Transport of fluorescent chenodeoxycholic acid via the human organic anion transporters OATP1B1 and OATP1B3

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Abstract This study sought to clarify the contributions of organic anion-transporting polypeptide (OATP) 1B1 and 1B3 to the liver uptake of chenodeoxycholic acid (CDCA). We synthesized a fluorescent version of CDCA, chenodeoxychilyl-(Nε-NBD)-lysine (CDCA-NBD), to characterize transporter-mediated uptake. CDCA-NBD is efficiently transported by OATP1B1 and OATP1B3 with high affinities. The Michaelis-Menten constants for CDCA-NBD uptake by OATP1B1 and OATP1B3 were 1.45 ± 0.39 µM and 0.54 ± 0.09 µM, respectively. By confocal laser scanning microscopy, CDCA-NBD, which is taken up by OATP1B1 and OATP1B3, was observed to localize to the cytosol. We also examined the transport of newly synthesized fluorescent bile acids. NBD-labeled bile acids, including cholic acid, deoxycholic acid, lithocholic acid, and ursodeoxycholic acid, were all transported by OATP1B1 and OATP1B3. CDCA-NBD exhibited the highest rate of transport of the five NBD-labeled bile acids examined in OATP1B1- and OATP1B3-expressing cells.■ Our results suggest that OATP1B1 and OATP1B3 play important roles in CDCA uptake into the liver. Fluorescent bile acids are useful tools to characterize the uptake properties of membrane transporters.—Yamaguchi, H., M. Okada, S. Akitaya, H. Ohara, T. Mikkaichi, H. Ishikawa, M. Sato, M. Matsuura, T. Saga, M. Unno, T. Abe, N. Mano, T. Hishinuma, and J. Goto. **Transport of fluorescent chenodeoxycholic acid via the human organic anion transporters OATP1B1 and OATP1B3.** *J. Lipid Res.* 2006. **47:** 1196–1202.

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INTRODUCTION

Organic anion-transporting polypeptides (OATPs) are sodium-independent organic anion transporters found in a variety of tissues, including liver, kidney, intestine, and brain. OATPs contribute to the transport of bile acids, thyroid hormones, steroid conjugates, anionic oligopeptides, eicosanoids, various drugs, and other xenobiotic compounds across membranes (1–3). OATP1B1 (LST-1/OATP2) and OATP1B3 (LST-2/OATP8) are members of the liver-specific subfamily of OATPs, which are localized to the basolateral membrane of hepatocytes (4–8). Although OATP1B3 shares 80% amino acid identity with OATP1B1, the substrate specificity differs slightly between these two transporters. OATP1B1 transports 2-D-penicillamine-5-D-penicillamine enkephalin, whereas OATP1B3 does not (9). The bile acids cholic acid (CA) and glycocholate are substrates of OATP1B1 but not OATP1B3 (10). In contrast, OATP1B3 transports the intestinal peptide cholecystokinin 8, the opioide peptide deltophin II, and the cardiac glycosides digoxin and ouabine (11, 12). In addition to the liver, OATP1B3 is also expressed in solid digestive organ cancers (9). Thus, the development of a specific substrate of OATP1B3 may be useful in cancer treatment.

Bile acids are amphipathic steroidal molecules derived from cholesterol catabolism. Bile acids play essential roles in the absorption of fats and contribute to the regulation of cholesterol metabolism. They are excreted into the intestinal lumen via the bile duct, reabsorbed in the ileum, and returned to the liver, forming the enterohepatic circulation (13, 14). The transport of CA has been well characterized. The uptake of CA into the liver is mediated by Na^+/taurocholate-cotransporting polypeptide and an additional Na^+-independent system (OATP1B1) (10, 15). Hepatic uptake of chenodeoxycholic acid (CDCA) is reported to be primarily mediated by a Na^+-independent process (16). CDCA is the most potent natural activator of the nuclear farnesoid X receptor (FXR) (17, 18). FXR regulates the expression of a wide variety of genes involved in bile acid and cholesterol homeostasis (19, 20). To date, there has not been any report demonstrating that the OATP contributes to the hepatic uptake of CDCA.

The aim of this study was to clarify the contribution of the Na^+-independent transporters OATP1B1 and OATP1B3 to the liver uptake of CDCA. We have generated 7-nitrobenz-2-oxa-1,3-diazole (NBD)-labeled and radioisotope-labeled bile acids to characterize their transport by membrane proteins visually. It is important to note that the transport properties of natural products and our synthetic NBD-labeled compounds were the same. Our results suggest that CDCA transport into hepatocytes is mediated by both OATP1B1 and OATP1B3. In addition, this work demonstrates that NBD-labeled bile acids are useful tools for the characterization of bile acid transport.

EXPERIMENTAL PROCEDURES

Materials

CDCA, CA, deoxycholic acid (DCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), and Nε-carbobenzoxy-L-lysine (Nε-CBZ-L-lysine) were purchased from Sigma (St. Louis, MO). NBD was obtained from Dojindo Laboratories (Kumamoto, Japan). Anti-OATP2 antibody (mouse monoclonal IgM) was purchased from Affinity BioReagents, Inc. (Golden, CO). All other chemicals were commercially available and of the highest purity possible.

Synthesis of fluorescent bile salt derivatives

Five fluorescent bile salt derivatives were generated for this study with the fluorophore attached to the side chain: chenodeoxycholyl-(Nε-NBD)-lysine (CDCA-NBD), cholyl-(Nε-NBD)-lysine (CA-NBD), deoxycholyl-(Nε-NBD)-lysine (DCA-NBD), lithocholyl-(Nε-NBD)-lysine (LCA-NBD), and ursodeoxycholyl-(Nε-NBD)-lysine (UDCA-NBD).

First, the carboxyl group of CDCA was chemically modified with *p*-nitrophenol. The *p*-nitrophenol group was then substituted with Nε-CBZ-lysine; the carboxyl group was converted into methyl ester. Nε-CBZ-lysine was then deprotected by catalytic transfer hydrogenation. After reduction of the chenodeoxycholyl-Nε-CBZ-lysine methyl ester to a chenodeoxycholyl-Nε-lysine methyl ester, the amino group was labeled with NBD. The methyl ester was converted into a carboxyl group by alkaline hydrolysis. The other NBD-labeled bile acids were also synthesized in the same manner. The chemical structure of CDCA-NBD is shown in **Fig. 1**.
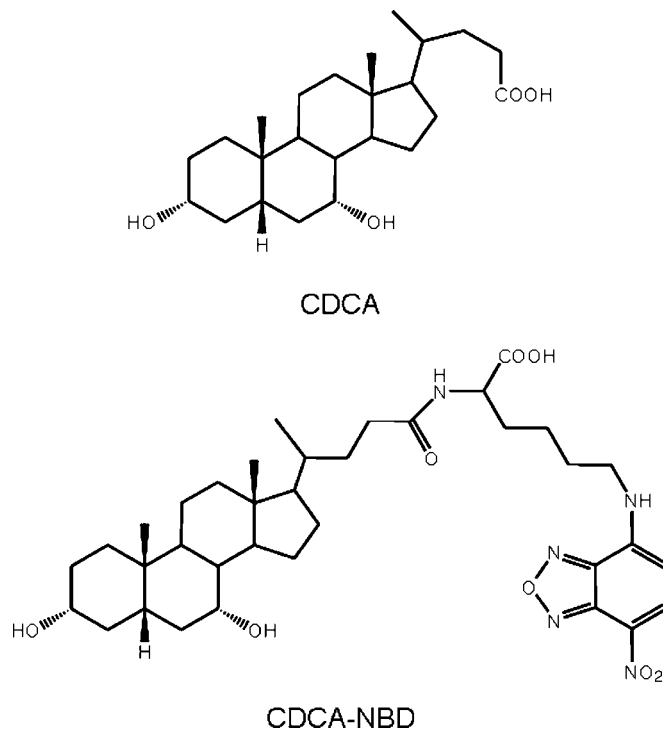

Fig. 1. Chemical structures of chenodeoxycholic acid (CDCA) and chenodeoxycholic acid 7-nitrobenz-2-oxa-1,3-diazole (CDCA-NBD).

Cell culture

HepG2 cells were acquired from the Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan). Cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, 60 μg/ml kanamycin, 2 mM L-glutamine, 1 mM pyruvic acid, and 0.2% glucose in a 5% CO_2 humidified atmosphere.

Expression of OATP1B1 and OATP1B3 in HepG2 cells

Recombinant adenoviruses were prepared by Dr. Michiaki Unno (unpublished data). OATP1B1 or OATP1B3 was exogenously expressed in HepG2 cells after infection with recombinant adenoviruses encoding OATP1B1 or OATP1B3 at multiplicities of infection of 300 or 10, respectively, 24 h before experimentation. The cells infected with recombinant adenoviruses encoding β-galactosidase were used as controls.

Immunoblot analysis

Total cell lysate proteins (50 μg/lane) were separated by SDS-PAGE (8%). OATP1B1 was detected by the monoclonal anti-OATP2 antibody diluted 1:1,000, and chemiluminescence detection was performed using alkaline phosphatase-labeled goat anti-mouse IgG+M (H+L) (Jackson Immunoresearch) diluted 1:5,000 as a second antibody. OATP1B3 was detected by the affinity-purified antibody against OATP1B3 (9) and detected using alkaline phosphatase-labeled goat anti-rabbit IgG (Biosource) diluted 1:3,000.

Transport study

Cellular uptake of fluorescent compounds was measured using monolayer cultures grown on 24-well plates. Cells expressing β-galactosidase were used as controls. To examine bile acid transport, culture dishes were washed three times and preincu-

bated in Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO$_3$, 4.83 mM KCl, 0.96 mM KH$_2$PO$_4$, 1.20 mM MgSO$_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM CaCl$_2$, adjusted to pH 7.4). Uptake was initiated by adding fluorescent ligand to the medium. At the designated times, uptake was terminated by aspiration of the uptake buffer and addition of ice-cold Krebs-Henseleit buffer containing 1% BSA. After washing three times in BSA-free ice-cold Krebs-Henseleit buffer, cells were lysed in lysis buffer (20 mM Tris and 0.4% Triton X-100, adjusted to pH 9.0). The fluorescence of the aliquots was measured using a microplate spectrofluorometer (SPECTRAmax GEMINI XS; Molecular Devices, Sunnyvale, CA). The protein content of the solubilized cells was determined with a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA).

Confocal imaging

Cells were grown on poly-L-lysine-coated cover slips in eight wells separated with flexiPERM (Vivascience AG, Hannover, Germany). Confocal fluorescent microscopic analysis used an LSM 5 PASCAL apparatus (Carl Zeiss, Overkochen, Germany) equipped with an inverted Axiovert 200M microscope and planneofluar 10× (numerical aperture, 0.3) and planneofluar 40× (numerical aperture, 1.3) oil-immersion objectives. The 488 nm line from an argon laser was used for excitation.

Statistical analysis

Data are expressed as means ± SEM. Where appropriate, the differences between groups were tested for significance using the unpaired Student's t-test. Statistical significance was indicated by $P < 0.05$.

RESULTS

Characterization of CDCA-NBD transport

We first examined CDCA-NBD uptake by OATP1B1- and OATP1B3-expressing HepG2 cells. We observed a time-dependent increase in CDCA-NBD uptake, determined from the fluorescence intensity seen after exposure to 0.5 μM CDCA-NBD in Krebs-Henseleit buffer (**Fig. 2**). OATP1B1- and OATP1B3-mediated CDCA-NBD uptake increased linearly up to 5 min. The protein expression of OATP1B1 and OATP1B3 in the cells infected with recombinant adenoviruses encoding OATP1B1 and OATP1B3 was verified by immunoblot analysis (Fig. 2, inset). OATP1B1 was detected in OATP1B1-expressing cells, and OATP1B3 was detected in OATP1B3-expressing cells. The β-galactosidase-expressing control cells did not show any OATP1B1 or OATP1B3 protein.

Kinetic parameters in OATP1B1- or OATP1B3-expressing HepG2 cells were determined after exposure to varying concentrations of CDCA-NBD. The apparent K_m values for OATP1B1- and OATP1B3-mediated CDCA-NBD uptake were 1.45 ± 0.39 μM (n = 4) and 0.54 ± 0.09 μM (n = 4), respectively (**Fig. 3**).

We next examined the inhibition of CDCA-NBD uptake by unlabeled CDCA. The estimated inhibition value (IC$_{50}$) of CDCA for OATP1B1-mediated CDCA-NBD uptake was 17 ± 4 μM, whereas that for OATP1B3-mediated CDCA-NBD uptake was 10 ± 2 μM (**Fig. 4**).
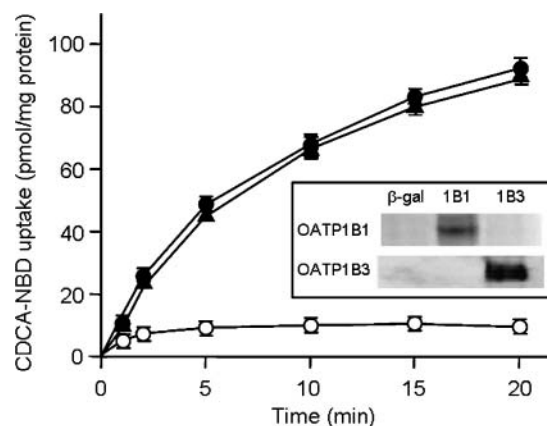

Fig. 2. Time course of CDCA-NBD uptake by organic anion-transporting polypeptide (OATP) 1B1- and 1B3-expressing HepG2 cells. HepG2 cells infected with recombinant adenoviruses encoding OATP1B1 (closed circles), OATP1B3 (closed triangles), or β-galactosidase (open circles) were incubated for the indicated times at 37°C with 0.5 μM CDCA-NBD. Each point represents the mean ± SEM of three determinations. The inset shows the immunoblot analysis of OATP1B1 and OATP1B3 in OATP1B1-expressing (1B1), OATP1B3-expressing (1B3), or β-galactosidase-expressing (β-gal) HepG2 cells.

Further inhibition studies were performed using various compounds (**Fig. 5**). Bromosulfophtalein (BSP), rifampicin, thyroxine, triiodothyronine (T$_3$), and estrone-3-sulfate (E3S) have been reported as substrates of OATP1B1 and OATP1B3 (4, 6, 9–11, 21, 22). Cyclosporin A (CsA) has been described as an inhibitor of both OATP1B1 and OATP1B3 (23–25). BSP, rifampicin, T$_3$, and CsA significantly inhibited CDCA-NBD uptake by OATP1B1 and OATP1B3. However, no significant inhibition was observed by thyroxine and E3S. Although transport of digoxin has been mediated by OATP1B3 (11) and pravastatin is a substrate for OATP1B1 (8), they had little effect on CDCA-NBD uptake. A typical substrate of organic anion transporter family members, p-aminohippuric acid, which is not transported by OATPs, had no significant effect on CDCA-NBD uptake by OATP1B1 and OATP1B3.

Visualization of CDCA-NBD uptake

To investigate the intracellular uptake and distribution of CDCA, we examined OATP1B1- and OATP1B3-expressing HepG2 cells by confocal microscopy after incubation for 20 min with 1 μM CDCA-NBD. Fluorescence of CDCA-NBD was observed in the cytosol of OATP1B1- and OATP1B3-expressing HepG2 cells, whereas only background fluorescence could be seen in β-galactosidase-expressing HepG2 cells (**Fig. 6**).

Transport of other NBD-labeled bile acids

To evaluate the correlation of transporter substrate specificities and the chemical structures of bile acids, we examined the uptake of NBD-labeled bile acids by OATP1B1- and OATP1B3-expressing HepG2 cells (**Fig. 7**). The uptake ratios, defined as the ratios of transporter-mediated uptake to control uptake, for CDCA-NBD,
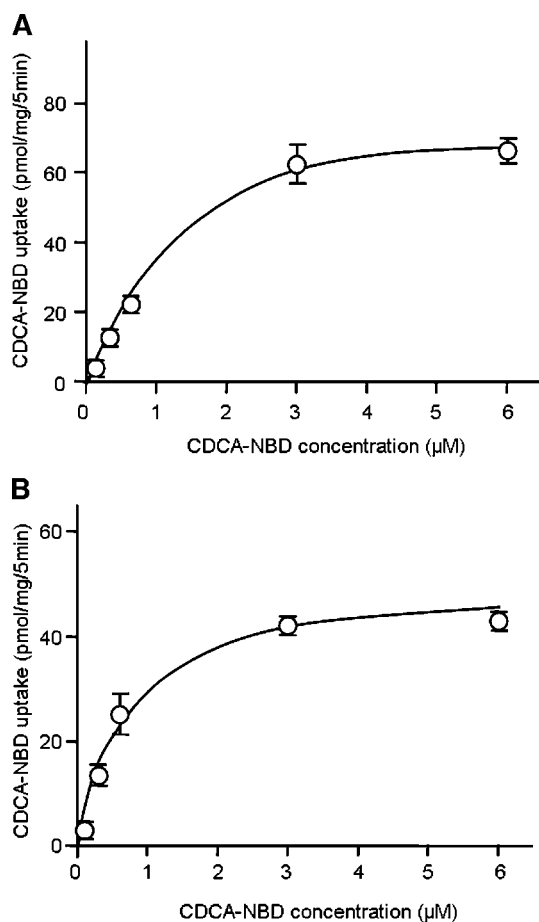
A

B

Fig. 3. Concentration dependence of CDCA-NBD uptake by OATP1B1-expressing (A) and OATP1B3-expressing (B) HepG2 cells. Cells were incubated at 37°C for 5 min with varying concentrations of CDCA-NBD. OATP1B1- and OATP1B3-mediated uptake was calculated after subtraction of the nonspecific uptake by β-galactosidase-infected cells. Each point represents the mean ± SEM of three determinations from a typical experiment.

CA-NBD, DCA-NBD, LCA-NBD, and UDCA-NBD were 12, 3, 12, 7, and 3 for OATP1B1 and 12, 6, 6, 4, and 5 for OATP1B3, respectively. Therefore, these transporters have different substrate specificities that depend on bile acid chemical structures.

DISCUSSION

Enterohepatic circulation of bile salts is essential for bile acid and lipid homeostasis (26). A number of bile acids are taken up by both Na^+-dependent and Na^+-independent processes in the liver, whereas others, such as CDCA, are mediated by Na^+-independent processes alone (16). In this study, we examined the contribution of the Na^+-independent hepatic transporters OATP1B1 and OATP1B3 to the liver uptake of CDCA using fluorescent bile acids.

CDCA-NBD was efficiently transported by both OATP1B1 and OATP1B3; both transporters exhibited high affinities for this bile acid [K_m values of 1.45 ± 0.39 μM and 0.54 ± 0.09 μM for OATP1B1 and OATP1B3 (means ± SEM),
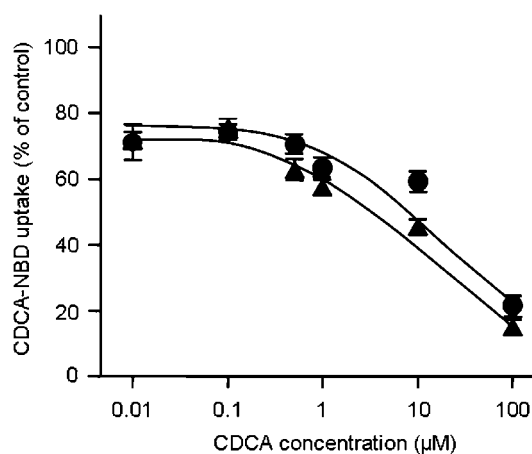

Fig. 4. Inhibition by unlabeled CDCA of CDCA-NBD uptake via OATP1B1 and OATP1B3. Cells were incubated with 0.5 μM CDCA-NBD at 37°C for 5 min in the presence of varying CDCA concentrations. OATP1B1-mediated (closed circles) and OATP1B3-mediated (closed triangles) uptake was calculated after subtraction of nonspecific uptake by β-galactosidase-infected cells. Each point represents the mean ± SEM of three determinations from a typical experiment.

respectively] (Figs. 2, 3). These values are comparable to those in a previous report using rat hepatocytes (16), suggesting that these transporters may play an important role in CDCA uptake by hepatocytes. Abe et al. (9) reported that the expression levels of OATP1B1 were higher than those of OATP1B3, leading to the hypothesis that OATP1B1 is the major transport system for CDCA in the liver. We also examined the concentration dependence of CA-NBD uptake in OATP1B1- and OATP1B3-expressing HepG2 cells. The apparent K_m values for CA-NBD uptake by OATP1B1 and OATP1B3 were 0.67 and 16.2 μM, respectively. The K_m value for [³H]CA uptake by OATP1B1 was 11.4 μM (10), and that for OATP1B3 was 85.3 μM. These results indicate that the conjugation of NBD results in a higher affinity of this molecule for the transporters. CA has a higher affinity for OATP1B1 than for OATP1B3, whereas CDCA exhibits a higher affinity for OATP1B3. It is likely that the apparent IC_{50} values of CDCA for CDCA-NBD uptake via OATP1B1 and OATP1B3 are greater than the apparent K_m values (Fig. 4).

It has been reported that the substrate specificity differs slightly between OATP1B1 and OATP1B3 (9–12). To further characterize this difference, we examined the inhibition effects of various compounds on CDCA-NBD uptake via OATP1B1 and OATP1B3 (Fig. 5). BSP, rifampicin, T_3, and CsA significantly inhibited the transport of CDCA-NBD by OATP1B1 and OATP1B3. BSP inhibited OATP1B1-mediated transport more strongly than OATP1B3-mediated transport, whereas OATP1B3 was inhibited more potently than OATP1B1 by rifampicin and CsA. BSP is known to show higher affinity for OATP1B1 than for OATP1B3 (10), and rifampicin exhibits higher affinity for OATP1B3 than for OATP1B1 (27). Our results are consistent with these reports. However, T_3 and E3S, which are substrates for OATP1B1 and OATP1B3 (6, 9, 11, 22),
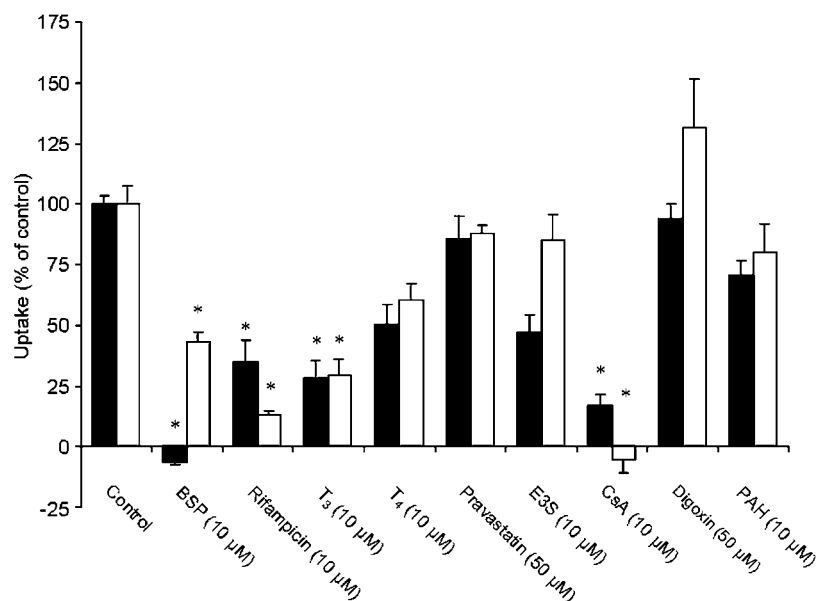
Fig. 5. Inhibition effects of various compounds on CDCA-NBD uptake via OATP1B1 and OATP1B3. The cells were incubated at 37°C for 5 min with 0.5 μM CDCA-NBD in the absence or presence of various compounds. OATP1B1-mediated (closed columns) and OATP1B3-mediated (open columns) uptake was calculated after subtracting nonspecific uptake by β-galactosidase-expressing cells. Data are expressed as percentage of the control value. Each column represents the mean ± SEM of three to six determinations. * $P < 0.05$, significantly different from control. BSP, bromosulfophtalein; T_3, triiodothyronine; T_4, thyroxine; E3S, estrone-3-sulfate; CsA, cyclosporin A; PAH, p-aminohippuric acid.

affected CDCA-NBD uptake only slightly. Moreover, we observed no inhibition effects by pravastatin and digoxin. Hsiang et al. (8) reported that pravastatin is a substrate for OATP1B1, but the inhibition effect by taurocholate on pravastatin transport is relatively weak. Similar phenomena were reported in OATP4C1, oatp2, and OAT-K1/K2 (28, 29). As discussed in those reports, these effects could be attributable to at least two substrate binding sites in these proteins.

We also examined the uptake of a variety of NBD-labeled bile acids by OATP1B1 and OATP1B3 (Fig. 7). All of the fluorescent bile acids tested were transported by these transporters in a similar pattern. OATP1B1 and OATP1B3 have been reported to differ in their substrate specificities (9–12). Cui et al. (10) demonstrated that taurocholate and CA at concentrations of 5 μM were substrates for OATP1B1 but not OATP1B3. However, we observed no differences in substrate specificity in our system. Kullak-Ublick et al. (11) demonstrated that taurocholate and glycocholate were substrates for both OATP1B1 and OATP1B3. These discrepancies may be explained by differences in the expression levels of the transporters in each experimental condition.

To visualize the transport process directly, we used confocal imaging to detect the real-time localization of a substrate in cells. Examination of the uptake of CDCA-NBD into OATP1B1- and OATP1B3-expressing cells revealed the accumulation of CDCA-NBD in the cytoplasm
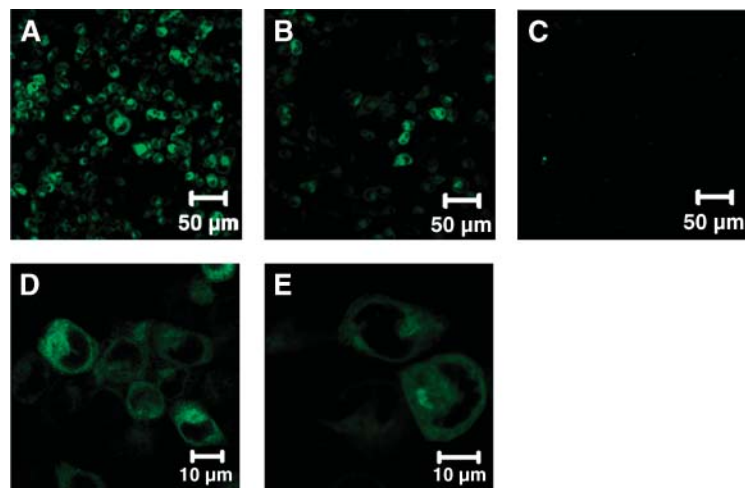

Fig. 6. Visualization of CDCA-NBD uptake by OATP1B1- and OATP1B3-expressing HepG2 cells. HepG2 cells infected with recombinant adenoviruses encoding OATP1B1 (A, D), OATP1B3 (B, E), or β-galactosidase (C) were incubated for 20 min at 37°C with 1 μM CDCA-NBD. The images were recorded using either 10× (A–C) or 40× (D, E) oil-immersion objectives.
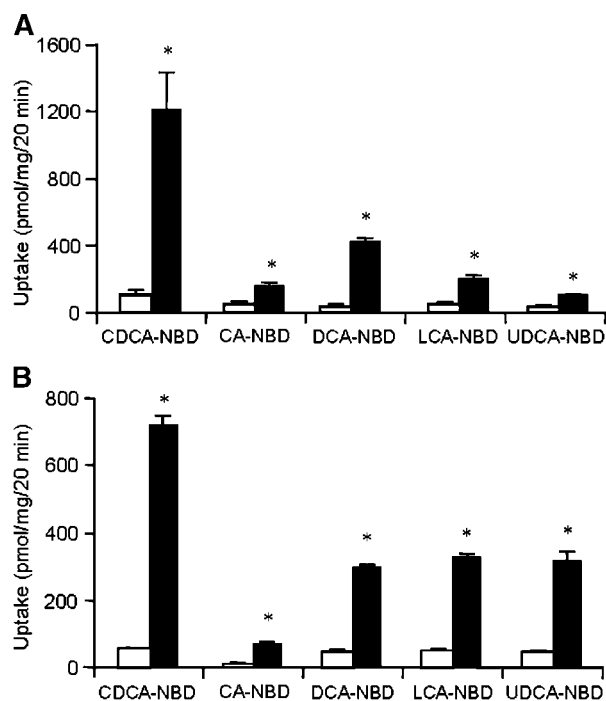
Fig. 7. Uptake of NBD-labeled bile acids by OATP1B1- and OATP1B3-expressing HepG2 cells. HepG2 cells infected with recombinant adenoviruses encoding OATP1B1 (A, closed columns), OATP1B3 (B, closed columns), or β-galactosidase (open columns) were incubated for 20 min at 37°C with 1 μM NBD-labeled bile acids. Each column represents the mean ± SEM of three determinations. * $P < 0.05$, significantly different from β-galactosidase-infected controls. CDCA-NBD, chenodeoxycholyl-(Nε-NBD)-lysine; CA-NBD, cholyl-(Nε-NBD)-lysine; DCA-NBD, deoxycholyl-(Nε-NBD)-lysine; LCA-NBD, lithocholyl-(Nε-NBD)-lysine; UDCA-NBD, ursodeoxycholyl-(Nε-NBD)-lysine.

(Fig. 6). Although CDCA acts as a ligand for the nuclear receptor FXR, we could not detect a CDCA-specific signal in the nucleus after internalization at a concentration of 1 μM.

Chemical modification of bile acids, however, may change the dynamics of the compound. Therefore, we selected the NBD fluorophore as a labeling compound because of its small size. We demonstrated that although NBD-labeled bile acids are taken up by the same system as the parental bile acids, the affinities for the transporters have changed. If this chemical modification used a more bulky fluorophore, such as fluorescein isothiocyanate, the molecules may behave as different compounds. To visualize the dynamics of small molecules more accurately, it is essential to develop a new fluorophore that does not affect the dynamics of the parental compound.

In conclusion, we examined the transport of fluorescent bile acids by OATP1B1- and OATP1B3-expressing cells. CDCA-NBD is efficiently transported by OATP1B1 and OATP1B3 with high affinities. Our results indicate that OATP1B1 and OATP1B3 play a critical role in CDCA uptake by the liver. In addition, fluorescent bile acids are useful tools to characterize the transport properties of membrane transporters.

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