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Efflux Mechanism of Taurocholate across the Rat Intestinal Basolateral Membrane

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Abstract: We investigated the contribution of multidrug resistance associated protein 3 (Mrp3/*ABCC3*) to the transport of bile acids across the rat intestinal basolateral membrane using the everted sacs. The permeability-surface area (PS) products of taurocholate in the everted sacs of rat jejunum, ileum, and colon were determined in the absence or presence of inhibitors for Mrp3. The results were analyzed to determine the PS product for the uptake across the apical membrane (PS1) and that for the efflux across the basolateral membrane (PS3). The mucosal-to-serosal transport of taurocholate in the ileum was the highest. The concentration-dependent inhibitory effects by all inhibitors in the ileum were observed on both PS1 and PS3 for taurocholate. However, even in the presence of 1 mM of each inhibitor, the decrease of PS3 was low. Additionally, PS3 in the colon, where Mrp3 is expressed at a high level, was not inhibited by MK571 and taurolithocholate-3-sulfate. Unlike PS1, PS3 did not exhibit saturation at the concentration examined. These results suggest that Mrp3 makes only a minor contribution to the efflux of bile acids across the basolateral membrane. Ostα−Ostβ heteromeric transporter is certainly one of the good candidates for such a transporter.

Keywords: Taurocholate; bile acid transporter; multidrug resistance associated protein (MRP); efflux; intestine; everted sac; PS product

Introduction

Bile acids are synthesized from cholesterol in the liver and excreted into the bile. Most of the bile acids excreted into the intestinal lumen are reabsorbed across enterocytes and then efficiently taken up by the liver from the portal vein. Such an extensive enterohepatic circulation is responsible for maintaining the homeostasis of bile acids. The enterohepatic circulation of bile acids also plays a central role in a number of physiologically essential functions, including promoting hepatic bile flow, the digestion and absorption of lipids, and cholesterol metabolism.[1−4]

In recent years, the transport of bile acids, which are required for the enterohepatic circulation, has been investigated in great detail.[5−13] Bile acids are taken up into hepatocytes by sodium-dependent and -independent uptake systems, which are mediated by Na^+-taurocholate cotransporting polypeptide (NTCP/*SLC10A1*) and organic anion transporting polypeptides (OATPs/*SLCO*), respectively, and secreted into the bile via an ATP binding cassette transmembrane transporter referred to as the bile salt export pump (BSEP/*ABCB11*). In the gut, particularly in the ileum, bile acids are taken up into enterocytes via an apical sodium-dependent bile acid transporter (ASBT/*SLC10A2*).

It has been shown that multidrug resistance associated protein 3 (MRP3/*ABCC3*), another basolaterally located ABC

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(1) Hofmann, A. F. Intestinal absorption of bile acids and biliary constituents. The intestinal component of the enterohepatic circulation and the integrated system. In *Physiology of the Gastrointestinal Tract*; Johnson, L. R., Ed.; Raven: New York, 1994; pp 1845−1867.

(2) Chawla, A.; Saez, E.; Evans, R. M. Don't Know Much Bile-ology. *Cell* **2000**, *103*, 1−4.

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transporter, is responsible for the cellular extrusion of monovalent and sulfated bile acids, such as taurocholate (TC) and taurolithocholate-3-sulfate (TLC-S), respectively.[14] Mrp3 was initially cloned from mutant rats (Eisai hyperbilirubinemic rats) whose apical expression of Mrp2/*ABCC2*, an ABC transporter for the biliary excretion of many kinds of organic anions, is hereditarily defective.[15] MRP3 is also induced on the basolateral membrane of hepatocytes in humans suffering from Dubin−Johnson syndrome which is caused by the defect in MRP2 expression.[16] Our liver perfusion studies have suggested the role of Mrp3 in exporting bile acids into the blood under conditions where Mrp3 is induced in rats.[17] Because Mrp3 is also expressed on the basolateral membrane of rat enterocytes,[18] and in human intestine,[19] it is possible that this transporter is responsible for the absorption of bile acids. Indeed, we have previously reported an ATP-dependent manner of TC and TLC-S, Mrp3 substrates, by basolateral membrane vesicles (BLMVs) isolated from rat intestine.[20] Western blot analysis demonstrated that the expression level of Mrp3 was highest in the colon, followed by the ileum and jejunum, and this rank order is in parallel with that of the extent of the transport of TC and TLC-S in BLMVs. These results suggest that MRP3 plays a role in the enterohepatic circulation of bile acids by transporting them from enterocytes into blood. However, it is not clarified that such an ATP-dependent transport is required for the absorption of bile acids.

In the present study, we investigated the contribution of Mrp3 to the transport of bile acids across the intestinal basolateral membrane using rat everted sacs. Then, we also examined the transport mechanism of bile acids across the basolateral membrane of rat enterocytes.

Experimental Section

Materials. [^{3}H]Taurocholate (74 GBq/mmol) and [^{3}H]-digoxin (585 GBq/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Taurocholate and taurolithocholate-3-sulfate were purchased from Sigma-Aldrich (St. Louis, MO). MK571 was purchased from Funakoshi (Tokyo, Japan). Quinidine sulfate and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Kanto Chemical (Tokyo, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. All other reagents were commercial products of analytical grade.

Male Sprague−Dawley rats at 7 weeks of age were obtained from Charles River Japan (Yokohama, Japan) and fed on standard laboratory animal chow (MF; Oriental Yeast, Tokyo, Japan). Rats were used in the experiments at 7−8 weeks of age and 220−270 g of body weight.

Preparation of Everted Sacs. The studies were carried out by using everted sacs prepared by a modification of the procedure described by Barr and Riegelman.[21] In brief, rats

(3) Sinal, C. J.; Tohkin, M.; Miyata, M.; Ward, J. M.; Lambert, G.; Gonzalez, F. J. Targeted Disruption of the Nuclear Receptor FXR/BAR Impairs Bile Acid and Lipid Homeostasis. *Cell* **2000**, *102*, 731−744.

(4) Russell, D. W. Nuclear Orphan Receptors Control Cholesterol Catabolism. *Cell* **1999**, *97*, 539−542.

(5) Meier, P. J.; Eckhardt, U.; Schroeder, A.; Hagenbuch, B.; Stieger, B. Substrate specificity of sinusoidal bile acid and organic anion uptake systems in rat and human liver. *Hepatology* **1997**, *26*, 1667−1677.

(6) Takikawa, H. Hepatobiliary transport of bile acids and organic anions. *J. Hepatobiliary Pancreat. Surg.* **2002**, *9*, 443−447.

(7) Kullak-Ublick, G. A.; Stieger, B.; Hagenbuch, B.; Meier, P. J. Hepatic transport of bile salts. *Semin. Liver Dis.* **2000**, *20*, 273−292.

(8) Meier, P. J.; Stieger, B. Bile salt transporters. *Annu. Rev. Physiol.* **2002**, *64*, 635−661.

(9) Byrne, J. A.; Strautnieks, S. S.; Mieli-Vergani, G.; Higgins, C. F.; Linton, K. J.; Thompson, R. J. The human bile salt export pump: Characterization of substrate specificity and identification of inhibitors. *Gastroenterology* **2002**, *123*, 1649−1658.

(10) Keppler, D.; Konig, J. Hepatic secretion of conjugated drugs and endogenous substances. *Semin. Liver Dis.* **2000**, *20*, 265−272.

(11) Gerloff, T.; Steiger, B.; Hagenbuch, B.; Madon, J.; Landmann, L.; Roth, J.; Hofmann, A. F.; Meier, P. J. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J. Biol. Chem.* **1998**, *273*, 10046−10050.

(12) Shneider, B. L.; Dawson, P. A.; Christie, D. M.; Hardikar, W.; Wong, M. H.; Suchy, F. J. Cloning and molecular characterization of the ontogeny of a rat ileal sodium-dependent bile acid transporter. *J. Clin. Invest.* **1995**, *95*, 745−754.

(13) Craddock, A. L.; Love, M. W.; Daniel, R. W.; Kirby, L. C.; Walters, H. C.; Wong, M. H.; Dawson, P. A. Expression and transport properties of the human ileal and renal sodium-dependent bile acid transporter. *Am. J. Physiol.* **1998**, *274*, G157−G169.

(14) Hirohashi, T.; Suzuki, H.; Takikawa, H.; Sugiyama, Y. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). *J. Biol. Chem.* **2000**, *275*, 2905−2910.

(15) Hirohashi, T.; Suzuki, H.; Ito, K.; Ogawa, K.; Kume, K.; Shimizu, T.; Sugiyama, Y. Hepatic Expression of Multidrug Resistance-Associated Protein-Like Proteins Maintained in Eisai Hyperbilirubinemic Rats. *Mol. Pharmacol.* **1998**, *53*, 1068−1075.

(16) Koenig, J.; Rost, D.; Cui, Y. H.; Keppler, D. Characterization of the human multidrug resistance protein isoform MRP3 localized to the basolateral hepatocyte membrane. *Hepatology* **1999**, *29*, 1156−1163.

(17) Akita, H.; Suzuki, H.; Sugiyama, Y. Sinusoidal efflux of taurocholate is enhanced in Mrp2-deficient rat liver. *Pharm. Res.* **2001**, *18*, 1119−1125.

(18) Rost, D.; Mahner, S.; Sugiyama, Y.; Stremmel, W. Expression and localization of the multidrug resistance-associated protein 3 in rat small and large intestine. *Am. J. Physiol.* **2002**, *282*, G720−G726.

(19) Kool, M.; Haas, M.; Scheffer, G. L.; Scheper, R. J.; van Eijk, M. J. T.; Juijn, J. A.; Baas, F.; Borst, P. Analysis of expression of cMoat (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res.* **1997**, *57*, 3537−3547.

(20) Shoji, T.; Suzuki, H.; Kusuhara, H.; Watanabe, Y.; Sakamoto, S.; Sugiyama, Y. ATP-dependent transport of organic anions into isolated basolateral membrane vesicles from rat intestine. *Am. J. Physiol.* **2004**, *287*, G749−G756.

(21) Barr, W. H.; Riegelman, S. Intestinal drug absorption and metabolism I: Comparison of methods and models to study physiological factors of in vitro and in vivo intestinal absorption. *J. Pharm. Sci.* **1970**, *59*, 154−163.

were fasted for 20−24 h prior to the experiments but given water ad libitum. They were anesthetized with sodium pentobarbital and sacrificed by exsanguination via the abdominal aorta. Then, segments of the jejunum, ileum, and colon were removed from the site 5 cm away from the ligament of Treiz, and above and below the cecum, respectively. Each segment was rinsed with cold saline, and then everted using a stainless steel rod. One end of the everted sac was ligated with silk thread, and a polyethylene tube connected to a silicon tube was inserted into the other end and tied.

Transport Study in Everted Sacs. The everted gut sacs of approximately 5 cm in length were filled with 0.5 mL of Krebs Ringer−Henseleit bicarbonate buffer (118 mM NaCl, 4.75 mM KCl, 2.50 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, and 25 mM $NaHCO_3$, pH 7.4). Subsequently, the sac was placed in 10 mL of Krebs Ringer−Henseleit bicarbonate buffer. After a 10 min preincubation at 37 °C, [^{3}H]taurocholate or [^{3}H]digoxin was added to the mucosal side to give a final concentration of 1 μM or 0.743 nM, respectively. In determining the concentration dependence of TC, the final concentrations of TC in the medium were set from 0.1 to 1200 μM. The solution was continually gassed with O_2/CO_2 (95/5) at 37 °C. Aliquots (100 μL) of serosal fluid were collected at designated times and then replaced by the same volume of buffer. At the end of the incubation period, the sac was quickly removed, and the wet tissue weight was measured. The radioactivity in mucosal fluid, serosal fluid, and sac was determined in a liquid scintillation counter (LS 6000SC, Beckman).

Inhibition Study in Everted Sacs. It has been shown that TLC-S potently inhibits the transport of [^{3}H]$E_2$17βG (estradiol 17β-D-glucuronide) and [^{3}H]taurocholate by membrane vesicles from rat Mrp3-transfected LLC-PK1 cells.[14] MK571 also inhibits MRP3-mediated etoposide glucuronide transport[22] and methotrexate transport[23] in a concentration-dependent manner. In addition, DNP-SG, which is formed from CDNB into the cells, inhibited ATP-dependent uptake of [^{3}H]$E_2$17βG by membrane vesicles from human MRP3 expressing Sf9 cells (K_i = 337 μM)[24] and rat Mrp3-transfected LLC-PK1 cells (K_i = 83.8 μM).[25] Thus, we used TLC-S, MK571, and CDNB (DNP-SG) as the inhibitors for MRP3.

The everted sacs were preincubated in the medium containing MK571 (2−1000 μM), TLC-S (20−1000 μM), CDNB (50−1000 μM), or quinidine (1.28 mM) for 10 min at 37 °C, and then the mucosal-to-serosal transport of [^{3}H]-taurocholate (1 μM) or [^{3}H]digoxin (0.743 nM) was examined under the presence of each inhibitor. Each inhibitor was added to the mucosal side.

Data Analysis. The transcellular transport of TC was analyzed to determine the permeability-surface area (PS) products for the influx of TC from the mucosal side to sacs (PS1), that for the efflux of TC from sacs to the mucosal side (PS2) and that for the efflux of TC from sacs to the serosal side (PS3). PS3 is given by the following equation according to its definition:

$$\mathrm{PS3} = \frac{\mathrm{d}C_s/\mathrm{d}t}{C_{sac}}$$

where C_s and C_{sac} represent the concentration of TC in serosal fluid and sacs, respectively.

Because it was considered that the mucosal-to-serosal transport of TC reached a steady state at 60 min from the present study and PS2 ≪ PS3 may hold, PS1 is given as follows:

$$\mathrm{PS1} = \frac{\mathrm{d}C_s/\mathrm{d}t}{C_m}$$

where C_m is the concentration of TC in mucosal fluid.

The concentration dependence of TC transport was described by one saturable and one nonsaturable component. The data were fitted to the following equation:

$$\mathrm{PS} = \frac{V_{max}}{K_m + C} + K_d$$

where V_{max} is the maximal influx rate (nmol min^{-1} (g of tissue)$^{-1}$), K_m is the half-saturation concentration of TC (μM), C is the TC concentration (μM) in the mucosal fluid, and K_d is the clearance corresponding to the nonsaturable component.

Statistical Analysis. All data represented at least three independent experiments and were expressed as the mean ± SD. Any statistical significance in the differences of the means was determined by ANOVA followed by Dunnet's test. $P < 0.01$ was considered to be statistically significant.

Results

TC Transport in Jejunum, Ileum, and Colon. The time-dependent mucosal-to-serosal and serosal-to-mucosal transport of TC (1 μM) in rat everted ileal sacs was observed. As shown in Figure 1, the amount of TC in the mucosal fluid decreased with time, and that in the serosal fluid increased linearly from 20 min up to 60 min. At 60 min, the concentration of TC on the serosal side was 2−3 times higher than that on the mucosal side (data not shown). Since the TC concentration in sacs at 60 min (5.80 ± 0.91 μM) was almost the same as that at 40 min (5.23 ± 1.60 μM), it was

(22) Zelcer, N.; Saeki, T.; Bot, I.; Kuil, A.; Borst, P. Transport of bile acids in multidrug-resistance-protein 3-overexpressing cells co-transfected with the ileal Na^+-dependent bile-acid transporter. *Biochem. J.* **2003**, *369*, 23−30.

(23) Zeng, H.; Chen, Z.-S.; Belinsky, M. G.; Rea, A. P.; Kruh, G. D. Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. *Cancer Res.* **2001**, *61*, 7225−7232.

(24) Akita, H.; Suzuki, H.; Hirohashi, T.; Takikawa, H.; Sugiyama, Y. Transport activity of human MRP3 expressed in Sf9 cells: Comparative studies with rat MRP3. *Pharm. Res.* **2002**, *19*, 34−41.

(25) Hirohashi, T.; Suzuki, H.; Sugiyama, Y. Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). *J. Biol. Chem.* **1999**, *274*, 15181−15185.

Table 1. The Values of PS1 and PS3 for Taurocholate in the Rat Jejunum, Ileum, and Colon[a]

segments	C_m[b] (nmol/mL)	C_{sac}[c] (nmol/(g of tissue))	dC_s[d] (nmol mL^{-1} (20 min)$^{-1}$)	PS1 (mL min^{-1} (g of tissue)$^{-1}$)	PS3 (mL min^{-1} (g of tissue)$^{-1}$)[e]
ileum	0.785 ± 0.112	5.80 ± 0.91	0.800 ± 0.153	0.0508 ± 0.0049	0.00671 ± 0.00051
jejunum	1.08 ± 0.11	1.21 ± 0.43	0.105 ± 0.044	0.00491 ± 0.00197*	0.00443 ± 0.00111*
colon	1.04 ± 0.09	0.550 ± 0.048	0.0614 ± 0.0082	0.00296 ± 0.00025*	0.00559 ± 0.00064

[a] Data represent the mean ± SD of four experiments. (*) Statistically significant difference compared with the PS value in the ileum ($P < 0.01$). [b] The concentration of TC in the mucosal side at 60 min. [c] The concentration of TC in the sac at 60 min. [d]Appearance rate. [e] Milliliters of water space per gram of tissue.

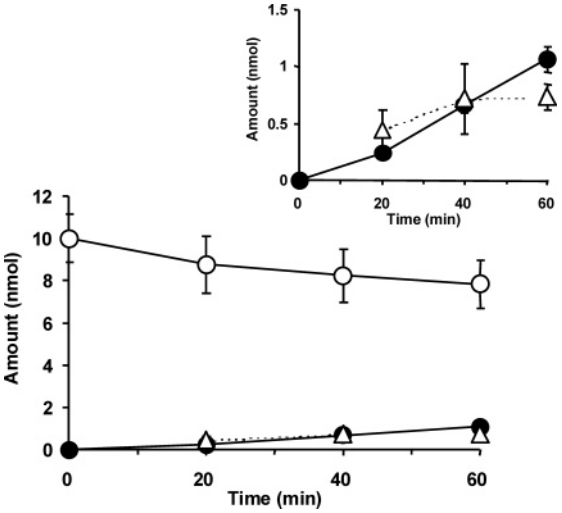

Figure 1. Transport of [^{3}H]taurocholate (1 μM) in rat everted ileal sacs. The data (nmol) represent the amount of taurocholate on the mucosal side (volume: 10 mL, open circle), the amount of taurocholate on the serosal side (volume: 0.5 mL, closed circle), and the amount of taurocholate in the sac (open triangle). The amount of TC in the sac and serosal side is also shown in a separate graph with a magnified *Y*-axis scale (from 0 to 1.5 nmol). Each point and vertical bar represents the mean ± SD of 3−11 experiments.

considered that the mucosal-to-serosal transport of TC reached a steady state at 60 min.

As shown in Table 1, the PS1 value for TC in the ileum was 0.0508 ± 0.0049 mL min^{-1} (g of tissue)$^{-1}$, and that in the jejunum and colon was approximately 10% and 6% of that in the ileum, respectively. Na^+-dependent transport of TC in the ileum was observed. When extracellular Na^+ was depleted, mucosal-to-serosal transport of TC in the ileum was markedly reduced, and the PS1 value (0.0047 mL min^{-1} (g of tissue)$^{-1}$) was much lower than that in the presence of Na^+ (data not shown). The PS3 value for TC in the ileum was 0.0067 ± 0.0005 mL min^{-1} (g of tissue)$^{-1}$ and similar to that in the colon. The PS3 value in the jejunum was much lower than that in the ileum.

Effects of Mrp3 Inhibitors on TC Transport. We examined the effect of Mrp3 inhibitors (MK571, TLC-S, and CDNB) on TC (1 μM) transport in the ileum. DNP-SG is formed intracellularly by incubating sacs in the presence of CDNB, and then Mrp3 function is inhibited. The inhibitory effect was observed on both PS1 and PS3 for TC in a concentration-dependent manner (Table 2). Although PS1 decreased to 9−19% of the control in the presence of 1 mM of inhibitors, PS3 decreased only to approximately 65% of the control.

Table 2. Effects of Mrp3 Inhibitors on Taurocholate Transport in Rat Everted Ileal Sacs[a]

Inhibitor	concn (μ M)	PS1 (% of control)	PS3 (% of control)
control		100	100
MK571	2	112 ± 24	95.6 ± 7.3
	5	52.5 ± 5.1	80.9 ± 6.9
	20	83.4 ± 13.6	77.3 ± 10.1
	50	39.0 ± 2.5	84.6 ± 7.6
	150	28.4 ± 3.8	73.6 ± 10.6
	1000	18.8 ± 5.6	65.4 ± 9.0
TLC-S	20	107 ± 14	115 ± 20
	50	61.4 ± 8.2	92.1 ± 9.5
	100	30.8 ± 7.2	88.5 ± 3.4
	300	16.4 ± 5.3	78.8 ± 10.3
	1000	9.38 ± 1.12	57.8 ± 6.5
CDNB	50	45.1 ± 11.1	111 ± 21
(DNP-SG)	100	62.4 ± 17.4	100 ± 15
	300	10.2 ± 1.8	77.3 ± 22.5
	1000	9.30 ± 2.92	68.7 ± 13.9

[a] Transport is expressed as a percentage of the control (absence of inhibitor) transport. The data represent the mean ± SD of four experiments.

Table 3. Inhibitory Effects of TLC-S and MK571 (1 mM) on Taurocholate (1 μM) Transport in Rat Everted Sacs[a]

	% of control			
	PS1		PS3	
segments	MK571	TLC-S	MK571	TLC-S
control	100	100	100	100
ileum	15.0 ± 3.1*	12.3 ± 4.2*	69.4 ± 4.5*	53.5 ± 6.8*
jejunum	112 ± 12	107 ± 18	116 ± 21	103 ± 9
colon	110 ± 20	127 ± 22	99.1 ± 14.9	127 ± 9

[a] The data represent the mean ± SD of four experiments. Transport is expressed as a percentage of the control (absence of inhibitor) transport. (*) Statistically significant difference compared with the PS value of the control ($P < 0.01$).

In addition to the inhibition study in the ileum, we studied the inhibitory effects of MK571 and TLC-S on TC transport in the jejunum and colon (Table 3). Neither PS1 nor PS3 was inhibited by these agents in either the jejunum or colon.

Concentration Dependence of TC Transport in the Ileum. Figure 2 shows the concentration dependence of TC transport in the rat everted ileal sac. PS1 values decreased gradually on increasing the TC concentration, suggesting a

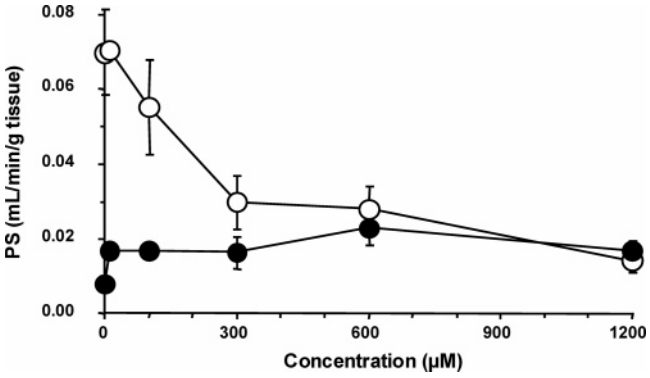

Figure 2. Concentration dependence of [^{3}H]taurocholate transport in rat everted ileal sacs. The results (mL min^{-1} (g of tissue)$^{-1}$) represent the PS1 value for taurocholate (open circle) and the PS3 value for taurocholate (closed circle). Each point and vertical bar represents the mean ± SD of three experiments.

saturable influx process. Nonlinear-least-squares analysis indicated a K_m of 299 ± 119 μM, a V_{max} of 20.5 ± 8.4 nmol min^{-1} (g of tissue)$^{-1}$, and a clearance for the nonsaturable component (K_d) of 0.0023 ± 0.0017 mL min^{-1} (g of tissue)$^{-1}$. On the other hand, PS3 values changed over the range 10 μM up to 1200 μM TC and no saturable efflux process was observed.

Effect of Quinidine on [^{3}H]Digoxin Transport in Everted Sacs. To evaluate the function of P-gp in order to confirm if the function of transporters in the everted sac is maintained during the experiments, we examined the effect of 1.28 mM quinidine on the transport of 0.743 nM [^{3}H]-digoxin in rat everted sacs. Figure 3 shows the mucosal-to-serosal transport of [^{3}H]digoxin in everted sacs of rat jejunum, ileum, and colon in the absence or presence of quinidine. In all segments, quinidine enhanced the transport of [^{3}H]digoxin to the serosal side. The function of transporters may be maintained in all segments during the present study, because the transport of P-gp was observed in the jejunum, ileum, and colon.

Discussion

In the present study, we studied the contribution of Mrp3 to the intestinal absorption of taurocholate using the rat everted sac method. The PS1 value for TC in the ileum was much higher than that in the jejunum and colon (Table 1). This result was consisted with the previous findings showing that the major site of bile acid absorption was in the ileum.[26,27] When extracellular Na^+ was depleted, mucosal-to-serosal transport of TC in the ileum was markedly reduced, and the PS1 value (0.0047 mL min^{-1} (g of tissue)$^{-1}$) was much lower than that in the presence of Na^+. In the rat intestine, ASBT is extensively expressed in the ileum and localized to the apical membrane of ileal enterocytes.[12] Therefore, this result demonstrated that the PS1 for TC in the ileum is sodium-dependent and reflects the uptake process by ASBT. Since most of bile acids in the jejunum present as the conjugated form and bile acids have negatively charged ions due to relatively low pK_a values, bile acid absorption in the jejunum was originally considered to occur only by passive diffusion across the brush-border membrane.[28] However, the recent study using the jejunal perfusion technique indicated that the steady-state absorption of conjugated bile acids was saturable and showed competitive inhibition.[29] In addition, the uptake of conjugated bile acids by rat jejunal brush-border membrane vesicles was saturable, temperature-dependent, and inhibited by an organic anion, bromosulfophthalein.[30] These results indicate that facilitated transport is present in the jejunum. Organic anion transporting polypeptide 1a5 (Oatp1a5) may facilitate carrier-mediated bile acid transport in the proximal intestine.[31] Oatp1a5 is localized to the apical brush-border membrane of rat jejunal enterocytes, suggesting that the uptake of bile acids takes place in enterocytes. In this study, in the jejunum, the mucosal-to-serosal transport of TC was very low (Table 1), and no Na^+-dependent TC transport was observed (data not shown). Moreover, PS1 in the jejunum was not inhibited by TLC-S (Table 3). These results suggest that the ileum is the major site of bile acid reabsorption and that the uptake mechanism of bile acids in the jejunum might be via passive diffusion and were the same as the results in the past.[26,27,32] In the intestine, bile acids are reabsorbed through a combination of passive absorption in the jejunum and active transport in the ileum.

The concentration of inhibitors (MK571, TLC-S, and CDNB) was examined up to high concentration (1 mM), because the inhibitors seemed to be taken up in the enterocyte by passive diffusion although they inhibit the transport via Mrp3 at lower concentration.[14,22] In the preliminary study, the mucosal-to-serosal transport of TLC-S in the jejunum, ileum, and colon was observed. The concentration of inhibitors in the sac has reached the concentration that can inhibit Mrp3. MK571, TLC-S, and CDNB (DNP-SG) inhibited the uptake from the mucosal side to the sac (PS1)

(26) Weiner, I. M.; Lack, L. Absorption of bile salts from the small intestine in vivo. *Am. J. Physiol.* **1962**, *202*, 155−162.
(27) Kramer, W.; Girbig, F.; Gutjahr, U.; Kowalewski, S.; Jouvenal, K.; Muller, G.; Tripier, D.; Wess, G. Intestinal Bile Acid Absorption. *J. Biol. Chem.* **1993**, *268*, 18035−18046.
(28) Dietschy, J. M. Mechanisms for the intestinal absorption of bile acids. *J. Lipid Res.* **1968**, *9*, 297−309.
(29) Amelsburg, A.; Schteingart, C. D.; Ton-Nu, H. T.; Hofmann, A. F. Carrier-mediated jejunal absorption of conjugated bile acids in the guinea pig. *Gastroenterology* **1996**, *110*, 1098−1106.
(30) Amelsburg, A.; Jochims, C.; Richter, C. P.; Nitsche, R.; Folsch, U. Evidence for an anion exchange mechanism for uptake of conjugated bile acid from rat jejunum. *Am. J. Physiol.* **1999**, *276*, G737−G742.
(31) Walters, H. C.; Craddock, A. L.; Fusegawa, H.; Willingham, M. C.; Dawson, P. A. Expression, transport properties, and chromosomal location of organic anion transporter subtype 3. *Am. J. Physiol.* **2000**, *279*, G1188−1200.
(32) Dietschy, J. M.; Salomon, H. S.; Siperstein, M. D. Bile acid metabolism. I. Studies on the mechanisms of intestinal transport. *J. Clin. Invest.* **1966**, *45*, 832−846.

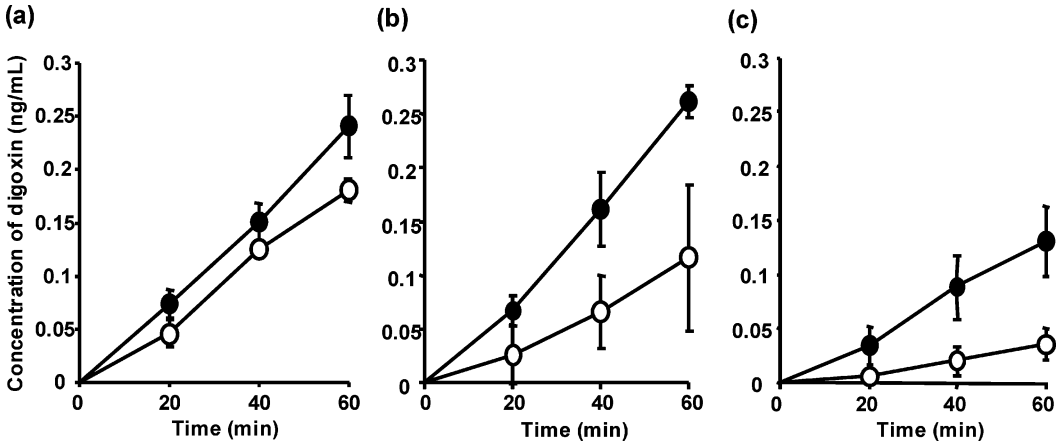

Figure 3. Effect of quinidine on the transport of [^{3}H]digoxin in rat everted sacs. The everted sacs of jejunum (a), ileum (b), and colon (c) were incubated in medium containing 0.743 nM [^{3}H]digoxin in the absence (open circle) and presence (closed circle) of 1.28 mM quinidine. Each point and vertical bar represents the mean ± SD of three experiments.

of TC in the ileum in a concentration-dependent manner (Table 2). The PS1 values decreased by approximately 90% of the control in the presence of 1 mM inhibitors and were similar to those (0.0047 mL min^{-1} (g of tissue)$^{-1}$) under Na^+-free conditions. This result demonstrates that the uptake of TC via ASBT in the ileum is completely inhibited by 1 mM inhibitors. Since chenodeoxycholate-3-sulfate (CDC-S) is an inhibitor for ASBT but not substrate,[13] TLC-S, which is a sulfate moiety of bile acid as well as CDC-S, may be an inhibitor for ASBT. In the case of MK571 and DNP-SG, both compounds may inhibit Na^+/K^+-ATPase because of the inhibitor for MRP family, which is an ATP-driven transporter. The reduction of Na^+ efflux from enterocytes via Na^+/K^+-ATPase may cause a disappearance of Na^+ concentration gradient between the inside and outside of the cell leading to the decrease of Na^+-dependent TC uptake via ASBT.

On the other hand, as far as the efflux from the sac to the serosal side (PS3) of TC was concerned, a concentration dependence of all inhibitors was also observed similar to that for PS1. However, the PS3 values were reduced to only approximately 65% of the control by 1 mM inhibitors (Table 2). Moreover, in the colon, where the expression level of Mrp3 protein was much higher than that in the ileum,[20] PS3 was not affected by 1 mM MK571 and TLC-S (Table 3). Recently, Zelcer et al. reported that human MRP3 transported bile acids with a low affinity and high capacity, and that human MRP3 was unlikely to be the main basolateral transporter of bile acids in the ileum.[22] Our conclusion was very similar to their result. Taken together, our results suggest that the contribution of Mrp3 to the efflux of bile acids from enterocytes to blood may be little. Mrp3 has the ability to transport bile acids in vitro, but its contribution in vivo may be very small.

In this study, we examined the concentration dependence of TC in rat everted ileal sacs to characterize the efflux mechanism of TC across the basolateral membrane. A concentration dependence in the mucosal-to-sac transport (PS1) of TC was observed (Figure 2), and the K_m value was approximately 300 μM. This value is comparable with the published K_m value of ASBT by everted sacs, isolated epithelial cells, and BBMVs from the rat ileum.[33,34] The sac-to-serosal transport (PS3) of TC did not show saturation at the concentrations examined. Additionally, the efflux of TC across the basolateral membrane in the ileum was inhibited by MK571, TLC-S, and CDNB (Tables 2 and 3). Therefore, an unidentified lower affinity and high capacity transporter(s) may be involved in the efflux of bile acids across the basolateral membrane of enterocytes. From a study using basolateral membrane vesicles in the small intestine, it was reported that the absorption of bile acids across the basolateral membrane could occur by anion exchange.[35] Moreover, a study using photoaffinity labeling has shown that the anion exchanger at the basolateral membrane is a 54 kDa protein.[36] An unknown low affinity transporter may be identified with an anion exchanger of 54 kDa protein. Recently, an organic solute transporter (OST) has been identified that is generated when two novel gene products are coexpressed, namely, human and mouse OSTα and OSTβ.[37] Ostα−Ostβ was able to transport taurocholate, estrone 3-sulfate, DHEAS, digoxin, and prostaglandin E2.[37,38] Uptake of [^{3}H]estrone 3-sulfate in human and mouse OSTα−

(33) Wilson, F. A.; Treanor, L. L. Characterization of the passive and active transport mechanisms for bile acid uptake into rat isolated intestinal epithelial cells. *Biochim. Biophys. Acta* **1975**, *406*, 280−293.

(34) Moyer, M. S.; Heubi, J. E.; Goodrich, A. L.; Balistreri, W. F.; Suchy, F. J. Ontogeny of bile acid transport in brush border membrane vesicles from rat ileum. *Gastroenterology* **1986**, *90*, 1188−1196.

(35) Weinberg, S. L.; Burckhard, G.; Wilson, F. A. Taurocholate transport by rat intestinal basolateral membrane vesicles. *J. Clin. Invest.* **1986**, *78*, 44−50.

(36) Lin, M. C.; Mullady, E.; Wilson, F. A. Timed photoaffinity labeling and characterization of bile acid binding and transport proteins in rat ileum. *Am. J. Physiol.* **1993**, *265*, G56−G62.

(37) Seward, D. J.; Koh, A. S.; Boyer, J. L.; Ballatori, N. Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OSTalpha-OSTbeta. *J. Biol. Chem.* **2003**, *278*, 27473−27482.

OSTβ expressing oocytes was sodium independent, saturable, and inhibited by a variety of bile salts, steroids, and other organic anions. The inhibitory effect of TLC-S on human and mouse OSTα−OSTβ-mediated transport of [^{3}H]estrone 3-sulfate was observed.[37] In addition, a possibility that MK571 and DNP-SG are inhibitors for OSTα−OSTβ may be very high because both compounds are organic anions such as estrone 3-sulfate, DHEAS, and so on. In the present study, PS3 in the ileum was inhibited by MK571, TLC-S, and DNP-SG (Tables 1 and 2). Thus, it is considered that PS3 of TC represents OSTα−OSTβ unlike Mrp3. The mRNA and protein levels of OSTα and OSTβ were most abundant in rat and mouse ileum,[38] and the mRNA expression level was also high in human small intestine.[38] By immunohistochemical analysis, Ostα−Ostβ protein localized specifically to the basolateral surface of the rodent and human ileal enterocytes[38] and that stable coexpression of Ostα−Ostβ in MDCK cells expressing Asbt increased the apical to basolateral transcellular transport of taurocholate.[38,39] In addition, the transcellular transport of taurine- and glycine-conjugate bile acids was observed in MDCK cells expressing ASBT, Ostα, and Ostβ.[38] These results indicate that Ostα−Ostβ heteromeric transporter may be an ileal basolateral bile acid transporter. Recently, Landrier et al. proposed the schematic model for the role of FXR in regulating bile acid transporter (apical influx and basolateral efflux) and intracellular transport.[40] In the future, further investigations are necessary to reveal the in vivo contribution of Ostα−Ostβ to the basolateral transport of bile acids out of the enterocyte in the ileum.

We considered the possibility of the loss of integrity of the Mrp3 function in the everted sacs as a reason for the small decrease of the PS3 value by inhibitors in the ileum and colon. Therefore, we evaluated the function of P-gp to confirm if the function of the transporters in the sac is maintained during the period of incubation. When 1.28 mM quinidine was added to the mucosal fluid, the mucosal-to-serosal transport of [^{3}H]digoxin was enhanced in all segments (Figure 3), as noted in a previous study.[41] This result supports the hypothesis that the function of transporters and ATP level in the jejunum, ileum, and colon sacs are maintained during the experiments. The enhancement of [^{3}H]digoxin transport by quinidine in the jejunum was lower than that in the ileum and colon. This is because the expression level of P-gp is as low as that in the upper part of the intestine.[42]

In conclusion, the results of the present study suggest that the contribution of MRP3 to the efflux of bile acids from enterocytes to blood may be little. Since an unknown transporter(s) having relatively low affinity for taurocholate may play a role in the enterohepatic circulation of bile salts by transporting from enterocytes into circulating blood, further investigations are required in order to elucidate the effect of this transport mechanism on the enterohepatic circulation of bile acids. As described above, the Ostα−Ostβ heteromeric transporter is certainly one of the good candidates for such a transporter.

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(38) Ballatori, N.; Christian, W. V.; Lee, J. Y.; Dawson, P. A.; Soroka, C. J.; Boyer, J. L.; Madejczyk, M. S.; Li, N. OSTalpha-OSTbeta: a major basolateral bile acid and steroid transporter in human intestinal, renal, and biliary epithelia.. *Hepatology* **2005**, *42*, 1270−1279.

(39) Dawson, P. A.; Hubbert, M.; Haywood, J.; Craddock, A. L.; Zerangue, N.; Christian, W. V.; Ballatori, N. The heteromeric organic solute transporter alpha-beta, Ostalpha-Ostbeta, is an ileal basolateral bile acid transporter. *J. Biol. Chem.* **2005**, *280*, 6960−6968.

(40) Landrier, J. F.; Eloranta, J. J.; Vavricka, S. R.; Kullak-Ublick, G. A. The nuclear receptor for bile acids, FXR, transactivates human organic solute transporter-alpha and -beta genes. *Am. J. Physiol.* **2006**, *290*, G476−G485.

(41) Su, S. F.; Huang, J. D. Inhibition of the intestinal digoxin absorption and exsorption by quinidine. *Drug Metab. Dispos.* **1996**, *24*, 142−147.

(42) Brady, J. M.; Cherrington, N. J.; Hartley, D. P.; Buist, S. C.; Li, N.; Klaassen, C. D. Tissue distribution and chemical induction of multiple drug resistance genes in rats. *Drug Metab. Dispos.* **2002**, *30*, 838−844.