Organic anion transporters play an important role in the uptake of p-cresyl sulfate, a uremic toxin, in the kidney

Yohei Miyamoto1, Hiroshi Watanabe1,2, Tsuyoshi Noguchi1, Shunsuke Kotani1, Makoto Nakajima3, Daisuke Kadowaki1,2, Masaki Otagiri1,4 and Toru Maruyama1,2

1Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan, 2Center for Clinical Pharmaceutical Sciences, School of Pharmacy, Kumamoto University, Kumamoto, Japan, 3Department of Organic Chemistry, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan and 4Laboratory of Pharmacokinetics, Faculty of Pharmaceutical Sciences, Sojo University, Kumamoto, Japan

Correspondence and offprint requests to: Toru Maruyama; E-mail address: tomaru@gpo.kumamoto-u.ac.jp

Abstract
Background. p-Cresyl sulfate (PCS), a recently identified anionic uremic toxin, is the main circulating metabolite of p-cresol. In cases of chronic kidney disease (CKD), it might be associated with cardiovascular outcomes and the progression of CKD. However, the renal excretion pathway of PCS is currently unknown. The objective of the present study was to determine whether organic anion transporters (OATs), which are renal tubular basolateral membrane transporters, play an important role in this process.

Methods. The uptake of PCS was investigated using rat renal cortical slices and human proximal tubular cells (HK-2). The active uptake velocity was calculated by subtracting the uptake velocity at 4°C (nonspecific uptake) from that at 37°C.

Results. As evidenced by renal cortical slice experiments, the uptake of PCS was saturable with a mean \( K_m \) of 231.6 \( \mu \)M, indicating that the active transport is involved in the basolateral uptake of PCS. Similar results were also observed in HK-2 cells. The active transport of PCS was significantly suppressed by inhibitors of OATs, such as probenecid, benzylpenicillin, p-aminomuconic acid and estrone sulfate. Similar inhibitions were observed in the presence of indoxyl sulfate and 3-carboxy-4-methyl-5-propyl-2-furanpropionate, OATs substrates among uremic toxins. In contrast, digoxin and tetraethylammonium that did not interact with OATs had little inhibitory effect.

Conclusions. The findings of the present study strongly suggest that PCS serves as a substrate for OATs, is preferentially recognized by OAT3 and plays a key role in the renal tubular secretion process.

Keywords: chronic kidney disease; human proximal tubular cells; organic anion transporter; p-cresyl sulfate; rat renal cortical slices; uremic toxins.

Introduction
In chronic kidney disease (CKD), metabolic changes and disturbances in the urinary excretion of metabolites cause the accumulation of uremic toxins in the body, resulting in a variety of pathological conditions [1]. Recent findings clearly indicate that these toxicities are associated with the progression of certain diseases. For example, indoxyl sulfate (IS), the most widely studied uremic toxin, has been shown by us and other research groups to cause an increase in free radical production and to induce the production of inflammatory cytokines in the kidneys and blood circulation [2, 3]. In addition, a recent clinical study reported that IS is a factor in the onset of complications in patients with chronic renal insufficiency who are on long-term hemodialysis treatment [4, 5].

p-Cresol, another frequently studied uremic toxin, has been reported to depress whole blood respiratory burst activity and myeloperoxidase activity [6]. However, recent advances in instrumental analytical technology have led to the conclusion that the majority of p-cresol that is biosynthesized from dietary tyrosine and phenylalanine undergoes sulfate conjugation by a sulfotransferase enzyme during passage through the intestinal membrane, resulting in the production of a conjugate, namely p-cresyl sulfate (PCS) [7], rather than p-cresol itself, in the blood circulation [8]. Scheper et al. [8] reported that PCS significantly increased free radical production by unstimulated leucocytes, although p-cresol had no effect on the leucocytes. In fact, Labeuf et al. [9] reported that high levels of PCS accumulate in CKD patients and concluded that this may well be associated with increased mortality rates and the onset of cardiovascular disease (CVD).

Most of the 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 [10, 11]. In fact, we previously demonstrated, for the first
time, that uremic toxins such as IS and 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF) are excreted from the kidneys via organic anion transporters (OATs) that are found in the renal tubular basolateral membrane [12, 13]. Based on this finding, Enomoto et al. [14] proposed a new mechanism for toxicity in which uremic toxins are taken up in the kidneys, blood vessels, bones and the blood-brain barrier (BBB) via OATs and subsequently induce the production of free radicals and the production of inflammatory cytokines in these organs. In fact, Nii-Kono et al. [15] reported that IS is taken up by osteoblastic cells and enhances reactive oxygen species production, resulting in resistance to the parathyroid hormone. It thus appears highly likely that PCS also accumulates in various organs, including the kidneys, via OATs and also could cause tissue damage. However, the relationship between PCS and OATs has not been clarified.

The purpose of the present study was to address the issue of whether OATs, which are renal tubular basolateral membrane transporters, play an important role in the renal basolateral uptake process using rat renal cortical slices and human proximal tubular cells (HK-2). Interactions between PCS and other uremic toxins that serve as substrates for OATs were also examined.

Materials and methods

Chemicals and materials

PCS was synthesized according to the method of Feigenbaum and Neuberg [16]. Identity and purity (>99%) were confirmed by nuclear magnetic resonance, IS, estrone sulfate (ES), probenecid, hippurate (HA) and digoxin were obtained from Sigma Chemical Co. (St Louis, MO). a-Aminohippuric acid (PAH) and indoleacetic acid (IA) were obtained from Nacalai Tesque (Kyoto, Japan). Benzyloxycarbonyl tryptophan-2-ethyl ester (BzTyrOEt) and S-carboxymethylcysteine (CMC) were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). Rabbit polyclonal anti-human OAT3 and OAT1 antibodies were purchased from TransGenic Inc (Kumamoto, Japan). Rabbit monoclonal anti-human Na+/K+-ATPase x1 antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Keratinocyte serum-free medium (K-SFM) and epidermal growth factor (EGF) were purchased from Invitrogen (Eugene, OR). Bovine pituitary extract was purchased from Kurabo (Kurashiki, Japan). SuperSignal West Pico Chemiluminescent substrate was purchased from Thermo Scientific (Yokohama, Japan). CMPF was synthesized as described previously [15]. All reagents from commercial sources were of the highest grade.

Uptake by rat renal cortical slices

The uptake of PCS by rat renal cortical slices was investigated using a procedure described in the literature [13]. Male Wistar rats (230–280 g) were anaesthetized and the kidneys promptly removed, decapsulated and placed in an ice-cold oxygenated incubation medium, consisting of 120 mM NaCl, 16.2 mM KCl, 1.0 mM CaCl2, 1.2 mM MgSO4 and 10 mM sodium phosphate buffer, at pH 7.4. Rat cortical slices (weight 10–20 mg/slice; ~0.5 mm in thickness) were cut freehand with Gillette valet strip blades about 7.60-cm long (Sabre International Products Ltd, Reading, UK). Two cortical slices were prepared from each half-kidney and were stored on ice in oxygenated incubation medium for not >15 min prior to incubation. Two slices were placed in each flask and the incubation medium containing PCS (50, 100, 250, 500, 750, 1000 and 2000 μM) thoroughly gassed with 100% oxygen during the incubation (10 min). The slices were then tightly sealed with rubber stoppers and incubated at 37°C in a shaking water bath at 60 cycles/min. To confirm the specificity of cellular transport, 1 μM inhibitors (probenecid, PCG, PAH, ES, digoxin and tetraethylammonium) and 1 μM urmic toxins (IS, IA, HA and CMPF) were added to the medium. After incubation, the slices were placed on ice, and the slices promptly removed, gently blotted and weighed. Individual slices were homogenized in 100 μL of phosphate-buffered saline (PBS). The homogenate (50 μL) was mixed with 100 μL of methanol for protein digestion. After centrifugation (3000 g for 10 min), the PCS concentration of the supernatant was determined by high-performance liquid chromatography (HPLC), as described below. PCS concentration in the incubation medium was also determined. Authentic PCS was recovered from the slice and medium in a yield of >95%. Tissue blanks were prepared by omitting PCS from the medium. The uptake of PCS is expressed as the S/M ratio (i.e. the concentration of PCS per gram of kidney tissue divided by the concentration of PCS per milliliter of medium). The active uptake velocity was calculated by subtracting the uptake velocity at 4°C (non-specific uptake) from that at 37°C.

Cell cultures

HK-2 cells were used as a model of human proximal tubular cells. HK-2 cells (American Type Culture Center, Manassas, VA) were cultured at 37°C in 5% CO2 in K-SFM, supplemented with 5 ng/mL human recombinant EGF and 0.05 μg/mL bovine pituitary extract.

Cellular uptake of PCS and other uremic toxins that serve as substrates for OATs were also examined.

HPLC conditions

The HPLC system consisted of an Agilent 1100 series intelligent pump and a fluorescence spectrophotometer. A LiChrosorb RP-18 column (Cica Merk, 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 reequilibration 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 [17].

Western blot analysis

HK-2 cells were seeded in a 12-well tissue culture plate at a density of 1 × 104 cells per well. One day after seeding, the cells were incubated in 1 mL of medium containing 1 mM PCS for 0–60 min at 37°C. After the incubation, the cells were washed three times with ice-cold PBS and once with PBS containing 1% bovine serum albumin. The cells were lysed by adding 150 μL of 0.2 M NaOH and then incubated for 30 min at room temperature. To adjust the pH, 9.5 μL of 3 M HCl was added and to maintain isotonicity, 6.5 μL of 1 M K2HPO4 was added. The amount of PCS in cellular extracts (100 μL) was determined by HPLC. In order to confirm the specificity of cellular transport, 10 mM inhibitors (probenecid, PCG, PAH, ES, digoxin and tetraethylammonium) were added to the medium. Active cellular uptake was calculated by subtracting the uptake velocity at 4°C from that at 37°C.

Kinetic analysis

In the renal cortical slice uptake study, kinetic parameters were obtained using the following equation:

\[ v = \frac{f_{\text{max}} \times S}{K_m + S} \]

where \( v \) is the velocity of the renal uptake of PCS (nmol/min/g kidney), \( S \) the PCS concentration in the medium (μM), \( K_m \) the Michaelis–Menten constant (μM) and \( f_{\text{max}} \) the maximum uptake rate (nmol/min/g kidney). Fitting was performed by the nonlinear least-squares method using the MULTI program and the Damping Gauss Newton method algorithm was used for fitting.

Statistical analysis

Statistical analyses were performed using the Student’s t-test. A probability value of \( P < 0.05 \) was considered to be significant.
Results

**Mechanism of uptake of p-cresyl sulfate by renal cortical slices**

The basolateral uptake of PCS by renal cortical slices was examined at both 4 and 37°C for 60 min. At 37°C, when 100 μM PCS was added to the medium, the slice-to-medium ratio (S/M) was 6.10 ± 0.52 mL/h/g kidney, whereas under anaerobic conditions at 4°C, this value was 0.95 ± 0.12 mL/h/g kidney. These results suggest that active transport is involved in the uptake of PCS by renal cortical slices. The active uptake of PCS was linear for at least 10 min (data not shown). To estimate the $K_m$ and $V_{max}$ values for the active uptake of PCS from the initial rate, the uptake velocity of PCS was measured over a range of concentrations (50–2000 μM) after incubation for 10 min at 37°C (Figure 1A and B). The mean $K_m$ and $V_{max}$ values were 231.6 ± 5.9 μM and 22.0 ± 1.7 nmol/min/g kidney, respectively.

To establish whether the uptake of PCS is mediated by the OATs system, the effects of various compounds on PCS uptake by rat renal cortical slices were examined. As shown in Figure 2, the active uptake of PCS was suppressed by OATs inhibitors, such as probenecid, PCG, PAH and ES. The inhibitory effects of PCG and ES were larger than that of PAH. In contrast, only a minor inhibitory effect was observed for digoxin and tetraethylammonium, typical inhibitors of the organic anion transporting peptide and the organic cation transporter, respectively. These data indicate that the OATs system may play an important role in the uptake of PCS in the kidney.

To examine the issue of whether other uremic toxins affect the uptake of PCS to the kidney, the inhibitory effect of other uremic toxins, IS, CMPF, IA and HA, which were taken up by OATs in renal proximal tubular tissue, was also investigated. As shown in Figure 3, IS and CMPF exhibited a significant inhibitory effect on the renal uptake of PCS (~80% inhibition). In addition, HA inhibited the uptake of PCS by 50%, whereas IA had no inhibitory effect.

**Mechanism of uptake of p-cresyl sulfate by human proximal tubular cells (HK-2 cells)**

To confirm species differences in the renal uptake of PCS, similar uptake experiments were also carried out using HK-2 cells. The expression of OAT1 and OAT3 in the absence of PCS was determined by western blotting. The expression of Na⁺/K⁺-ATPase was also determined as a standard membrane protein. The results indicate that these proteins were expressed in HK-2 cells (Figure 4A). As shown in Figure 4B, when 1 mM PCS was added to the medium, intracellular PCS levels were increased in a time-dependent manner and were consistent with a saturable process. These data also indicate that the active uptake of PCS by HK-2 cells is linear for at least 15 min.

As shown in Figure 4C, the uptake of PCS by HK-2 cells was strongly inhibited by probenecid and PCG (~80% inhibition). This indicates that an OATs system is likely involved in the uptake of PCS in the human kidney as well as the rat. Furthermore, PAH and ES also inhibited the
uptake of PCS, while digoxin and tetraethylammonium had no inhibitory effect. Again, the inhibition of uptake by PAH was less than that by PCG and ES. These observations are consistent with the results for rat renal cortical slices, strongly suggesting that PCS is taken up via the OATs system in humans.

**Discussion**

The serum levels of the uremic toxin, PCS, are elevated (~30 times higher than in healthy subjects as renal dysfunction progresses, based on extrapolation of the p-cresyl sulfate data [18]). The ratio is even higher (100 or more) for the free fraction. A recent study revealed that this accumulation can possibly be associated with increased mortality rates and the onset of CVD in CKD patients [9]. 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.

Generally, many organic anionic uremic toxins such as IS, CMPF, IA and HA bind to serum protein with a high affinity. They are efficiently excreted from the kidneys via a tubular secretion process. This is because OATs play a key role in this process. Additionally, a model of tissue accumulation via transporters such as OATs has recently attracted interest as an important mechanism for explaining the toxicities of uremic toxins [14]. Because it is an organic anion containing a sulfate group in its molecular structure, a PCS, like IS, would be expected to serve as a substrate for OATs.

To clarify this possibility, we conducted uptake experiments using rat renal cortical slices and HK-2 cells. Active transport of PCS was observed at 37°C but, in contrast, no uptake activity was noted at 4°C. Thus, saturation of the uptake process of PCS over 500 μM was evident, strongly suggesting the presence of active uptake process via transporters. Judging from these data, combined with previous findings that the serum protein binding of PCS is ~90% [19], and that serum PCS concentrations in CKD patients are 100–400 μM [7], it would appear that, in CKD patients, the uptake of PCS by the kidney is not saturated and reductions in renal clearance due to an impaired renal function are intimately involved in the accumulation of PCS. On the other hand, the removal of PCS could proceed via another mechanism, namely break down, e.g. by a sulfatase. To exclude this, we measured PCS levels in supernatants from the slice experiments. As a result, p-cresol has not been detected in these samples (data not shown). Thus, such a mechanism is less likely to be operative in the experiments dealing with the intracellular content of PCS.

To identify which transporters are involved in the active uptake of PCS, we carried out inhibition experiments for the uptake of PCS using inhibitors that are relatively specific for various OATs. The uptake of PCS in rat renal cortical slices and HK-2 was efficiently inhibited by probenecid, PCG, PAH and ES. These results strongly suggest that OATs play a key role in the renal uptake of PCS. Interestingly, the inhibitory effect of PCG and ES was larger than that of PAH. Earlier reports have shown that PAH is a selective substrate for OAT1 [20] and PCG and ES for OAT3 [21, 22]. Therefore, it is likely that both OAT1 and OAT3 are involved in the renal uptake of PCS, with a greater contribution by OAT3. To quantitatively estimate the contribution of OAT3 in the renal uptake of PCS, we carried out inhibition experiments for the uptake of PCS using an inhibitor that is relatively specific for OAT3 [21, 22]. Therefore, it is likely that both OAT1 and OAT3 are involved in the renal uptake of PCS, with a greater contribution by OAT3. To quantitatively estimate the contribution of OAT3 in the renal uptake of PCS, it will be necessary to perform a kinetic analysis using the kidney uptake index method in the future.

We also examined the interactions between PCS and uremic toxins that serve as substrates for OATs. As a result, the basolateral uptake of PCS was strongly inhibited by IS and CMPF but only partially inhibited by HA. IA showed no inhibitory effect. We previously reported that IS and CMPF are taken up mainly via OAT3 and IA and HA by OAT1 [23]. These results strongly support the above results as shown in Figures 2 and 4, demonstrating the possibility that PCS undergoes renal excretion mainly via OAT3. Since the K_m value of PCS for OATs was larger than those of IS and CMPF previously reported [12, 13], the affinity of PCS is thought to be weaker than that of IS and CMPF. If this is true, the plasma-free concentration of PCS may be elevated as a result of competitive inhibition during the urinary secretion of PCS by coexisting IS or CMPF in CKD patients. In this regard, it will be necessary to perform a kinetic analysis of the process of PCS uptake using a model system that expresses OAT3 alone.

![Fig. 4. (A) Expression of OAT1 and OAT3 proteins in HK-2 cells. (B) Time dependence of p-cresyl sulfate uptake by HK-2 cells. (C) Effect of various compounds on p-cresyl sulfate uptake by HK-2 cells. Values are expressed as the mean ± SD (n = 3). *P < 0.05, **P < 0.01 in comparison to the control.](https://academic.oup.com/ndt/article-abstract/26/8/2498/1915365/82x410)
via OATs to exhibit its toxicity in these organs. The findings reported herein are of great significance in terms of elucidating the disposition of PCS and the mechanism of its toxicity in CKD.

References


Received for publication: 14.10.10; Accepted in revised form: 2.12.10