Human Renal Organic Anion Transporter 4 Operates as an Asymmetric Urate Transporter

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Human organic anion transporter 4 (hOAT4) is located at the apical membrane of proximal tubule cells and involved in renal secretion and reabsorption of endogenous substances as well as many drugs and xenobiotics. This study reevaluated the physiologic role, transport mode, and driving forces of hOAT4. 6-Carboxyfluorescein (6-CF) uptake into HEK293 cells that stably expressed hOAT4 was saturable, resulting in a K_m of 108 μM. 6-CF as well as [³H]estrone sulfate ([³H]ES) accumulation by HEK293-hOAT4 cells were abolished by ES, dehydroepiandrosterone sulfate, sulfinpyrazone, benzbromarone, and probenecid, whereas several OA, including p-aminohippurate (PAH), lactate, pyrazinoate, nicotinate, glutarate, and the diuretic hydrochlorothiazide (HCTZ) exhibited a slight or a NS inhibitory effect. PAH and glutarate are not taken up by HEK293-hOAT4 cells, but they trans-stimulated 6-CF and [³H]ES uptake, indicating an asymmetric interaction of hOAT4 with these substrates. In chloride-free medium, HEK293-hOAT4–mediated [³H]PAH efflux was almost abolished, whereas addition of ES restored it comparable to Ringer solution, consistent with a physiologic ES/PAH or PAH/Cl⁻ exchange mode of hOAT4. Moreover, an acidification of the uptake medium increased 6-CF as well as [³H]ES uptake, which was reduced by nigericin, suggesting that hOAT4 also can operate as an OA/OH⁻ exchanger. hOAT4 facilitates substantial uptake of [¹⁴C]urate, which was elevated 2.6-fold by intracellular HCTZ. Thus, hOAT4 is the long-postulated, low-affinity apical urate anion exchanger that facilitates HCTZ-associated hyperuricemia.

Active tubular transport processes determine secretion or reabsorption of a large variety of substances, a substantial fraction of which is represented by organic anions (OA), such as antibiotics, diuretics, antiviral drugs, cytostatics, and several endogenous metabolites. Proteins that are known to deal with these multiple compounds belong to the family of organic anion transporters (OAT) (1–3). They are expressed along the proximal tubule of the kidneys and in other bordering epithelia as such blood-brain barrier, choroid plexus, and placenta. OAT1 and OAT3 are located at the basolateral membrane of renal proximal tubule cells. Both transporters show overlapping substrate specificities and share the mode of transport and driving force, exchanging OA against α-ketoglutarate (4), representing the first step of renal OA secretion. The luminal exit of OA is not well characterized. Besides OAT2 (luminal localization in rodents [5]), the voltage-dependent OAT_v1 (related to NPT1 in human [6,7]), and the multidrug resistance–associated proteins 2 and 4 (8), other OAT members, namely hOAT4, OAT5, and URAT1, have been documented to be involved in apical OA release as well as in reabsorption processes (9–12).

hURAT1 was discovered recently as apical urate transporter, playing a pivotal role in urate homeostasis (11,13,14). Mutations in the hURAT1 gene diminish renal urate reabsorption, resulting in hypouricemia (11). hURAT1 operates as an exchanger, which takes up urate from the primary urine in exchange for intracellular nicotinate or lactate but not α-ketoglutarate, indicating different driving forces for OAT at the luminal and basolateral side of proximal tubule cells. Accordingly, the apically expressed rOAT5 exchanges organic anions against succinate, a dicarboxylate that is not accepted by OAT1 and OAT3 (10).

hOAT4 was identified as apical transporter in the proximal tubule cells (15), which is expressed only in higher primates, including human. It facilitates the reabsorption of conjugated steroids such as estrone sulfate (ES) or dehydroepiandrosterone sulfate with high affinity (12). Moreover, hOAT4 was detected in placenta, where it may be involved in the uptake of fetal-derived steroid sulfates (16). The driving force of hOAT4-mediated transport is controversial, and its physiologic role is still unknown. hOAT4 is closely related to hURAT1, and their genes are located as a tandem on chromosome 11q13 (17). Recently, Eraly et al. (17) postulated that OAT, which are genomically clustered in pairs, should have similar functional characteristics. This assumption holds for OAT1 and OAT3, but published functional data of hOAT4 and URAT1 so far do not support this hypothesis (11,12).

We re-investigated the transport mechanism and substrate specificity of hOAT4. We describe for the first time that hOAT4 transports urate. Urate/hydrochlorothiazide (HCTZ) or urate/OH^- exchange by hOAT4 may be involved in HCTZ-associated hyperuricemia (18). In addition, we provide evidence that hOAT4 operates in an asymmetric manner and interacts with inorganic anions such as chloride and hydroxyl ions. Furthermore, our data prove that hOAT4 facilitates one-way *p*-aminohippurate (PAH; and probably drug) excretion into the urine and that it may represent the formerly postulated low-affinity urate/OH^- exchanger in the human brush border membrane (19).

Materials and Methods
Reagents
Materials used included FBS, trypsin, and PBS (Invitrogen, Groningen, The Netherlands). All chemicals were of analytic grade and purchased from Sigma-Aldrich (Deisenhofen, Germany). 6-Carboxyfluorescein (6-CF) was purchased from Molecular Probes (Leiden, The Netherlands). [^{3}H]ES (ES, ammonium salt, [6,7-^{3}H(N)], 43.5 Ci/mmol) was purchased from New England Nuclear (Boston, MA). [^{14}C]uric acid (ARC-513, [8-^{14}C], 50 to 60 mCi/mmol) was obtained from Biotrend (Cologne, Germany). [^{3}H]PAH (3.25 Ci/mmol) was provided by Perkin Elmer Life Sciences (Boston, MA). [^{14}C]glutarate (GA), (glutaric acid, [1,5-^{14}C], 30.8 mCi/mmol) was obtained from ICN (Costa Mesa, CA).

cDNA Clone
hOAT4 (GeneBank accession no. AL514126) was obtained from Invitrogen cloned into pSPORT6 expression vector. The clone was sequence-verified using an automated sequencer (ABI, Weiterstadt, Germany). Sequence analysis was done by online services (*e.g.*, MAP, http://genome.cs.mtu.edu/map.html [20]).

Stable Transfection of hOAT4 into HEK293 Cells
The stably transfected human epithelial kidney cell line T-REx-HEK293-hOAT4 was established by using the Flp-In expression system (Invitrogen) as described previously (21). Cells were grown in flasks in DMEM (high glucose; Invitrogen) supplemented with 10% FCS, 1% penicillin/streptomycin, and blasticidine (5 mg/L; Sigma-Aldrich, Schnelldorf, Germany). Cultures were maintained in a humidified atmosphere that contained 5% CO_2 at 37°C.

In Vitro cRNA Synthesis
The pSPORT6-hOAT4 construct was used for cRNA synthesis. After NotI linearization of the plasmid, *in vitro* transcription was carried out using the SP6 mMessage mMachine kit (Ambion, Austin, TX) according to the manufacturer's protocol. The cRNA was resuspended in water and adjusted to a final concentration of 1 μg/μl.

Expression and Transport Measurements of hOAT4 in Xenopus laevis Oocytes
Oocytes from *Xenopus laevis* ovaries (Nasco, Fort Atkinson, WI) were prepared, injected, and cultivated as previously detailed (22). Transport experiments were carried out 3 d after injection at room temperature for the time periods indicated in oocyte Ringer's solution that contained (in mM) 90 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES/Tris (pH 7.6), unless otherwise stated in the figure legends. Oocytes then were washed in ice-cold uptake buffer and dissolved. For *trans*-stimulation experiments, *X. laevis* oocytes were injected with 1 mM HCTZ or oocyte Ringer as a control, and the uptake of 400 μM (100 μM [^{14}C]uric acid +

300 μM cold uric acid) uric acid was determined for 1 h at room temperature. Efflux of [^{3}H]PAH was measured for 30 min at room temperature after injection of 46 nl of [^{3}H]PAH using oocyte Ringer's solution (as a control), chloride-free oocyte Ringer's solution, and chloride-free oocyte Ringer's solution plus 500 μM ES as extracellular incubation solutions.

Transport Measurements in HEK293 Cells
HEK293-hOAT4 and HEK293-control cells were harvested and plated into 24-well plastic dishes (Sarstedt, Nümbrecht, Germany) at a density of 2×10^5 cells/well. Transport assays were performed 48 h after seeding in mammalian Ringer solution that contained (in mM) 130 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 20 HEPES, and 18 glucose at a pH of 7.4. The cells were washed twice with 500 μl of buffer and incubated in buffer that contained 10 nM [^{3}H]ES or 20 μM 6-CF for 2 min or 30 s, respectively. In some cases, the solutions also included additional test substances as described in the figure legends. For *trans*-stimulation experiments, cells were preloaded for 2 h with 10 mM test substances before uptake. The incubation was stopped and the extracellular tracer was removed by washing the monolayer twice with 750 μl of ice-cold PBS. Subsequently, cells were dissolved in 0.5 ml of 1 N NaOH, and for assessment of 6-CF accumulation, fluorescence was measured in a fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at 492/512 nm (excitation/emission). The kinetics of 6-CF uptake was described adequately by the following Michaelis-Menten equation fitted by nonlinear regression with SigmaPlot 2001 (SPSS Science, Chicago, IL):

$$J = \frac{J_{max}[S]}{K_m + [S]} \tag{1}$$

where J is the rate of transport of a labeled organic anion, J_{max} is the maximum rate of mediated transport, and K_m is the substrate concentration that resulted in half-maximal transport. The ^{3}H and ^{14}C contents of cells, oocytes, or supernatants were determined by liquid scintillation counting (Canberra-Packard, Dreieich, Germany).

Statistical Analyses
Statistical analysis and graphical layout were performed with Microsoft Excel (Microsoft, Unterschleißheim, Germany) and SigmaPlot 2001 (SPSS Science, Chicago, IL).

Results
Kinetics of hOAT4-Mediated ES and 6-CF Transport
Basic functional properties of hOAT4 were evaluated and confirmed by expression in *X. laevis* oocytes. hOAT4-expressing oocytes showed a 10-fold higher ES uptake (15.2 ± 1.5 fmol/oocyte × 30 min; $n = 3$; $P < 0.001$) than water-injected oocytes (1.5 ± 0.2 fmol/oocyte × 30 min; $n = 3$). A K_m determined for ES in hOAT4-expressing oocytes revealed a value of 1.1 ± 0.2 μM ($n = 3$), which is in good agreement with published results for hOAT4 (12). 6-CF uptake mediated by HEK293-hOAT4 cells was approximately linear up to 60 s (Figure 1A). Increasing concentrations of 6-CF exhibited saturation with a K_m of 108.3 ± 9.6 μM (Figure 1B).

cis-Inhibition Studies on hOAT4-Mediated 6-CF and ES Uptake
Several OA were used to test the inhibitory potency on hOAT4-mediated 6-CF uptake. In seven independent experiments with quadruplicate determinations, 6-CF uptake (60.8 ± 6.4 pmol/mg protein × 30 s) without inhibitor (control) into
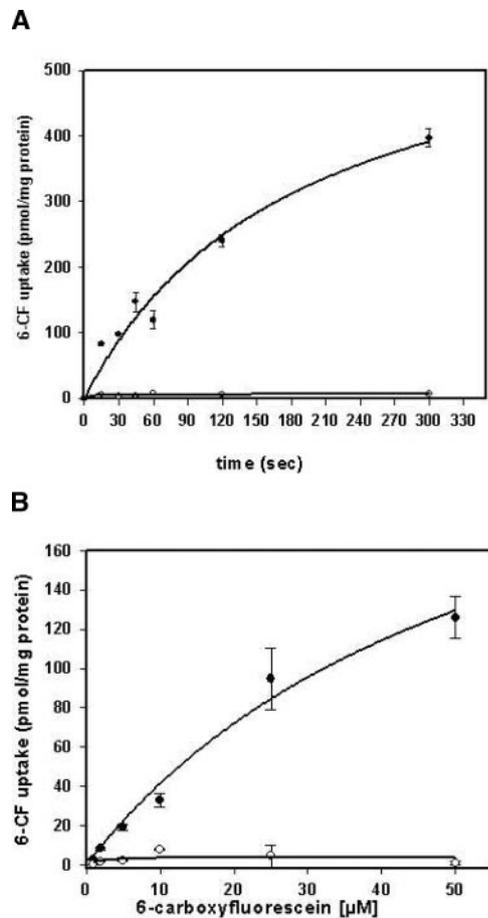
Figure 1. Time- and concentration-dependent uptake of 6-carboxyfluorescein (6-CF) (A) Time course of 20 μM 6-CF uptake into HEK293 cells that stably expressed human organic anion transporter 4 (hOAT4). (B) Concentration-dependent uptake of 6-CF into HEK293-hOAT4 cells measured 30 s at room temperature. ●, HEK293-hOAT4–expressing cells; ○, nontransfected cells. Data are means ± SEM of three independent experiments with three repeats each. The lines were based on a nonlinear regression algorithm.

HEK293-hOAT4 cells was more than 10-fold higher than into mock cells (4.1 ± 0.4 pmol/mg protein × 30 s; $P < 0.00001$). hOAT4-mediated 6-CF uptake was inhibited significantly by 500 μM of each ES to 5.5 ± 0.9% ($P < 0.0001$), dehydroepiandrosterone sulfate to 6.0 ± 1.0% ($P < 0.0001$), and probenecid to 9.8 ± 1.3% ($P < 0.0001$) compared with control, which was set to 100 ± 3.7%. A total of 500 μM urate in the uptake buffer significantly reduced hOAT4-mediated 6-CF uptake to 61.0 ± 7.8% ($P < 0.01$), suggesting that hOAT4 transports urate. Oxaloacetate, succinate, fumarate, α-ketoglutarate, and glutarate reduced 6-CF uptake slightly but significantly ($P < 0.05$), to 67.9 ± 3.7, 68.5 ± 2.9, 68.7 ± 5.9, 74.1 ± 7.4, and 74.5 ± 10.9%, respectively. All other tested OA, such as PAH, pyrazinoate, nicotinate, orotate, citrate, pyruvate, malate, or L-lactate did not show any alteration of 6-CF or ES uptake by hOAT4 (Figure 2). The uricosuric agents benzbromarone and sulfinpyrazone abolished HEK293-hOAT4–mediated ES uptake, whereas HCTZ showed a slight but significant reduction of ES uptake.
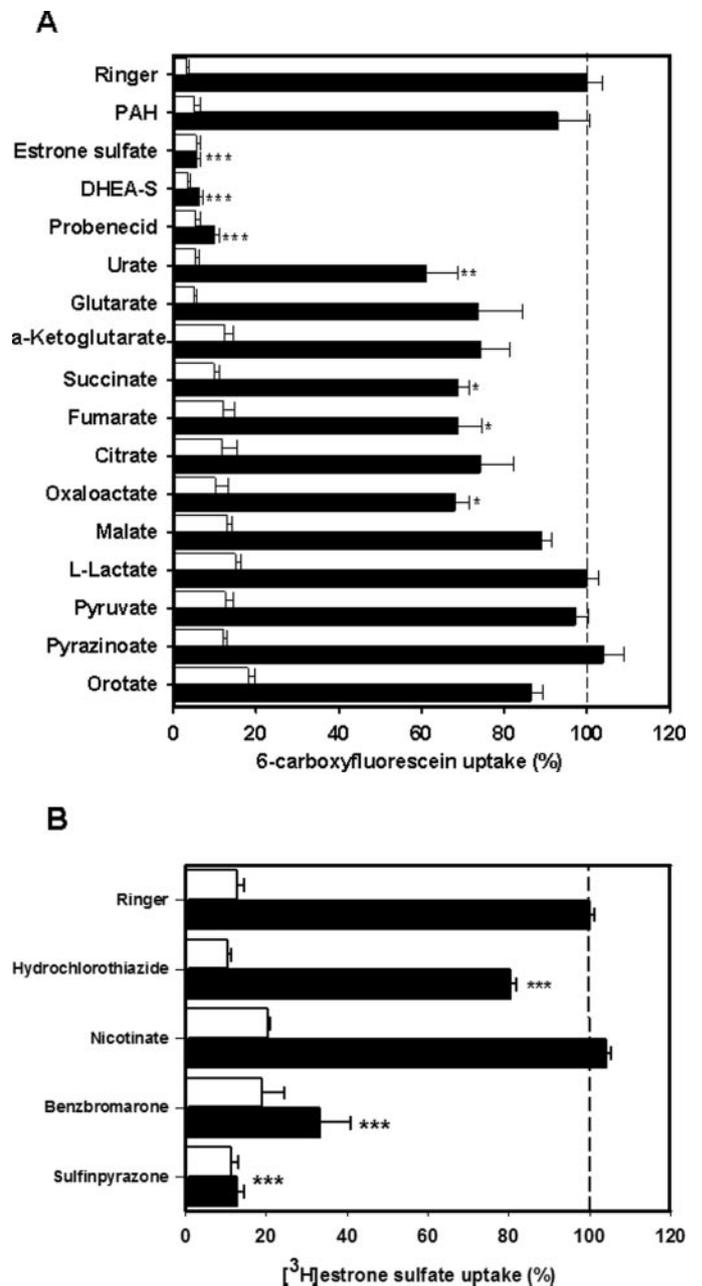

Figure 2. cis-Inhibition of hOAT4-mediated 6-CF or estrone sulfate (ES) uptake. (A) Inhibition of hOAT4-mediated 6-CF uptake was performed using 50 μM 6-CF with or without 500 μM of several organic anions. (B) Inhibition of hOAT4-mediated ES uptake was performed using 10 nM [³H]ES with or without 50 μM benzbromarone or 500 μM of several OA. □, mock cells; ■, hOAT4-expressing cells. All experiments were standardized by setting the control (without inhibitor) of each experiment to 100%. Data are means ± SEM of three independent experiments with four repeats each. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Uptake of Radioactively Labeled ES, PAH, Glutarate, and Urate by hOAT4

The *cis*-inhibitory effect of ES, urate, and glutarate on 6-CF uptake by hOAT4 indicates an interaction of these substances.

To prove their transport by hOAT4, we performed tracer experiments with [³H]PAH, [¹⁴C]glutarate, and [¹⁴C]urate. [³H]ES uptake served as a control.

HEK293-hOAT4 cells accumulated 10-fold more ES (185.5 ± 14.2 fmol/mg protein × 2 min; $n = 6$) than mock cells (12.03 ± 1.4 fmol/ mg protein × 2 min; $n = 6$; $P < 0.0001$; Figure 3A), but they showed virtually no uptake of [³H]PAH or [¹⁴C]glutarate compared with mock cells (Figure 3, B and C). These results suggest that hOAT4 does not mediate uptake of PAH or glutarate from the extra- to the intracellular compartment. HEK293-hOAT4 cells showed a higher urate uptake than mock cells (1.4-fold; $P < 0.01$; Figure 3D). hOAT4-mediated urate accumulation amounted to 7.7 ± 2.4 pmol/mg protein × 5 min in comparison with mock cells with an uptake of 4.5 ± 1.0 pmol/mg protein × 5 min (Figure 3D). Similarly, hOAT4-expressing oocytes showed a urate uptake of 1.9 ± 2.4 pmol/oocyte × 30 min as compared with water-injected oocytes with 0.9 ± 0.33 pmol/oocyte × 30 min ($n = 3$) using 100 μM [¹⁴C]urate (data not shown). A direct comparison of urate uptake using plasma-equivalent urate concentrations (100 μM [¹⁴C]uric acid + 300 μM cold uric acid) by hOAT4- and hURAT1-expressing oocytes revealed a substantial (almost 5×) accumulation of urate mediated by hOAT4 over mock, which was doubled in hURAT1-expressing oocytes (Figure 4A).

Figure 4. Urate uptake in *Xenopus laevis* oocytes. A total of 23 ng of cRNA encoding hURAT1 or hOAT4 were injected into each oocyte, and the same volume of water was injected for the mock oocytes. After 3 d, uptake experiments were carried out with 100 μM [¹⁴C]urate plus 300 μM unlabeled urate for 60 min. (A) Comparison of hURAT1- and hOAT4-mediated urate uptake (one representative experiment is shown, demonstrating highly significant urate accumulation [$P < 0.001$]). (B) Influence of 1 mM hydrochlorothiazide (HCTZ), injected into hOAT4-expressing oocytes and mock oocytes, on urate uptake. Data are means ± SEM of three independent experiments with six to 10 oocytes each. The uptakes were standardized by setting the uptake of hOAT4-expressing cells without HCTZ to 100%.
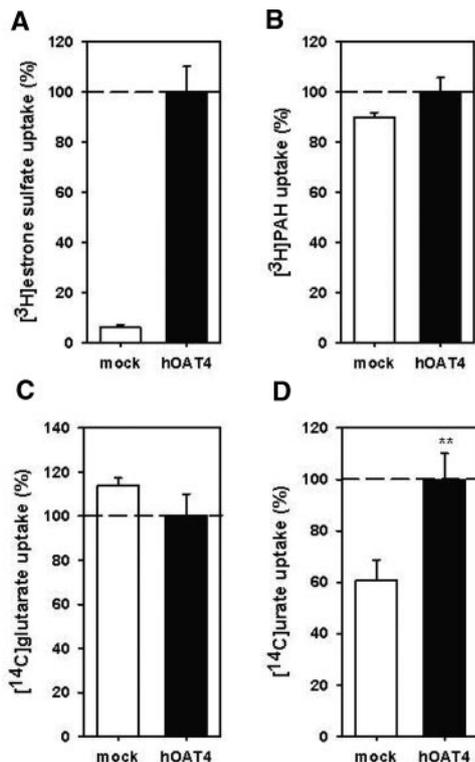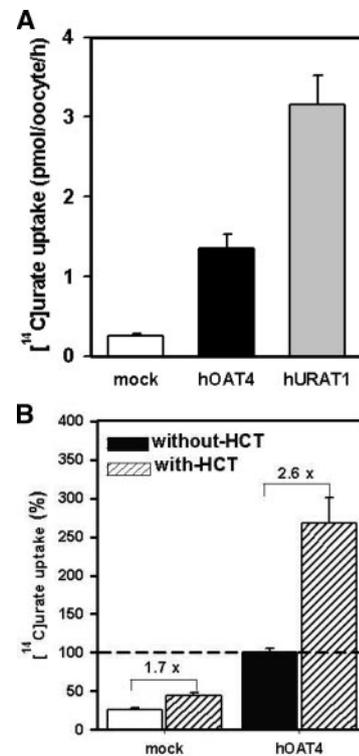

Figure 3. Uptake of radioactively labeled ES, *p*-aminohippurate (PAH), glutarate, and urate by hOAT4. hOAT4-expressing or mock HEK293 cells were incubated in 10 nM [³H]ES (A), 20 μM [³H]PAH (B), 10 μM [¹⁴C]glutarate (C), or 50 μM [¹⁴C]urate (D). □, mock cells; ■, hOAT4-expressing cells. All experiments were standardized by setting the uptake of hOAT4-expressing cells of each day to 100%. Data are means ± SEM of three independent experiments with four repeats each. **$P < 0.01$.

The commonly used diuretic HCTZ is known to cause a decrease in urate excretion and hyperuricemia in patients (23,24). Injecting 1 mM HCTZ into *X. laevis* oocytes resulted in a significant 2.6-fold increase of hOAT4-mediated uric acid uptake compared with Ringer-injected hOAT4-expressing oocytes. Therefore, efflux of HCTZ can drive urate uptake (Figure 4B).

Trans-*Stimulation of hOAT4 Transport Activity*

For further characterization of transport mode and driving forces of hOAT4, *trans*-stimulation experiments were carried out by preloading the cells for 2 h with test substrates. Subsequently, 6-CF as well as [³H]ES uptake were measured. Figure 5A demonstrates a 59.8 ± 9.6 and 51.3 ± 9.3% increased uptake of 6-CF into cells that were preloaded with PAH and glutarate, respectively. Urate, nicotinate, and pyrazinoate had no *trans*-effect, whereas L-lactate significantly inhibited 6-CF uptake to 62.8 ± 3.3% as compared with control hOAT4 cells (100 ± 8.7%; $P < 0.001$). Studies that were performed with ES revealed a
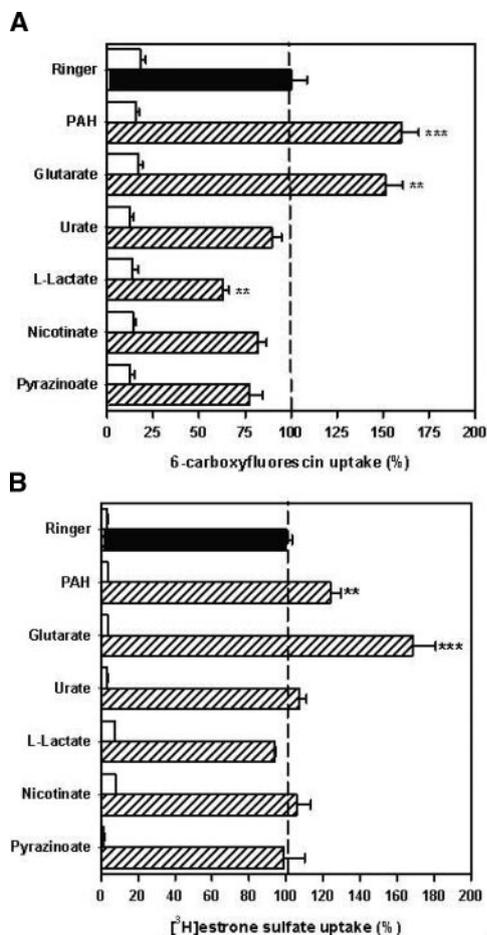
Figure 5. trans-Stimulation of HEK293-hOAT4–mediated 6-CF and ES uptake. The uptakes of 6-CF and ES were *trans*-stimulated by preloading the cells with 10 mM each of PAH, glutarate, L-lactate, nicotinate, and pyrazinoate and 1 mM urate for 2 to 3 h. □, mock cells; ■, cells that were not preloaded, as a control; ▨, preloaded hOAT4-expressing cells. After preloading, the uptakes of 50 μM 6-CF (A) or 10 nM [³H]ES (B) were measured. Data are means ± SEM of three independent experiments with four repeats each. All experiments were standardized by setting the uptake of hOAT4-expressing cells without inhibitor of each experiment to 100%. **$P < 0.01$; ***$P < 0.001$.

comparable *trans*-stimulation pattern as observed with 6-CF uptake (PAH 23.9 ± 3.6%; glutarate 68.4 ± 3.5%; Figure 5B). Thus, PAH and glutarate are not taken up by hOAT4 but *trans*-stimulate ES and 6-CF uptake.

Efflux of [³H]PAH Mediated by hOAT4-Injected X. laevis *Oocytes*

To confirm and explore further the mechanism of PAH release *via* hOAT4, we determined [³H]PAH efflux by hOAT4-expressing oocytes providing oocyte Ringer's solution (as a control) and chloride-free oocyte Ringer's solution with or without 500 μM ES. Oocyte Ringer's solution revealed an almost two-times higher [³H]PAH efflux than mock oocytes (Figure 6). This hOAT4-mediated efflux was abolished using chloride-free oocyte Ringer's solution but restored in the presence
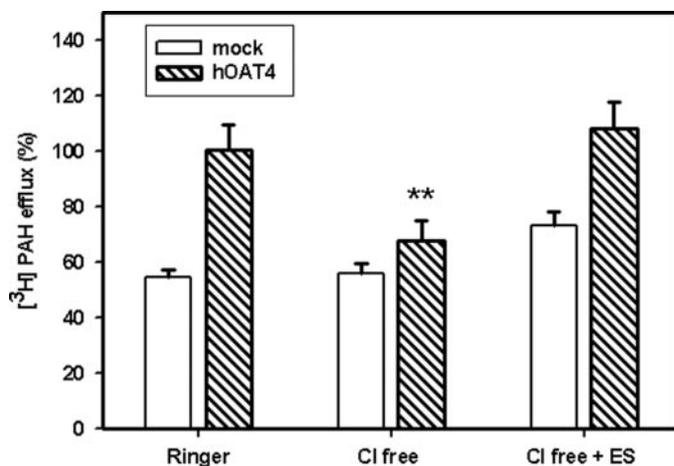

Figure 6. [³H]PAH efflux studies on *X. laevis* oocytes that were injected with water (mock) or hOAT4-cRNA. Three days after cRNA or water injection, 46 nl of a 200-μM [³H]PAH solution was injected into the oocytes, and efflux of [³H]PAH was measured, providing oocyte Ringer's solution, chloride-free oocyte Ringer's solution, and chloride-free oocyte Ringer's solution plus 500 μM ES. The efflux was calculated as percentage of ³H content recovered in the supernatant compared with the sum of total radioactivity recovered in the medium and that remaining in the oocytes at the conclusion of a 30-min efflux period at room temperature, where hOAT4-expressing oocytes in oocyte Ringer's solution were set to 100%, Data are means ± SEM or three independent experiments with five to seven oocytes each. **$P < 0.01$.

of ES, indicating hOAT4-mediated PAH/Cl⁻ or PAH/ES exchange.

pH-Dependent 6-CF and ES Uptake by HEK293-hOAT4 Cells

Next, we examined the pH dependence of hOAT4-mediated transport. Acidification of the uptake medium to pH 5.5 led to a 3.6-fold (360.8 ± 30.8%) increase of 6-CF uptake and an elevation of ES uptake to 156 ± 4.2% in comparison with hOAT4-mediated uptake at pH 7.4. Alkalization of the medium to pH 8.0 led to a significant ($P < 0.001$) inhibition of 6-CF and ES uptake to 49.8 ± 4.0 and 75.2 ± 3.3%, respectively (Figure 7). hOAT4 mediated [³H]ES uptake at pH 5.5 in the presence of nigericin was decreased significantly by 23.8 ± 1.6% ($P < 0.001$) in comparison with [³H]ES uptake in the absence of nigericin. These results document a marked pH dependence of hOAT4 and suggest a hOAT4-mediated exchange of organic anions against OH⁻ ions.

A possible functional coupling of hOAT4 to NHE3 was examined by sodium-dependent ES uptake. The replacement of sodium by choline should inhibit the activity of the Na⁺/H⁺ exchanger and consequently lower the in > out OH⁻ ion gradient. As shown in Figure 8A, we observed a significant reduction of hOAT4-mediated ES uptake to 71.5 ± 2.3%, when sodium was replaced by choline. An exchange of sodium by potassium resulted in the same reduction of ES uptake (68.4 ± 5.0%). To test more directly an influence of the Na⁺/H⁺ ex-
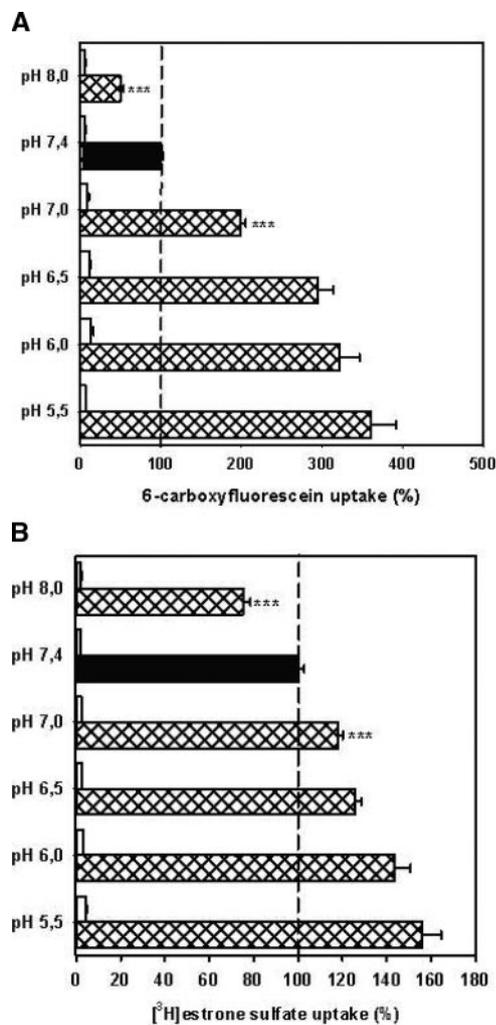
Figure 7. pH dependence of HEK293-hOAT4–mediated 6-CF and ES uptake. The uptake of 50 μM 6-CF (A) and 10 nM [^{3}H]ES (B) was examined at various pH conditions of the transport medium. $\square$, mock cells; $\blacksquare$, $\boxtimes$, hOAT4-expressing cells. The data points were normalized by setting the values of hOAT4-expressing cells at pH 7.4 as a control to 100%. Data are means $\pm$ SEM of three independent experiments with four repeats each. ***$P < 0.001$.

changer, we used the NHE inhibitor amiloride (25) and measured ES uptake by HEK293-hOAT4 cells in the absence or presence of 1 mM amiloride. As shown in Figure 8B, the presence of 1 mM amiloride significantly reduced ES uptake to 67.2 $\pm$ 5.6% ($P < 0.001$) compared with ES uptake in the absence of amiloride (100.0 $\pm$ 3.4%). This result is similar to the finding of Na$^+$ removal (Figure 8A), indicating a functional coupling of hOAT4 to the Na$^+$/H$^+$ exchanger.

Inorganic Ion-Dependent hOAT4-Mediated ES Uptake

hURAT1 as the closely related transporter to hOAT4 uses chloride as a driving force for uric acid uptake (11). To examine whether hOAT4 transport activity is chloride dependent, we replaced chloride in the transport buffer by gluconate and performed ES uptake. In chloride-free medium, ES uptake by
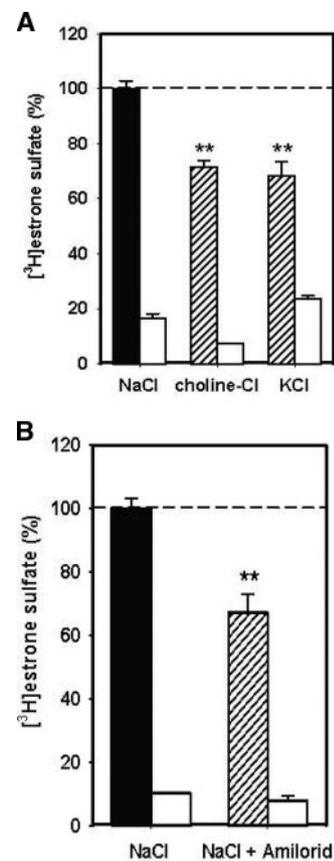

Figure 8. Sodium dependence of HEK293-hOAT4–mediated ES uptake. For examination of the sodium dependence of the [^{3}H]ES uptake by HEK293-hOAT4 cells, sodium was replaced in the uptake medium by choline or potassium (A), or the transport medium was supplemented with 1 mM amiloride (B). $\square$, mock cells; $\blacksquare$, $\boxtimes$, hOAT4-expressing cells. All experiments were standardized by setting the uptake of hOAT4-expressing cells without inhibitor or under normal mammalian Ringer conditions of each experiment to 100%. Data are means $\pm$ SEM of two independent experiments with four repeats each. **$P < 0.01$.

HEK293-hOAT4 cells was increased remarkably up to four-fold, consistent with hOAT4 facilitating OA/chloride exchange (Figure 9).

Discussion

The genes of hOAT4 and hURAT1 are speculated to be derived from one ancestor in the late phase of higher primate evolution (17). Although a physical coupling and regulation of both transporters via PDZK1 and NHERF1 proteins was reported recently (26), current functional characteristics of hOAT4 and hURAT1 illustrate different physiologic roles (11,12).

In earlier vesicle studies, Roch-Ramel et al. (19) postulated the existence of two different urate exchangers, one with high affinity for urate and aromatic molecules, such as nicotinate, pyrazinoate, and orotate, which now is represented by hURAT1, and a second, low-affinity urate transporter that exchanges urate against OA or chloride (27). Moreover, they
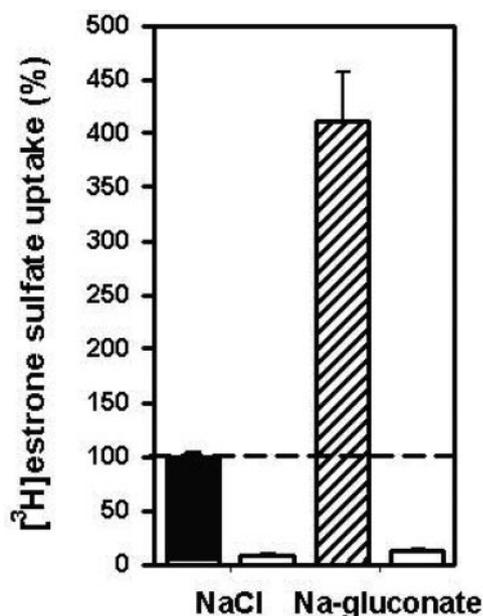
Figure 9. Chloride dependence of HEK293-hOAT4 mediated ES uptake. For evaluation of the chloride dependence of [^{3}H]ES uptake by HEK293-hOAT4 cells, chloride was replaced in the uptake medium by gluconate. □, mock cells; ■, ▨, hOAT4-expressing cells. All experiments were standardized by setting the uptake of hOAT4-expressing cells under normal mammalian Ringer conditions of each experiment to 100%. Data are means ± SEM of three independent experiments with four repeats each.

demonstrated that an OH$^-$ ion gradient provokes urate uptake into human brush border membrane vesicles (BBMV), a transport mode, which is not covered by hURAT1 (11). The molecular identity of this transporter so far is unknown.

To reevaluate the physiologic role and mode of action of hOAT4, we generated a HEK293-hOAT4 stably transfected cell line. 6-CF uptake into HEK293-hOAT4 cells was time and concentration dependent with a K_m of 108 μM, which qualifies hOAT4 as a low-affinity transporter for 6-CF compared with hOAT1 with a K_m value of 3.9 μM (28). *Cis*-inhibition studies revealed almost total inhibition by ES, dehydroepiandrosterone sulfate, and probenecid, consistent with the hallmarks of hOAT4 transport characteristics (12). In addition, urate significantly reduced 6-CF uptake, whereas all other tested compounds, including PAH, glutarate, and the hURAT1 substrates L-lactate, pyrazinoate, orotate, and nicotinate, exhibited only a slight or no significant alteration of 6-CF or ES uptake, indicating a clear difference in the specificities of hOAT4 and hURAT1.

It is interesting that urate preloading of the cells did not result in a significant increase of 6-CF or ES uptake, suggesting an affinity of hOAT4 for urate only from the extracellular side. Further evidence for an asymmetric mode of action of hOAT4 was provided by experiments with radioactively labeled PAH, glutarate, and urate. hOAT4 did not show any significant [^{3}H]PAH or [^{14}C]glutarate uptake, but intracellular PAH and

glutarate *trans*-stimulated ES uptake. These observations are supported further by efflux studies using [^{3}H]PAH. OAT4-mediated PAH efflux (and therefore secretion of OA into the urine) takes place in the presence of ES or a physiologically inwardly directed Cl$^-$ gradient. The lack of interaction of PAH with hOAT4 from the extracellular side was observed already by Ugele *et al.* (16) in hOAT4-expressing human mononuclear trophoblasts, documenting a cell system–independent property of hOAT4. Cha *et al.* (12) found no inhibition of hOAT4-mediated ES uptake by 0.5 mM PAH but a low [^{14}C]PAH uptake by hOAT4, which, however, was not statistically significant. Almost the same results were reported by Zhou *et al.* (29) regarding no PAH interaction with hOAT4. These observations are in good accordance with our data. However, recently, Ekaratanawong *et al.* (15) reported a two-fold PAH and glutarate uptake by hOAT4 expressed in mouse proximal tubule cells. They also found a *trans*-stimulation of [^{14}C]PAH uptake by intracellular glutarate and of [^{14}C]glutarate uptake by intracellular PAH. We repeated these experiments using the same concentrations of substrates on our HEK293-hOAT4 cells but did not observe any significant uptake of PAH or glutarate. The reason for these discrepancies is unknown.

[^{14}C]Urate uptake by HEK293-hOAT4 cells as well as hOAT4-expressing oocytes using plasma-equivalent (up to 400 μM) concentrations demonstrate for the first time that hOAT4 is a urate transporter that shares this important physiologic function with hURAT1. Urate is the end product of purine metabolism in higher primates, including human, and is excreted mainly *via* the kidneys. The fractional excretion of urate in these species is low (FE$_{urate}$ approximately 10%). The resulting high urate plasma levels are discussed to offer an advantage during hominoid evolution, because of the compensatory effect of urate for blood pressure regulation under low-salt-diet conditions (30–32). hURAT1 possesses an intermediate affinity for urate of $K_m = 371$ μM and is held to be responsible for 50% of renal urate reabsorption (11,33). This assumption is supported by the fact that mutations in the hURAT1 gene substantially elevate FE$_{urate}$ up to 60% and lead to the clinical manifestation of idiopathic hypouricemia (11,34). It is interesting that according to the recently postulated four-component model of urate handling in the kidneys (33,35) and that patients without any mutations in the hURAT1 gene show hypouricemia, other apical urate transporters were postulated (36), one of which might be hOAT4. We determined a concentration-dependent inhibition of hOAT4-mediated ES uptake (IC$_{50}$) by urate of 925 μM (data not shown), consistent with hOAT4 representing a low-affinity urate transporter compared with hURAT1 (11).

Urate is a potent endogenous free radical scavenger that protects the body and, most important, the brain against oxidative damage (37). Besides the kidneys, hOAT4 is expressed in placenta (12), where hURAT1 was not detected and where urate plays an important role, especially within the first trimester, a time when antioxidant enzyme activity and the levels of reducing agents such as glutathione or α- and γ-tocopherol are low (38). In this most vulnerable phase of fetal development, when oxidative stress has a substantial impact on normal organ

growing, hOAT4 may be responsible for the placental urate homeostasis and consequently for the protection of the fetus from oxidative damage.

The interaction of diuretics such as carbonic anhydrase inhibitors, loop diuretics, and thiazides with human OAT is well documented (18). hOAT1 and hOAT3 facilitate the basolateral entry of these drugs into the proximal tubule cell and therefore are responsible for the first step of active secretion of many of these compounds. hURAT1 was shown to interact with the loop diuretics bumetanide and furosemide (11), whereas hOAT4 was noted to interact preferentially with furosemide and ethacrynic acid but possesses virtually no affinity for carbonic anhydrase inhibitors and thiazides (18). It is widely known that patients who are treated with thiazides become hyperuricemic (24). We tested the inhibitory and *trans*-stimulatory effect of HCTZ on HEK293-hOAT4–mediated ES and urate uptake and found little interaction of hOAT4 with HCTZ from the extracellular (*cis*-) side. Importantly, we found that urate uptake *via* hOAT4 is elevated substantially (2.6-fold) by intracellular HCTZ. Consequently, HCTZ-induced hyperuricemia could be explained by the following mechanism: HCTZ enters the proximal tubule cell at the basolateral side through hOAT1 and is released into the urine *via* hOAT4, driving the reabsorption of urate.

An increasing body of evidence supports the notion that gout should not be considered as the only consequence of hyperuricemia. A higher risk for development of cardiovascular diseases and hypertension (30,31) as a result of urate-induced vascular smooth muscle cell proliferation and endothelial cell dysfunction (39,40) is currently discussed in the context of elevated urate plasma levels. Hence, urate is postulated to be a risk factor for the development of essential hypertension (41), and hOAT4, therefore, may play an important role in maintaining high urate levels.

Urate uptake into human renal BBMV was noted to be strongly stimulated by an outwardly directed Cl^- gradient (19). We found that ES uptake is enhanced with the removal of extracellular chloride, suggesting that hOAT4, like hURAT1 (11) and NPT1 (6), can use chloride as an exchange anion for ES as well as urate (data not shown) uptake. In view of the physiologically inwardly directed Cl^- gradient in the proximal tubule, our PAH efflux experiments clearly show that hOAT4 represents the apical one-way efflux avenue for PAH and consequently several drugs *via* PAH/Cl^-, PAH/ES, and possibly PAH/urate exchange.

In human, dog, and rat BBMV, urate uptake was stimulated by an outwardly directed OH^- gradient (42,43). hURAT1 was shown not to operate as a urate/OH^- exchanger (11). By acidification of the extracellular medium, in which 6-CF and ES are independent of the applied pH still in its anionic form, hOAT4-mediated uptake of both substrates was stimulated. This effect was reduced significantly in the presence of the proton-ionophore nigericin, supporting the idea of a 6-CF/OH^-, ES/OH^-, and urate/OH^- exchange as possible transport modes of hOAT4. A measurement of the intracellular pH values using a fluorescence tracer revealed a drop of the intracellular pH value in HEK293-hOAT4 cells in the presence of ES (data not shown),

confirming ES/OH^- exchange mediated by hOAT4. The intracellular pH of the proximal tubule cells and, thereby, the OH^- gradient is maintained by the luminal sodium-proton exchanger NHE3 (44). We observed a significant decrease of ES uptake in HEK293-hOAT4 cells after removal of sodium or by addition of the NHE3-specific inhibitor amiloride, suggesting a possible coupling of hOAT4 transport to the action of NHE3. The expression of NHE3 in HEK cells is well documented (45). Whereas hURAT1 is functionally connected to the recently identified sodium-monocarboxylate transporter 1 (46), which facilitates sodium-dependent reabsorption of L-lactate, pyrazinoate, and nicotinate, hOAT4 rather is coupled to the action of NHE3 and the sodium-dicarboxylate transporter 1, which is involved in the maintenance of the intracellular α-ketoglutarate (47). This again becomes important if patients are treated long term with thiazides. Recently, Nijenhuis *et al.* (48) provided evidence that the thiazide-induced hypocalciuria is due to an increased Na^+ reabsorption *via* NHE3 in the proximal tubule caused by the thiazide-induced contraction of the extracellular volume. Consequently, we speculate that another mechanism for a thiazide-induced hyperuricemia is given by the higher activity of NHE3, which drives urate/OH^- exchange specifically through hOAT4.

Conclusion

We provide evidence that hOAT4, like hURAT1, is a urate transporter that operates as an asymmetric exchanger. We document two possible ways for a reasonable involvement of hOAT4 in HCTZ-associated hyperuricemia. Moreover, the observed chloride dependence of hOAT4 is well reflected in a PAH/Cl^- exchange mode, facilitating the secretion of PAH and consequently a wide array of OA by using the physiologic chloride gradient into the urine. Finally, we conclude that hOAT4 represents the long-postulated apical low-affinity urate/OH^- exchanger.

Acknowledgments

Disclosures

None.

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