

Human organic anion transporters function as a high-capacity transporter for *p*-cresyl sulfate, a uremic toxin

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Received: 22 August 2013 / Accepted: 21 October 2013 / Published online: 2 November 2013
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Abstract

Background Recent clinical studies have shown that increased serum levels of *p*-cresyl sulfate (PCS), a uremic toxin, are associated with the progression of chronic kidney disease (CKD) and cardiovascular outcomes. Using rat renal cortical slices, we previously reported that the rat organic anion transporter (OAT) could play a key role in the renal tubular secretion of PCS. However, no information is currently available regarding the transport of PCS via human OAT (hOAT) isoforms, hOAT1 and hOAT3.

Methods Uptake experiments of PCS were performed using HEK293 cells, which stably express hOAT1 or hOAT3.

Results PCS was taken up by hOAT1/HEK293 and hOAT3/HEK293 cells in a time- and concentration-dependent manner. The apparent K_m for the hOAT1-mediated transport of PCS was 128 μ M, whereas in hOAT3/HEK293, saturation was not observed at the highest tested PCS concentration of 5 mM. Probenecid, an OAT inhibitor, inhibited PCS transport by hOAT1 and hOAT3. The uptake of *p*-aminohippurate by hOAT1 and estron-3-sulfate

by hOAT3 was decreased with increasing PCS concentration. The apparent 50 % inhibitory concentrations for PCS were 690 and 485 μ M for hOAT1 and hOAT3, respectively.

Conclusion PCS is a substrate for hOAT1 and hOAT3, and hOAT1 and hOAT3 appear to play a physiological role as a high-capacity PCS transporter. Since hOATs are expressed not only in the kidneys, but also in blood vessels and osteoblasts, etc., these findings are of great significance in terms of elucidating the renal clearance, tissue disposition of PCS and the mechanism of its toxicity in CKD.

Keywords Chronic kidney disease · *p*-Cresol sulfate · High-capacity · Organic anion transporter · Uremic toxin

Introduction

Chronic kidney disease (CKD) patients have higher risks of cardiovascular disease (CVD) and mortality than the

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normal population. The characteristics of the pathophysiology of the cardiorenal syndrome is bidirectional cross-talk; mediators/substances that are activated by a diseased state of one organ and play a role in worsening a dysfunction of another by exerting their biologically harmful effects, leading to the progression of the syndrome [1]. A recent study reported that uremic toxins may play an important role in the cardiorenal syndrome [1].

p-Cresyl sulfate (PCS) was identified as a protein-bound uremic toxin in 2005 [2, 3]. Recent clinical studies have shown that accumulation of PCS is related to the progression of CKD. Indeed, a build-up of PCS appears to be associated with reduced cardiovascular survival and all-cause survival of CKD patients [4–6]. As such, it is expected that PCS would be a good predictor for the prognosis of CKD patients. In addition, accumulated evidence from basic research indicates that PCS plays significant pathological roles in CKD, both in vitro and in vivo. In fact, we and other researchers demonstrated that PCS causes renal toxicity by activating the renin–angiotensin–aldosterone system [7] and NADPH oxidase [8], or suppressing Klotho expression [9]. In these cases, it is assumed that the intracellular accumulation of PCS via transporters could become a trigger for exerting its toxic effects.

Most low-molecular-weight uremic toxins are excreted into the urine. In this excretion process, protein-bound uremic toxins are minor contributors to glomerular filtration, but substantially contribute to tubular secretion. In fact, it was previously demonstrated that protein-bound uremic toxins such as indoxyl sulfate, 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF), indoleacetate and hippurate are excreted from the kidneys via organic anion transporters (OATs), especially rat Oat1/Oat3 and human OAT1/OAT3 (hOAT1/hOAT3), which are found in the renal tubular basolateral membrane [10, 11]. Since OATs appear to be expressed by the kidneys [12], blood vessels [13, 14] and osteoblasts, etc. [15, 16], it has been proposed that those uremic toxins could be transported via OATs and accumulated into various tissues, thereby exerting their biological activity. Recent findings indicating that the renal clearance of indoxyl sulfate was three times larger than that of PCS, may be due to the difference in their affinity for the renal transporter between the 2 toxins [17]. Thus, to understand the processes associated with the urinary excretion and toxicity of PCS in tissues in more detail, it is important to identify the transporter that contributes to the renal uptake of PCS in humans and to evaluate its properties, such as affinity and capacity. We recently reported that PCS, as well as indoxyl sulfate, could be a substrate for rat Oat and human OAT, based on experiments using rat renal cortical slices and human proximal tubular cells (HK-2) [18, 19]. In addition, Mutsaers et al. [20] also proposed

the involvement of OATP4C1 in the uptake of PCS in HK-2. However, no information is currently available regarding whether PCS is actually transported via the hOAT isoforms, hOAT1 and hOAT3.

The purpose of this study was to examine the interaction or transport of PCS via hOAT1 and hOAT3 using HEK293 cells, which stably express hOAT1 and hOAT3. The results would provide useful information related not only to the human renal basolateral uptake process, but also to the toxicity of PCS in human kidneys, blood vessels and osteoblasts, etc., which also expresses hOATs.

Materials and methods

Chemicals and materials

PCS was synthesized according to the method of Feigenbaum and Neuberg [21]. Identity and purity (>99 %) were confirmed by nuclear magnetic resonance. [³H]*p*-Aminohippurate (NET053: 1.0 mCi/mL) and [³H]estron-3-sulfate (NET203: 1.0 mCi/mL) were obtained from Perkin Elmer (Boston, MA, USA). Probenecid (P8761) was obtained from Sigma Chemical Co. (St Louis, MO, USA). All reagents from commercial sources were of the highest available grade.

Uptake experiment using HEK293 cells stably expressing hOAT1 and hOAT3

The open reading frame coding for hOAT1 and hOAT3 were amplified from human kidney total RNA by a reverse transcription-polymerase chain reaction method using polymerase chain reaction primers based on sequences in the DNA Data Bank of Japan/European Bioinformatics Institute/GenBank DNA databases under accession number NM_004790 (hOAT1) and NM_004254 (hOAT3), and then subcloned into a pCI-neo mammalian expression vector. The constructs were transfected into human embryonic kidney 293 (HEK293) cells by using FuGENE HD (Promega, WI, USA) according to the manufacturer's protocol. HEK293 cells stably expressing hOAT1 and hOAT3 were cloned by limiting dilution technique. The functional expressions of transporter genes were confirmed by estimating the uptake of [³H]*p*-aminohippurate ($K_m = 97.9 \mu\text{M}$; $V_{\text{max}} = 5560 \text{ pmol/mg protein/min}$) for hOAT1 [22] and [³H]estron-3-sulfate ($K_m = 16.8 \mu\text{M}$; $V_{\text{max}} = 289 \text{ pmol/mg protein/min}$) for hOAT3 [23].

HEK293 cells stably expressing hOAT1 or hOAT3 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum, 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin and 10 $\mu\text{g/mL}$ blasticidin at 37 °C in 5 % CO₂. For the transport assays, hOAT1/

HEK293 or hOAT3/HEK293 cells were seeded in collagen-coated (Toyobo, Tokyo, Japan) 12-well plates (4.0×10^5 cells/well) and incubated for 24 h. The medium was then changed to DMEM without fetal bovine serum and antibiotics, and incubated for 24 h. For the transport assay, the cells were preincubated for 5 min at 37 °C and 5 % CO₂ with 1.0 mL of HEPES-buffered Krebs–Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.20 mM MgSO₄, 1.2 mM KH₂PO₄, 6.3 mM HEPES, 11.0 mM glucose and 25.0 mM NaHCO₃, pH7.4). The cells were then further incubated at 37 °C with 1 mL of PCS solution. The surface of the cells was washed three times with 1 mL of ice-cold HEPES-buffered Krebs–Henseleit solution, and the cells were then lysed by treatment with 150 µL of 0.2 M NaOH at room temperature for 30 min. After neutralization by adding 10 µL of 3 M HCl, the cell extract was centrifuged (10,000g, 4 °C, 10 min). A 100 µL aliquot of the extract was transferred to a tube containing 200 µL of methanol. After deproteination, the concentration of PCS was measured using the HPLC system. The protein contents of the lysate were determined using a 25 µL aliquot of the extract. The uptake of PCS by the cells was calibrated against the protein content of the cells. For the inhibition experiments using probenecid, 1, 10 or 100 µM of probenecid was co-incubated with PCS.

For the uptake experiment using [³H]p-aminohippurate for hOAT1 and [³H]jestron-3-sulfate for hOAT3, the neutralized cell lysate was transferred to a scintillation counting vial. Scintillation cocktail (5 mL) (Hionic-Fluor; Perkin Elmer) was then added to each tube, and radioactivity was determined by liquid scintillation counting (Aloka LSC-5121).

HPLC conditions

The HPLC system consisted of an Agilent 1100 series intelligent pump and a fluorescence spectrophotometer. A Capcell Pak C-18 column (Shiseido, Tokyo, Japan) was used as the stationary phase. The mobile phase consisted of (A) 100 % methanol and (B) 50 mM ammonium formate using a gradient elution of 65–25 % B at 0–15 min, 25–65 % B at 15–20 min and the re-equilibration time for the gradient elution was 2 min. The flow rate was 1.0 mL/min. PCS was detected by means of a fluorescence monitor. The excitation/emission wavelengths were 214/306 nm [18].

Kinetic analysis

The kinetic parameters of PCS transport by hOAT1 and hOAT3 were calculated using a non-linear least squares regression analysis from the following Michaelis–Menten equation: $V = V_{\max} \times [S]/(K_m + [S])$, where V is the

transport rate (nmol/mg protein/min), V_{\max} is the maximum velocity by the saturable process, $[S]$ is the concentration of PCS (µM), K_m is the Michaelis–Menten constant (µM). The data were fitted using the Kaleida Graph package (Synergy Software, Reading, PA, USA).

The apparent 50 % inhibitory concentration IC₅₀ (inhibitory concentration at 50 %) for hOAT1 and hOAT3, derived from the dose–response curve, was plotted against the corresponding PCS concentration and calculated from the four-parameter logistic model using the following equation:

$$IC_{50} = 10^{f(\chi)}$$

$$f(\chi) = \log(A/B) * (50 - C) / (D - C) + \log(B)$$

Two plot points corresponding to an inhibitory concentration at 50 % were used. A is the concentration of PCS that exceeded the amount required to give a 50 % inhibitory effect. B is the concentration of PCS lower than the amount required to give a 50 % inhibitory effect. C is the inhibitory effect (%) at B. D is the inhibitory effect (%) at A.

Statistical analysis

Data were analyzed statistically by analysis of one-way factorial ANOVA with post hoc comparison by Tukey's multiple comparison tests to evaluate the significance of differences between groups. A p value of ≤ 0.05 was considered significant.

Results

Transport characteristics of PCS by hOAT1 and hOAT3

To examine the issue of whether hOAT1 and hOAT3 recognize PCS as a substrate, the amounts of PCS taken up by HEK293 cells, which stably express hOAT1 or hOAT3, were measured. As shown in Fig. 1, the uptake of PCS was increased markedly in the presence of hOAT1 and hOAT3, compared with mock-transfected cells, and this increase was time-dependent. The uptake of PCS by hOAT1 and hOAT3 was linear for periods of up to 3 min. These findings indicate that PCS is, in fact, a substrate for hOAT1 and hOAT3.

Figure 2 shows the concentration-dependent uptake of PCS by hOAT1 and hOAT3. hOAT1 and hOAT3 transported PCS in a dose-dependent manner. Saturation was observed in hOAT1/HEK293. The initial velocity data were visualized by Eadie–Hofstee plots (Fig. 2a; inset). The apparent K_m and V_{\max} values for the hOAT1-mediated transport of PCS were calculated to be 127.8 µM and 9.8 nmol/min/mg protein, respectively. In hOAT3/

Fig. 1 Time-dependent uptake of PCS by hOAT1 and hOAT3. **a** hOAT1/HEK293 (closed circle) or mock/HEK293 (open circle) was incubated with 100 μ M PCS for the indicated periods. **b** hOAT3/HEK293 (closed circle) or mock/HEK293 (open circle) was incubated with 500 μ M PCS for the indicated periods. The amounts of PCS taken up by each type of cell were determined by HPLC as described in 'Materials and methods'. Values are expressed as the mean \pm SD ($n = 4$)

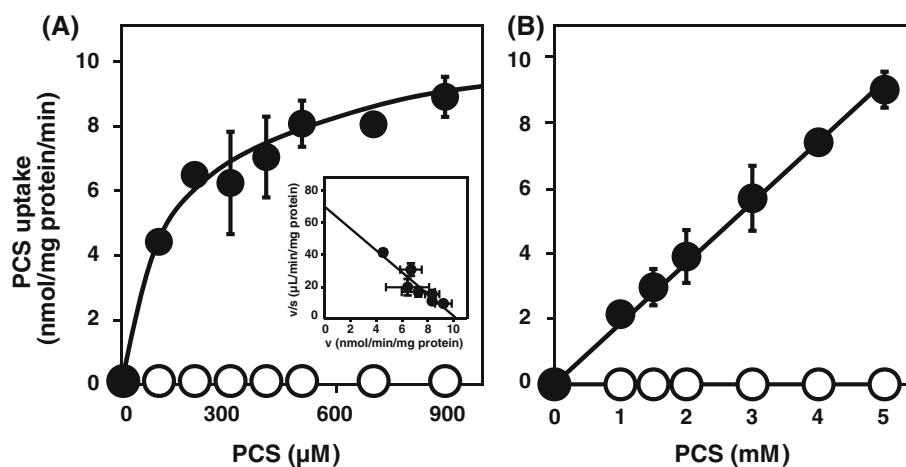
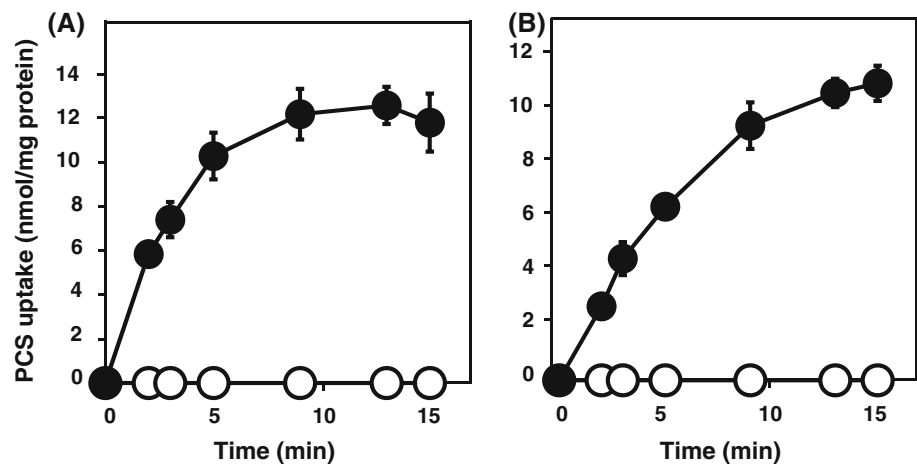


Fig. 2 Concentration-dependent uptake of PCS by hOAT1 and hOAT3. **a** hOAT1/HEK293 (closed circle) or mock/HEK293 (open circle) was incubated with PCS at various concentrations for 3 min. **b** hOAT3/HEK293 (closed circle) or mock/HEK293 (open circle) was incubated with PCS at various concentrations for 3 min. (**a** inset)

HEK293, saturation was not observed at the highest tested PCS concentration of 5 mM.

Next, we investigated the effect of probenecid, a well-known inhibitor of OAT, on PCS transport by hOAT1 and hOAT3. As shown in Fig. 3, the uptake of PCS by hOAT1 and hOAT3 decreased with increasing concentration of probenecid. Probenecid (100 μ M) inhibited the transport of PCS by hOAT1 and hOAT3 by >90 %.

Inhibitory effect of PCS on hOAT1 and hOAT3

The inhibitory effect of PCS on hOAT1 and hOAT3 was also investigated. Figure 4 provides information on the concentration-dependent inhibitory effect of PCS on hOAT1 and hOAT3. The uptake of *p*-aminohippurate by hOAT1 and estron-3-sulfate by hOAT3 decreased with increasing PCS concentration. The apparent 50 %

An Eadie–Hofstee plot analysis was performed for this experiment. The amounts of PCS taken up by each type of cell were determined by HPLC as described in 'Materials and methods'. Values are expressed as the mean \pm SD ($n = 4$)

inhibitory concentrations of PCS were determined to be 690 μ M for hOAT1 and 485 μ M for hOAT3.

Discussion

The serum levels of PCS become \sim 20 times elevated as renal dysfunction progresses [24]. A recent clinical study revealed that this accumulation is associated with increased mortality rates and the onset of CVD in CKD patients [4–6]. For this reason, interest has arisen in elucidating the disposition of PCS and the mechanism of its toxicity, as well as in its pathophysiological significance.

A wide variety of organic anionic protein-bound uremic toxins such as indoxyl sulfate, CMPF, indoleacetate and hippurate bind to serum albumin with a high affinity [25, 26]. They are efficiently excreted from the kidneys via a

Fig. 3 Effect of probenecid on PCS uptake by (a) hOAT1 and (b) hOAT3. hOAT1/HEK293 or hOAT3/HEK293 was incubated with 100 or 500 μM PCS in the absence or presence of probenecid at 1, 10 and 100 μM for 3 min. The amounts of PCS taken up by each type of cell were determined by HPLC as described in the 'Materials and methods'. Values are expressed as the mean \pm SD ($n = 6$). ** $P < 0.01$, significantly different

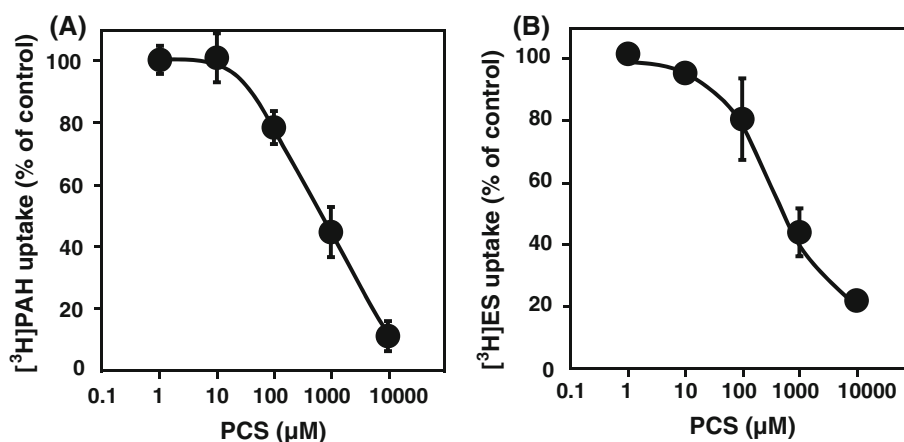
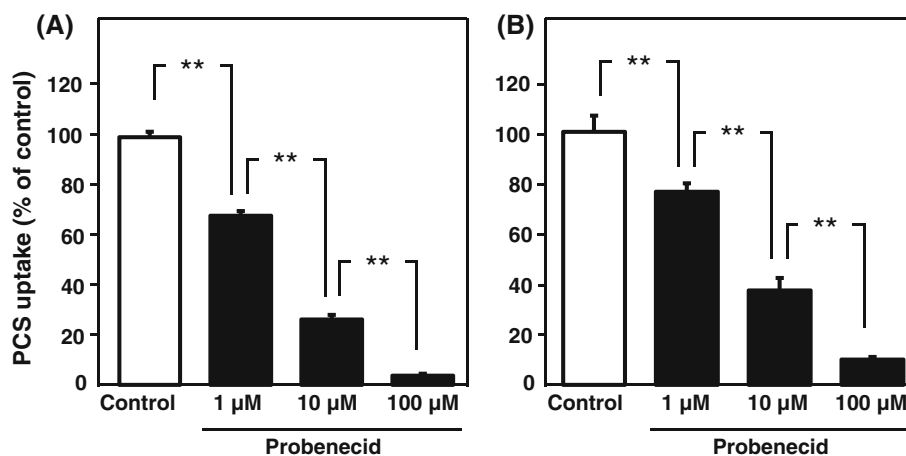


Fig. 4 Concentration-dependent inhibitory effect of PCS on a *p*-aminohippurate uptake by hOAT1 and on b estron-3-sulfate uptake by hOAT3. a hOAT1/HEK293 was incubated with 2.0 μM [^3H] *p*-aminohippurate in the absence (control) or presence of PCS at various concentrations for 3 min. b hOAT3/HEK293 was incubated with 10

nM [^3H] estron-3-sulfate in the absence (control) or presence of PCS at various concentrations for 3 min. The uptake amount of [^3H] *p*-aminohippurate or [^3H] estron-3-sulfate in each cell was determined and shown as a percentage of the control. Values are expressed as the mean \pm SD ($n = 4$)

tubular secretion process. This is because OATs play a key role in this process [11]. Additionally, a model of tissue accumulation via transporters such as OATs has recently attracted interest as an important mechanism for explaining the toxicity of uremic toxins [12]. Our recent study, using rat renal cortical slices and HK-2 cells, indicated that PCS appears to be a substrate for rat Oat and human OAT, based on the use of various transporter inhibitors. In addition, Mutsaers et al. [20] also proposed the involvement of OATP4C1 in PCS uptake in HK-2. However, no direct evidence is available as to whether PCS can be transported via hOAT isoforms, hOAT1 and hOAT3.

To clarify this, we conducted transport and inhibition experiments for PCS using HEK293 cells stably expressing hOAT1 and hOAT3. From these experiments, it was clearly concluded that PCS is, in fact, a substrate for hOAT1 and hOAT3, but PCS had a lower potency in inhibiting the

transport of *p*-aminohippurate by hOAT1 and estron-3-sulfate by hOAT3. The uptake of PCS by hOAT1/HEK293 and hOAT3/HEK293 were both time- and concentration-dependent, but the characteristics of the transport between hOAT1 and hOAT3 were somewhat different (Figs. 1, 2). The apparent K_m for the hOAT1-mediated transport of PCS was 128 μM . In contrast, in the case of hOAT3/HEK293, saturation was not yet observed at the highest tested PCS concentration of 5 mM. Judging from these data, combined with previous findings that the serum protein binding of PCS is $>90\%$ [26], and that serum PCS concentrations in CKD patients are $\sim 200\ \mu\text{M}$ [24], it would appear that, in CKD patients, the transport of PCS via hOAT1 and hOAT3 should occur under linear conditions. Based on the kinetic parameters (K_m and V_{max}), it appears that PCS is a high-capacity (low affinity) substrate for hOATs, especially hOAT3. These data, therefore, suggest that hOAT1/hOAT3

mediated the transport of PCS, even under conditions of high PCS levels in the body. Similar behavior between an endogenous substrate and a transporter has been shown in the urate ABCG2/BCRP system [27]. Urate was identified as a high-capacity (low affinity) substrate of ABCG2/BCRP (K_m , 8.2 mM; V_{max} , 7.0 nmol/min/mg protein), indicating that ABCG2/BCRP could play a physiological role as a high-capacity urate exporter [27].

In our previous study [18], we reported that the uptake of PCS by renal cortical slices and HK-2 cells, which express both OAT1 and OAT3, was increased in a time-dependent manner and was consistent with a saturable process. We also described that PCS is preferentially recognized by OAT3 because the inhibitory effect of estron-3-sulfate on PCS uptake was larger than that of *p*-aminohippurate in HK-2 cells. However, in this study, specific transport of PCS by both hOAT1 and hOAT3 was observed (Figs. 1, 2). The saturation was not observed in hOAT3/HEK293 cells. In addition, PCS showed a similar inhibitory effect on *p*-aminohippurate and estron-3-sulfate uptake in hOAT1/HEK293 and hOAT3/HEK293 cells, respectively (Fig. 4). These data indicated that PCS is a substrate for both hOAT1 and hOAT3. The differences between the two studies may be attributed to species difference and/or the difference in the expression levels of hOAT1 and hOAT3. To extrapolate the contribution of hOAT1 and hOAT3 for the uptake of PCS in human kidney from the data using the hOAT1/hOAT3 HEK293 cell system, studies possibly using freshly prepared human kidney slices in which the protein expression of hOAT1 and hOAT3 were determined, are needed in the future.

It was previously reported that the uptake of indoxyl sulfate, CMPF, indoleacetate and hippurate by hOAT1 was saturable, with K_m values of 21, 141, 14 and 24 μ M, respectively, whereas a significant uptake of indoxyl sulfate and CMPF, but not of indoleacetate or hippurate, was observed in hOAT3-expressing cells with K_m values of 263 and 27 μ M, respectively [11]. Since the K_m value of PCS for both hOAT1 and hOAT3 was larger than that of indoxyl sulfate, the affinity of hOAT1 and hOAT3 for PCS was expected to be weaker than that for indoxyl sulfate. This finding is supported by a report from Poesen's group, in which the renal clearance of indoxyl sulfate in CKD patients exceeded the clearance of PCS by approximately three-fold, indicating that substantial differences exist between PCS and indoxyl sulfate in terms of their affinity for the tubular transporter [17]. In addition, our data also indicated that the plasma-free concentration of PCS may be elevated as a result of competitive inhibition during the urinary secretion of PCS when indoxyl sulfate or CMPF are also present in CKD patients, which may lead to PCS being distributed to other organs such as blood vessels and osteoblasts, etc., and could lead to tissue damage.

In the renal uptake process, another kidney-specific basolateral transporter that has been demonstrated to be involved in the removal of uremic toxins is the organic anion transporting polypeptide (OATP4C1) [28, 29]. Mutsaers et al. [20] also proposed the involvement of OATP4C1 in the uptake of PCS in HK-2 cells. Therefore, it would be of interest to quantitatively compare the affinity or capacity (K_m and V_{max} values) between hOATs isoforms and hOATP4C1 for PCS, or the interaction between PCS and other uremic toxins/or anionic drugs via hOATP4C1 in the future. In addition, to achieve the vectorial transport of PCS, it is likely that transporter(s) are involved in the secretion across the brush border membrane of the proximal tubule. For example, PCS may be secreted by hOAT4 [30], human sodium phosphate transporter 4 (hNPT4) [31] and/or human multidrug resistance associated protein 2 or 4 (MRP2/4) [32]. Further studies are necessary to identify transporters responsible for the luminal excretion of PCS.

Conclusion

The present study demonstrates that PCS is a substrate for hOAT1 and hOAT3, and suggests that hOAT1 and hOAT3 could play a physiological role as a high-capacity PCS transporter, which could play a key role in the tubular secretion process. In addition, since hOATs are expressed not only in the kidneys, but in other tissues as well, including blood vessels and osteoblasts, etc., PCS may accumulate via hOATs to toxic levels in these tissues. The findings reported here are of great significance in terms of elucidating the renal clearance, tissue disposition of PCS and the mechanism of its toxicity in CKD.

Acknowledgments This work was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS) [KAKENHI 23790187].

Conflict of interest The authors declare no conflict of interest.

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