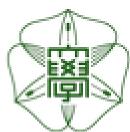

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Short Communication

Transport of Estrone 3-sulfate Mediated by Organic Anion Transporter OATP4C1: Estrone 3-sulfate binds to the Different Recognition Site for Digoxin in OATP4C1

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Summary: Human organic anion transporter OATP4C1 is a member of the OATP family predominantly expressed in the kidney, and contributes to the renal secretion of digoxin. However, little is known about the characteristics of OATP4C1-madiated transport. We examined the transport of estrone 3-sulfate, which is known as a substrate for other OATPs, by OATP4C1-expressing cells. Estrone 3-sulfate was efficiently transported by OATP4C1. The Michaelis-Menten constant for estrone 3-sulfate uptake by OATP4C1 was 26.6 ± 4.9 μM. Transport of estrone 3-sulfate was significantly inhibited by triiodothyronine, chenodeoxycholic acid, bromosulfophtalein, and cyclosporine, whereas known substrates of OATP4C1, digoxin and ouabain, did not change OATP4C1-mediated transport. We further examined the mutual inhibition study between estrone 3-sulfate and digoxin. Digoxin partially inhibited the estrone 3-sulfate transport, and estrone 3-sulfate did not significantly inhibit digoxin transport. The estimated IC_{50} value of digoxin for OATP4C1-mediated estrone 3-sulfate transport was 119 μM. This value is not comparable with the Michaelis-Menten constant for digoxin uptake by OATP4C1 (7.8 μM) reported by Mikkaichi *et al.*[1] In conclusion, we found that estrone 3-sulfate is a novel substrate for OATP4C1. Moreover, our results indicate that estrone 3-sulfate does not bind to the recognition site for digoxin in OATP4C1.

Keywords: OATP4C1; estrone 3-sulfate; digoxin; mutual inhibition; recognition site

Introduction

The kidney is one of the most important tissues for the excretion of endogenous and exogenous compounds from circulation into urine. Urinary excretion involves glomerular filtration and tubular secretion. Renal tubular secretion is mediated by various membrane transporters expressed in the proximal tubules including the organic anion transporting polypeptide (OATP) family, the organic anion transporter (OAT) family, organic cation transporter (OCT) family, multidrug and toxic compound extrusion (MATE) family, and ATP-binding cassette (ABC) transporters.

OATPs are sodium-independent organic anion transporters found in a variety of tissues, including the liver, kidney, intestine, and brain. OATPs contribute to the transport of bile acids, thyroid hormones, steroid conjugates, anionic oligopeptides, eicosanoids, various drugs,

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and other xenobiotic compounds across membranes.[2–5] OATP4C1 is the first member of the OATP family predominantly expressed in the kidney. OATP4C1 is localized in the basolateral membrane of the proximal tubule, and transports cardiac glycoside (digoxin and ouabain), thyroid hormones (triiodothyronine and thyroxine), cAMP, methotrexate, and sitagliptin.[1,6] Recently, Toyohara *et al.* reported that OATP4C1 decreased plasma levels of uremic toxins (guanidino succinate, asymmetric dimethylarginine, and the newly identified trans-aconitate), and suggest that OATP4C1 might have therapeutic potential for patients with chronic kidney disease.[7] However, characteristics of OATP4C1-madiated transport are not sufficiently understood. In the present study, we characterized the transport property of the novel OATP4C1 substrate estrone 3-sulfate.

Materials and Methods

Materials: [^{3}H]Estrone 3-sulfate (2.10 TBq/mmol), [^{3}H]digoxin (1.37 TBq/mmol), [^{3}H]estrone (3.70 TBq/mmol), and [^{3}H]testosterone (4.54 TBq/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{14}C]Chenodeoxycholic acid (1.85 GBq/mmol) and [^{14}C]glycocholic acid (2.03 GBq/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were commercially available and of the highest purity possible.

Cell culture and transfection studies: MDCKII cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in an atmosphere of 5% CO_2, 95% air at 37°C. Cells were transfected with a pcDNA3.1(+) plasmid vector (Invitrogen) encoding OATP4C1 using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. After three weeks of selection in G418 (0.5 mg/mL), we screened single colonies for OATP4C1 expression by transport studies. Cells transfected with the empty vector were used as controls.

Transport experiment: We measured the cellular uptake of radiolabeled compounds in monolayer cultures grown on 24-well plates. After washing once, cells were preincubated in Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM $CaCl_2$, pH 7.4). Uptake was initiated by adding radiolabeled compounds to the medium. At the indicated times, uptake was terminated by replacement of the uptake buffer with ice-cold Krebs-Henseleit buffer containing 1% BSA. After washing two times in ice-cold BSA-free Krebs-Henseleit buffer, the cells were lysed in 1 M NaOH. The radioactivity remaining in the cells was determined.

The protein content of the solubilized cells was determined using a Protein Assay kit (Bio-Rad Laboratories Inc., Hercules, CA).

Statistical analysis: Data are expressed as means ± S.E. When appropriate, differences between groups were tested for significance using the non-paired Student's *t*

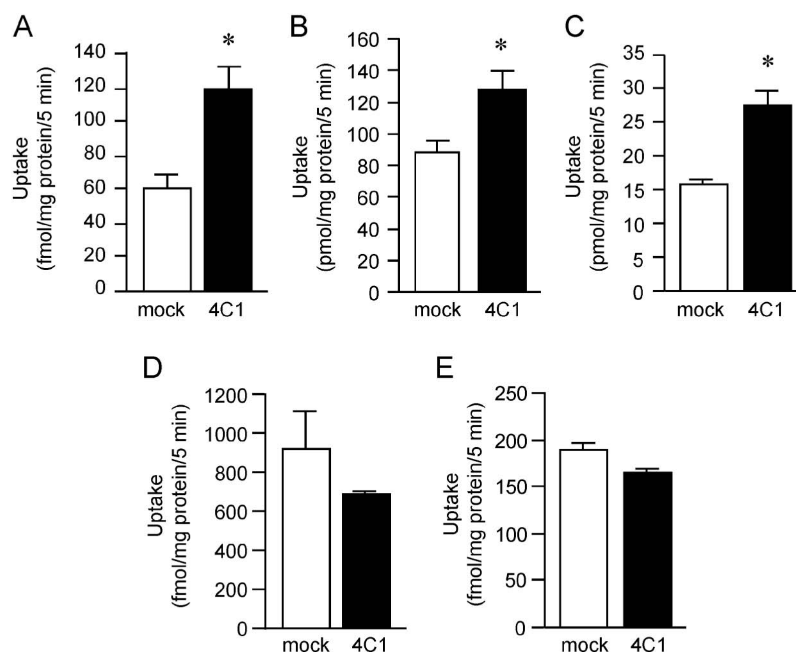

Fig. 1. Substrates screening of OATP4C1

OATP4C1/MDCKII and mock cells were incubated for 5 min at 37°C with (A) 17.5 nM [^{3}H]estrone 3-sulfate, (B) 2.0 μM [^{14}C]chenodeoxycholic acid, (C) 1.8 μM [^{14}C]glycocholic acid, (D) 10 nM [^{3}H]estrone, and (E) 9.5 nM [^{3}H]testosterone. Each column represents the mean ± S.E. of three independent determinations. *$P < 0.05$, significantly different from mock cells.

test. Statistical significance was indicated by *P* values less than 0.05.

Results and Discussion

First we established a stable clone overproducing OATP4C1. We examined the transport of steroidal compounds by OATP4C1-expressing cells. [^{3}H]Estrone 3-sulfate, and [^{14}C]chenodeoxycholic acid, and [^{14}C]glycocholic acid were significantly transported by OATP4C1 compared with the control (**Fig. 1**). Neither [^{3}H]estrone nor [^{3}H]testosterone were transported by OATP4C1. Negative charge might be one of the key factors for the recognition by OATP4C1 as a substrate. Estrone 3-sulfate is known as a substrate for other OATPs involved in OATP1B1, OATP1B3, and OATP2B1. We tried to characterized the transport of estrone 3-sulfate by OATP4C1 for comparison with data previously reported.

Transport amount of [^{3}H]estrone 3-sulfate by OATP4C1-exressing cells was significantly higher than that by mock cells (**Fig. 2A**). At 10 minutes, levels of cellular accumulation of [^{3}H]estrone 3-sulfate into OATP4C1-expressing cells (114 fmol/mg protein) were 6.6-fold greater than that into mock cells (17 fmol/mg protein). The apparent Michaelis-Menten constant (K_m) for OATP4C1-mediated estrone 3-sulfate uptake was 26.6 ± 4.9 μM (**Fig. 2B**). This value is comparable to the values reported other OATPs (OATP1A2; 59 μM, OATP1B1; 12.5 μM, OATP2B1; 12.5 μM, respectively).[8–10] OATP4C1 is the sole OATP expressed in the kidney, and estrone 3-sulfate is reported to be excreted into urine. Nozaki *et al.* demonstrated that uptake of estrone 3-sulfate by human kidney slices was saturable with K_m value of 9.2 to 11 μM.[11] They concluded that organic anion transporter (OAT) 3 contributed to the renal uptake of estrone 3-sulfate. On the other hand, our results indicate the possibility of partial involvement of OATP4C1 in renal estrone 3-sulfate uptake.

Next, we performed inhibition study. *p*-Aminohippuric acid (PAH) did not inhibit [^{3}H]estrone 3-sulfate uptake (**Fig. 2C**). Triiodothyronine (T3), chenodeoxycholic acid (CDCA), bromosulfophtalein (BSP), and cyclosporine A (CsA) significantly decreased estrone 3-sulfate transport, while known substrates of OATP4C1, digoxin and ouabain, did not change OATP4C1-mediated transport. Previously, we showed that transport of T3 was not inhibited by digoxin and vice versa.[1] This result suggests that OATP4C1 possesses multiple substrate recognition sites. Thus, our present data strongly support previous suggestion. We further examined the mutual inhibition assay between estrone 3-sulfate and digoxin. Digoxin partially inhibited [^{3}H]estrone 3-sulfate transport, and estrone 3-sulfate did not significantly inhibit [^{3}H]digoxin transport (**Fig. 3**). The estimated IC_{50} value of digoxin for OATP4C1-mediated [^{3}H]estrone 3-sulfate transport was 119 μM. Because the K_m value of OATP4C1-mediat-

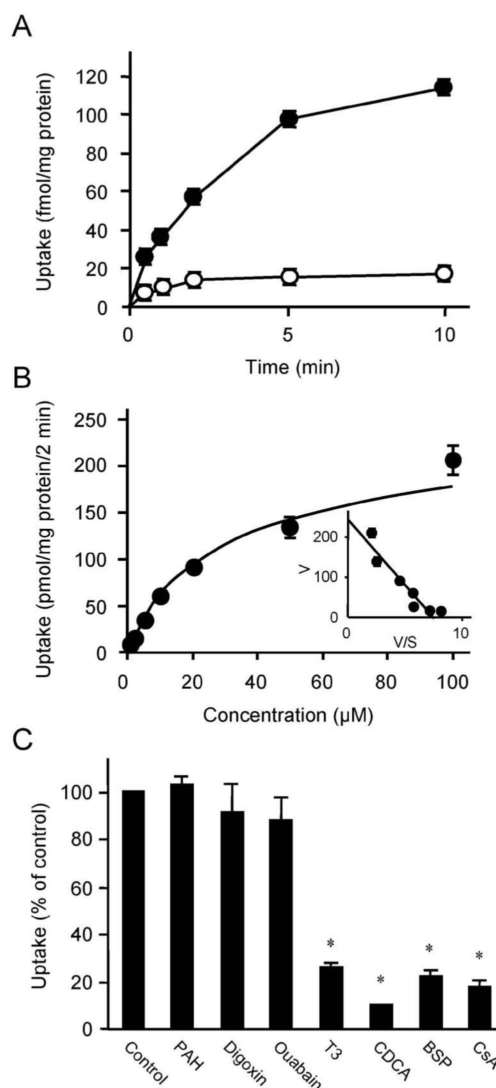

Fig. 2. Characteristics of estrone 3-sulfate transport by OATP4C1

(A) Time course of [^{3}H]estrone 3-sulfate uptake via OATP4C1. OATP4C1/MDCKII (closed circle) and mock cells (open circle) were incubated at 37°C with 8.7 nM [^{3}H]estrone 3-sulfate for designated time. Each point represents the mean ± S.E. of three independent determinations. (B) Kinetic analysis of estrone 3-sulfate uptake via OATP4C1. OATP4C1/MDCKII cells were incubated with varying concentrations of estrone 3-sulfate for 2 min. OATP4C1-mediated uptake was calculated after subtraction of nonspecific uptake by mock cells. Inset: Eadie-Hofstee plot. V, velocity; V/S, velocity per concentration of substrate. Each point represents the mean ± S.E. of three independent experiments. (C) Inhibition of various compounds to [^{3}H]estrone 3-sulfate uptake via OATP4C1. The OATP4C1-mediated 8.7 nM [^{3}H]estrone 3-sulfate uptake was determined in the presence of various inhibitors (PAH: 100 μM, digoxin: 10 μM, ouabain: 10 μM, T3: 10 μM, CDCA: 10 μM, BSP: 10 μM, CsA: 10 μM) for 2 min. OATP4C1-mediated uptake was calculated after subtraction of nonspecific uptake by mock cells. PAH: *p*-aminohippuric acid, T3: Triiodothyronine, CDCA: chenodeoxycholic acid, BSP: bromosulfophtalein, CsA: cyclosporin A. Each column represents the mean ± S.E. of three independent determinations. $^{*}P < 0.05$, significantly different from control.

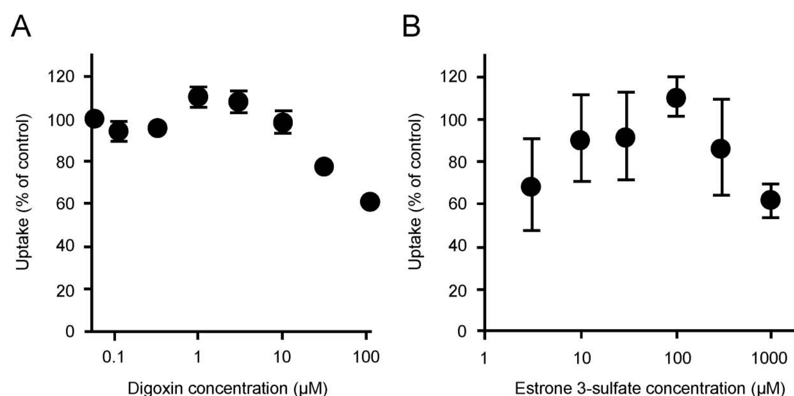

Fig. 3. Mutual inhibition between [^{3}H]estrone 3-sulfate and [^{3}H]digoxin uptake via OATP4C1
OATP4C1/MDCKII and mock cells were incubated with (A) 8.7 nM [^{3}H]estrone 3-sulfate or (B) 42.6 nM [^{3}H]digoxin for 2 min in the presence of varying concentrations of digoxin or estrone 3-sulfate. OATP4C1-mediated uptake was calculated after subtraction of nonspecific uptake by mock cells. Each point represents the mean ± S.E. of three independent determinations.

ed digoxin transport was reported as 7.8 μM, it is not likely that estrone 3-sulfate and digoxin share the same recognition site, although both compounds possess a steroid ring. Further studies are needed to elucidate the substrate recognition mechanism of OATP4C1.

In conclusion, we established OATP4C1 overexpressing-MDCKII cells, and it would be a useful experimental model for the analysis of the renal handling of compounds via OATP4C1. We found that estrone 3-sulfate, chenodeoxycholic acid, and glycocholic acid were novel substrates for OATP4C1. Moreover, our data suggest that estrone 3-sulfate does not bind to the recognition site for digoxin in the OATP4C1 molecule.

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