocytes. The transport of TC across the LLC-PK1, LLC-NTCP, and LLC-BSEP was small and symmetrical. In contrast, the basal to apical flux of TC was greatly stimulated in LLC-NTCP/BSEP. Basal to apical and apical to basal transport by LLC-NTCP/BSEP was 5.13- and 0.6-fold that by LLC-NTCP, respectively (Fig. 3). These results indicate that NTCP and BSEP have a cooperative role in its transport in this expression system. The intracellular concentration of TC in LLC-NTCP/BSEP at steady state was 18% of that in LLC-NTCP. This result suggests that the cells expressing BSEP account for 80% of LLC-NTCP/BSEP monolayers, indicating that the expression efficiency was higher than that inferred from the immunofluorescence studies (positive cell: ~50%).

Kinetic analysis of the transport of TC and GUDC revealed that basal to apical transport across LLC-NTCP/BSEP was saturable with a $K_m$ value of $20 \pm 1$ and $16 \pm 7 \mu M$, respectively (Table 1). These $K_m$ values are similar to the reported $K_m$ values of TC for uptake into human hepatocytes [34–62 (19, 20) and 84–34 \mu M (unpublished observations)] and NTCP [6–8 (6) and 25 \mu M (unpublished observations)], which agrees with the hypothesis that the basal to apical flux of TC across LLC-NTCP/BSEP is predominantly by influx clearnace mediated by NTCP (MATERIALS AND METHODS, Eq. 2).

Transcellular transport of other primary bile salts, GC, CA, TCDC, GCDC, and CDCA, secondary bile salts, TDC and GDC, and tertiary bile salts, TUDC, GUDC, and UDCA, was also observed (Figs. 4–6). However, there was no clear transport of LCA (Fig. 6D). Recently, it was reported that CDCA strongly upregulated BSEP through nuclear receptor farnesoid X receptor (15). Therefore, the observation that BSEP can efficiently transport CDCA suggests that BSEP is responsible
for the homeostasis of intracellular concentration of bile salts via its regulation expression level.

The basal to apical transport by LLC-NTCP/BSEP was comparable among the primary bile salts GC, CA, TCDC, GCDC, and CDCA and the tertiary bile salts TUDC, GUDC, and UDCA. The transport of the secondary bile salts, TDC and GDC, was less efficient, and no significant transport of LCA was observed.

The fact that unconjugated CDCA and UDCA were transported by BSEP disagrees with our recent result from an uptake experiment using membrane vesicles prepared from human BSEP-expressing HEK-293 cells, where significant ATP-dependent transport of CDCA and UDCA was rarely observed (9a). This contradiction implies the possibility that BSEP can transport these substrates only when a certain kind of endogenous factor helps the function of BSEP. Therefore, it is essential to evaluate the substrate specificity of efflux transporter in intact mammalian cells.

Thus NTCP/BSEP doubly transfected cells are suitable for defining the structure-transport properties of bile salts as well as characterizing the transport function of other organic anions. Furthermore, as shown in the data analysis, the respective function of NTCP and BSEP can be calculated using transcellular transport data. In this study, using this method, the species function of NTCP and BSEP can be calculated using transcellular transport data. The solid lines represent the fitted line. CL, clearance; V, uptake.

In conclusion, we have established that LLC-PK1 cells expressing both NTCP and BSEP transport conjugated and unconjugated bile salts vectorially and have identified some new substrates for NTCP and BSEP. In this study, it was demonstrated that the NTCP and BSEP transporters of humans and rats have similar substrate specificity for the bile salts examined. This model should be applicable for the study of the transport of bile salts and other organic anions. In the future, this model could also be used as a screening system for drugs that inhibit or enhance the function of NTCP and/or BSEP. It has been suggested that inhibition of bile salt transporters by drugs is associated with drug-induced cholestasis (2, 11, 13). Because the NTCP- and BSEP-expressing system may be
useful for evaluating the inhibitory effects of such drugs on the transporters, this system would be a good screening system during the early phase of drug development for eliminating compounds that could have potential cholestatic and, therefore, hepatotoxic effects.

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