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Research Paper

Differential Contributions of rOat1 (Slc22a6) and rOat3 (Slc22a8) to the *in Vivo* Renal Uptake of Uremic Toxins in Rats

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Purpose. Evidence suggests that uremic toxins such as hippurate (HA), indoleacetate (IA), indoxyl sulfate (IS), and 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF) promote the progression of renal failure by damaging tubular cells via rat organic anion transporter 1 (rOat1) and rOat3 on the basolateral membrane of the proximal tubules. The purpose of the current study is to evaluate the *in vivo* transport mechanism responsible for their renal uptake.
Methods. We investigated the uremic toxins transport mechanism using the abdominal aorta injection technique [i.e., kidney uptake index (KUI) method], assuming minimal mixing of the bolus with serum protein from circulating serum.
Results. Maximum mixing was estimated to be 5.8% of rat serum by measuring estrone sulfate extraction after addition of 0–90% rat serum to the arterial injection solution. Saturable renal uptake of *p*-aminohippurate (PAH, K_m = 408 μM) and benzylpenicillin (PCG, K_m = 346 μM) was observed, respectively. The uptake of PAH and PCG was inhibited in a dose-dependent manner by unlabeled PCG (IC_{50} = 47.3 mM) and PAH (IC_{50} = 512 μM), respectively, suggesting that different transporters are responsible for their uptake. A number of uremic toxins inhibited the renal uptake of PAH and PCG. Excess PAH, which could inhibit rOat1 and rOat3, completely inhibited the saturable uptake of IA, IS, and CMPF by the kidney, and by 85% for HA uptake. PCG inhibited the total saturable uptake of HA, IA, IS, and CMPF by 10%, 10%, 45%, and 65%, respectively, at the concentration selective for rOat3.
Conclusions. rOat1 could be the primary mediator of the renal uptake of HA and IA, accounting for approximately 75% and 90% of their transport, respectively. rOat1 and rOat3 contributed equally to the renal uptake of IS. rOat3 could account for about 65% of the uptake of CMPF under *in vivo* physiologic conditions. These results suggest that rOat1 and rOat3 play an important role in the renal uptake of uremic toxins and the induction of their nephrotoxicity.

KEY WORDS: chronic renal failure; kidney uptake index; nephrotoxicity; organic anion transporters; uremic toxins.

INTRODUCTION

Uremic syndrome, which is a complex form of organ dysfunction, causes the retention of waste products that, under normal conditions, should be eliminated from the body by the kidneys (1). Organic anions, such as indoxyl sulfate (IS), hippurate (HA), indoleacetate (IA), and 3-carboxy-4-methyl-5-propyl-2-furanpropionate (CMPF), which are derived from dietary protein, are mainly excreted into the urine, but they accumulate to a high degree in uremic plasma (2,3). Recent findings suggest that uremic toxins promote the progression of renal failure by damaging tubular cells. In previous studies, overload of IS, HA, and IA accelerated the loss of kidney function in 5 of 6 nephrectomized rats (4,5). In another study, administration of AST-120, an oral absorbent, decreased the intensity of IS staining in the proximal tubules as well as the serum and urinary concentrations of IS, and it prevented the progression of renal dysfunction by reducing the gene expression of TGF-β1, TIMP-1, and pro-α1(I) collagen in the kidney (6). IS and IA induce free-radical production and activate NF-κB to upregulate plasminogen activator inhibitor-1 (PAI-1) expression in renal tubular cells (7). These findings indicate that increased concentrations of uremic toxins in the renal tubules can exacerbate the deterioration of renal function (8,9).

In renal tubules, membrane transport systems mediate the tubular secretion of endogenous and exogenous

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ABBREVIATIONS: CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate; ES, estrone sulfate; HA, hippurate; IA, indoleacetate; IS, indoxyl sulfate; KUI, kidney uptake index; PAH, *p*-aminohippurate; PCG, benzylpenicillin; rOat, rat organic anion transporter.

organic anions, including various drugs, toxins, and endogenous metabolites (10). We recently demonstrated that rat and human organic anion transporter 1 (rOat1/hOAT1; Slc22a6/SLC22A6) and rOat3/hOAT3 (Slc22a8/SLC22A8) are responsible for the renal uptake of uremic toxins on the basolateral membrane of the proximal tubules (11). Transport studies using cDNA-transfected cells and rat kidney slices have revealed that both rOat1 and rOat3 contribute to IS transport in the kidney. rOat1 is predominantly responsible for the renal uptake of HA and IA, while rOat3 plays a major role in the renal uptake of CMPF.

The abdominal aorta injection technique, which is also known as the kidney uptake index (KUI) method, is useful for *in vivo* examination of transport mechanisms of plasma membrane at the vascular side of tissue separated from the luminal side (12). Previously, using the KUI method, we obtained data suggesting that rOat3 is the main contributor to the renal uptake of IS (13). However, *p*-aminohippurate (PAH) and benzylpenicillin (PCG), which are inhibitors of rOat1 and rOat3, have been shown to inhibit the renal uptake of IS by 75% and 50%, respectively, suggesting that both rOat1 and rOat3 are involved in the renal uptake of IS. However, there is no conclusive evidence about the contributions of rOat1 and rOat3 to the renal uptake transport of IS or other uremic toxins under physiologic conditions. Additionally, previous reports revealed that the activity of rOats was modulated by protein kinases, hormones, and xenobiotics, endogenous driving forces and cellular stress, suggesting that their function could be affected by various physiologic conditions in which rOats are expressed (14). Therefore, further quantitative studies are needed to indicate whether findings from *in vitro* studies are applicable to *in vivo* conditions. On the other hand, a crucial element of this technique is the assumption that there is minimal mixing of the injection solution bolus with the circulating serum after rapid arterial injection. IS, IA, and CMPF have high affinity for albumin, with apparent association constants (K_a) of 1.6×10^6, 2.1×10^5, and 1.3×10^7 M^{-1}, respectively (15). Thus, with the KUI technique, mixing may produce artifacts in estimates of the transport properties of such highly protein-bound substances. Furthermore, endogenous compounds may inhibit transport of test compounds, resulting in underestimation of their renal uptake clearance. However, there has been no quantification of the potential for bolus mixing after bolus injection. Previous KUI study has suggested that serum protein binding has variable effects on the *in vivo* influx of circulating estrone sulfate (ES), which is strongly bound to plasma protein, from the microvasculature into the kidney (16). Thus, in the current study, the effects of serum dilution on the renal uptake of ES were used to quantify the mixing of bolus with circulating rat serum.

The purpose of the current study was to investigate the contributions of rOat1 and rOat3 to the *in vivo* renal uptake of uremic toxins, using the KUI method. The potential for bolus mixing after the abdominal aorta injection was quantified by measuring extraction of radiolabeled ES after the addition of 0 to 90% rat serum to the arterial injection solution. Also, the effects of inhibitors relatively selective for rOat1 and rOat3 on the uptake of uremic toxins were examined.

MATERIALS AND METHODS

Materials

CMPF was synthesized as previously described (17). [^{3}H]IS (2.78 Ci/mmol) and [^{3}H]CMPF (78.9 Ci/mmol) were synthesized and purified by PerkinElmer Life Sciences (Boston, MA, USA). [^{3}H]ES (46.0 Ci/mmol) and [^{3}H]PAH (4.54 Ci/mmol) were purchased from PerkinElmer Life Sciences. [^{3}H]IA (26.0 Ci/mmol) and [^{3}H]PCG (20.0 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). [^{14}C]PAH (55.0 mCi/mmol), [^{14}C]HA (55.0 mCi/mmol), and [^{14}C]butanol (1.0 mCi/mmol) were purchased from American Radiolabeled Chemicals (St.Louis, MO, USA), and [^{3}H]water (1.0 mCi/g) was purchased from Moravek Biochemical (Brea, CA, USA). All other chemicals were of analytical grade and commercially available.

Animals

Adult male Wistar rats (230 to 270 g) were housed in an air-conditioned room with free access to commercial feed and water, and fasted for 16 h before the abdominal aorta injection. All animal experiments were conducted according to the guideline principle and procedures of Kumamoto University for the care and use of laboratory animals.

Kidney Uptake Index (KUI) Experiment

Abdominal aorta injection for the KUI experiment was performed as described in previous reports (12). Briefly, rats were placed in the supine position, and a laparotomy was performed after the animals had been anesthetized with an intramuscular injection of a mixture of ketamine (235 mg/kg) and xylazine (2.3 mg/kg). The upper part of the abdominal aorta was isolated, and the origin of the left renal artery was identified. The aorta was cannulated with a 27-gauge needle adjacent to the site of the origin of the left renal artery. About 300 μl of buffered Ringer's solution (10 mM HEPES, 141 mM NaCl, 4 mM KCl, and 2.8 mM $CaCl_2$, pH 7.4) containing both test compounds and a freely diffusible reference ([^{14}C]butanol or [^{3}H]water) was rapidly injected (<0.5 s). At 5 s after injection, the entire left kidney was removed. This kidney was minced, and about 200 mg of tissue was solubilized in 2.0 ml of Soluene-350 at 55°C for 3 h. After the addition of 300 μl of hydrogen peroxide, 10 ml of Hionic-flour (PerkinElmer Life Sciences) was added, followed by measurement of double-isotope radioactivity with a liquid scintillation counter. The radioactivity of the injected solution was measured simultaneously.

Determination of KUI

The KUI was defined by Eq. (1) and determined using Eq. (2) (12,13,16):

$$\mathrm{KUI} = \frac{E_T}{E_R} \tag{1}$$

$$\mathrm{KUI} = \frac{\dfrac{\text{Amount of test compound in the kidney}}{\text{Amount of reference in the kidney}}}{\dfrac{\text{Amount of test compound injected}}{\text{Amount of reference injected}}} \times 100 \tag{2}$$

where E_T and E_R are the extraction of the test and reference compounds, respectively.

The E_R value of [^{14}C]butanol and [^{3}H]water in the kidney was determined as described previously (12). In order to estimate the extraction of reference compounds, a combination of [^{14}C]butanol and [^{3}H]PAH or [^{3}H]water and [^{14}C]PAH was injected into the abdominal aorta, and the left kidney was removed at 5 s after aortic injection. Due to the activity of a specific carrier-mediated system in renal tubular cells, PAH is almost completely extracted by the kidney and efflux is negligible for at least 30 s (16). Thus, it was assumed that $E_{PAH} = 1$ and that the E_R value of [^{14}C]butanol and [^{3}H]water is given by the following equation:

$$E_R = KUI_R \times E_{PAH} \tag{3}$$

where KUI_R, E_R, and E_{PAH} are the KUI of reference relative to radiolabeled PAH, the extraction of the reference compounds, and PAH, respectively. The extraction of [^{14}C]butanol and [^{3}H]water relative to radiolabeled PAH obtained were 80.6 ± 1.9 and 46.9 ± 0.9% (mean ± SE, n = 3), respectively, and used for the E_R of kidney. Because E_T can be estimated from experimentally determined values of KUI and E_R, the following equation is valid:

$$E_T = E_R \times KUI \tag{4}$$

Estimation of Kinetic Parameters

The apparent extraction of test compounds consists of intracellular uptake, distribution to the interstitial space, retention in the vascular space and glomerular filtration. Therefore, intracellular extraction of test compounds is obtained by the following equation:

$$E = \frac{(E_T - E_{ns})}{(100 - E_{ns})} \tag{5}$$

where E represents the intracellular extraction (extraction due to cellular uptake only), and E_{ns} includes the extraction of the drug for distribution in the vascular and extracellular space, and for glomerular filtration. In a previous study, an E_{ns} value of 26.2% was found for the kidney (12).

Kinetic parameters were obtained using the following equation (Michaelis-Menten equation):

$$V = \frac{V_{max} \times C'}{K_m + C'} + CL_{non} \times C' \tag{6}$$

where V is the intracellular uptake rate (μmol/min/g of kidney), K_m is the Michaelis-Menten constant (mM), V_{max} is the maximal intracellular uptake rate (μmol min^{-1} g^{-1} of kidney), C′ is the mean capillary concentration of substrate (mM), and CL_{non} is the nonsaturable uptake clearance (ml min^{-1} g^{-1} of kidney). The number of components involved in the uptake by the kidney was determined based on Akaike's Information Criterion values (18).

Because the capillary concentration cannot be used to approximate the concentration of the injection solution when the extravascular extraction exceeds 35% (19), as in the current case, the mean capillary concentration (C′) was used for the estimation of the kinetic parameters. C′ and V were obtained using the following equation (19):

$$V = \frac{E \times F \times C_{in}}{100} \tag{7}$$

$$C' = \frac{C_{in} \times (-E)}{\ln(1 - E)} \tag{8}$$

where F, C_{in}, and E are the blood flow rate, the concentration in the injection solution, and the intracellular extraction (which was calculated using Eq. 5), respectively. In this calculation, we used the reported renal blood flow of 4.0 ml min^{-1} g^{-1} of kidney (16).

Half-maximal inhibitory concentrations (IC_{50}) of a series of compounds were obtained by examining their inhibitory effects on the intracellular uptake under conditions in which the substrate concentration was much lower than its K_m value, using the following equation:

$$CL_{+I} = \frac{CL}{1 + I/IC_{50}} + CL_{non} \tag{9}$$

where CL represents the uptake clearance, and the subscript (+I) represents the value in the presence of the inhibitor. I represents the concentration of the inhibitor (μM).

The following equation for the expected extraction of test compounds by the kidney at any given percentage of rat serum (S_T) was derived in a previous report (20), and was fit to the current extraction data using the nonlinear least-squares technique:

$$E = 1 - (1 - E'_{NS})\exp\left\{\frac{\overline{K}'_m \ln[(1 - E'_0)/(1 - E'_{NS})]}{(\overline{K}'_m + S_T)}\right\} \tag{10}$$

This model assumes no bolus mixing, and the estimable parameters are as follows: $\overline{K}'_m$, the weighted harmonic mean of half-saturation constants for rat serum; E'_0, the maximal extraction in the absence of addition of rat serum to the injection solution; and E'_{NS}, the nonsaturable extraction at large concentrations of S_T. The units of $\overline{K}'_m$ are the same as those of S_T.

If bolus mixing does occur, then E'_0 estimated from Eq. (10) is an underestimate of the true maximal extraction (E_0) in the absence of rat serum component, and $\overline{K}'_m$ is an overestimate of the true $\overline{K}'_m$ value. The dilution of the bolus with circulating rat serum may be expressed as an equivalent fraction X of native (100%) rat serum. The relationships among X, E_0, and the regression estimates of E'_0, E'_{NS}, and $\overline{K}'_m$ obtained using Eq. (10) are given in the following equation:

$$X = \frac{1}{1 + \dfrac{A/\overline{K}'_m}{1 - \dfrac{\ln[(1 - E'_0)/(1 - E'_{NS})]}{\ln[(1 - E'_0)/(1 - E'_{NS})]}}} \tag{11}$$

where A is set to 100% because of the choice of units for S_T and $\overline{K}'_m$. Therefore, the bound for X may be estimated using a range of physiologically reasonable values for E_0. The absolute upper bound for X is obtained from the following equation:

$$X < 1/(1 + 1/\overline{K}'_m) \tag{12}$$

Data Analysis

Unless otherwise indicated, all data represent the mean ± SE. Statistical significance among means of more than two groups was determined by one-way ANOVA followed by the modified Fisher's least squares difference method. Fitting was performed by the nonlinear least-squares method using a MULTI program and the Damping Gauss Newton Method algorithm (18).

RESULTS

Quantification of Mixing Effect

The effects of addition of rat serum to the injection solution on the KUI values of [^{3}H] ES and [^{3}H] PAH are shown in Table I. At concentrations of rat serum in the injection solution ranging from 5% to 90%, when the concentration of rat serum was increased, extraction of ES significantly decreased, but extraction of PAH did not significantly change. Equation (10) was fit to the extraction data of ES by nonlinear regression methods to estimate the E'_0 and $\overline{K}'_m$ values under the assumption of no bolus mixing in anesthetized rats (Table II). The E'_0 and $\overline{K}'_m$ estimates were then entered into Eq. (11) along with hypothetical values of E_0 to estimate X, the equivalent fraction of bolus mixing with native rat serum. The data in Table II indicate that if the permeability of the kidney for the test compounds is so high that maximal substrate extraction in the absence of any mixing effects is 90%, then the estimated bolus mixing is 3.3%. The absolute upper bound for bolus mixing using Eq. (12) is 5.8%, but this corresponds to the limit of reasonable physiologic conditions, E_0 = 100% and $\overline{K}'_m$ = 0%.

Concentration-Dependent Uptake of PAH and PCG by the Kidney and Mutual Inhibitory Effects

The intracellular uptake clearances of PAH and PCG decreased in a dose-dependent manner (Fig. 1, A and B), and the Michaelis-Menten constants (K_m) for their uptake are summarized in Table III. Kinetic analysis of the uptake of PAH and PCG by the kidney revealed one saturable component and one nonsaturable component (PAH, AIC = –13.1; PCG, AIC = –21.2), whereas it did not appear to fit two saturable component model well (PAH, AIC = –4.20; PCG, AIC = –17.4). The apparent K_m and V_{max} values of PAH for the saturable component and uptake clearance for the nonsaturable component were 7.44 ± 2.94 mM, 23.8 ± 8.3 μmol min^{-1} g^{-1} of kidney, and 0.926 ± 0.119 ml min^{-1} g^{-1} of kidney, respectively. The apparent K_m and V_{max} values of PCG for the saturable component and uptake clearance for the nonsaturable component were 1.37 ± 0.33 mM, 3.98 ± 0.75 μmol min^{-1} g^{-1} of kidney, and 0.751 ± 0.038 ml min^{-1} g^{-1} of kidney, respectively.

The renal uptake of PAH and PCG was inhibited by the unlabeled PCG and unlabeled PAH, respectively (Figs. 1C and 1D). The IC_{50} values of PAH and PCG for the renal uptake of PCG and PAH were 1.35 ± 0.54 and 62.7 ± 28.5 mM, respectively (Table III). Also, the following kinetic parameters were estimated from mean capillary concentration. K_m for the renal uptake of PAH and PCG was 408 ± 142 and 346 ± 93 μM, respectively. IC_{50} values of PAH and PCG for the renal uptake of PCG and PAH were 512 ± 245 μM and 47.3 ± 24.1 mM, respectively.

Table I. Effects of Addition of Rat Serum to Injection Solution on KUI and Intracellular Extraction (E) of [^{3}H]ES and [^{3}H] PAH

Substrate	S_T (%)	KUI (%)	E (%)
[^{3}H]ES	0	93.3 ± 5.2	66.4 ± 5.7
	5	72.1 ± 4.4	43.3 ± 4.8
	10	67.4 ± 3.8	38.1 ± 4.2
	30	55.3 ± 6.3	24.9 ± 6.8
	90	43.1 ± 3.2	11.5 ± 3.5
[^{3}H]PAH	0	124 ± 5	100 ± 5
	5	115 ± 5	89.9 ± 5.1
	10	116 ± 4	91.1 ± 4.0
	30	107 ± 2	81.2 ± 1.7
	60	105 ± 2	78.7 ± 2.3
	90	106 ± 2	80.6 ± 1.7

Intracellular extractions of ES and PAH were obtained using Eq. (5). The S_T is percentage rat serum in the injection solution. Date are means ± SE (n = 3 to 6).

Table II. Summary of Parameter Estimates and Bounds for Mixing Fraction (X)

Parameters	
E'_0	65.6 ± 3.5%
$\overline{K}'_m$	6.19 ± 1.97%
E'_{NS}	7.88 ± 4.56%
X (if E_0 = 100%)	5.83%
X (if E_0 = 90%)	3.34%
X (if E_0 = 80%)	2.17%
X (if E_0 = 70%)	0.77%

Estimates for E'_0, $\overline{K}'_m$, and E'_{NS} of ES were determined by fitting Eq. (10) to the extraction data of ES (Table I). X was estimated using Eq. (11) at hypothetical E_0 values. Absolute upper bounds for X were estimated using Eq. (12). Each value represents the mean ± SD (n = 3 to 6).

Inhibitory Effect of Uremic Toxins on the Intracellular Uptake of PAH and PCG by the Kidney

To examine the possibility that uremic toxins share the PAH- or PCG-sensitive transport system, we analyzed the inhibitory effect of uremic toxins on the renal uptake of [^{3}H]PAH and [^{3}H]PCG using the KUI method (Table IV). All uremic toxins tested significantly inhibited the PAH uptake by the kidney. On the other hand, HA, IS and CMPF significantly inhibited renal PCG uptake, while IA did not. These results suggest that these uremic toxins would share transport systems involved in PAH or PCG uptake at the basolateral membrane in the kidney. In contrast, the transport system for IA would be different from that for PCG.

Effects of Inhibitors on the Uptake of Uremic Toxins into the Kidney

The effects of inhibitors of rOat1 and rOat3 on the renal uptake of uremic toxins were examined (Fig. 2), and their IC_{50} values are summarized in Table V. Probenecid, a nonspecific inhibitor of organic anion transporters, markedly inhibited the uptake of uremic toxins by the kidney. PAH preferentially inhibited the uptake of HA, IA, and IS into the

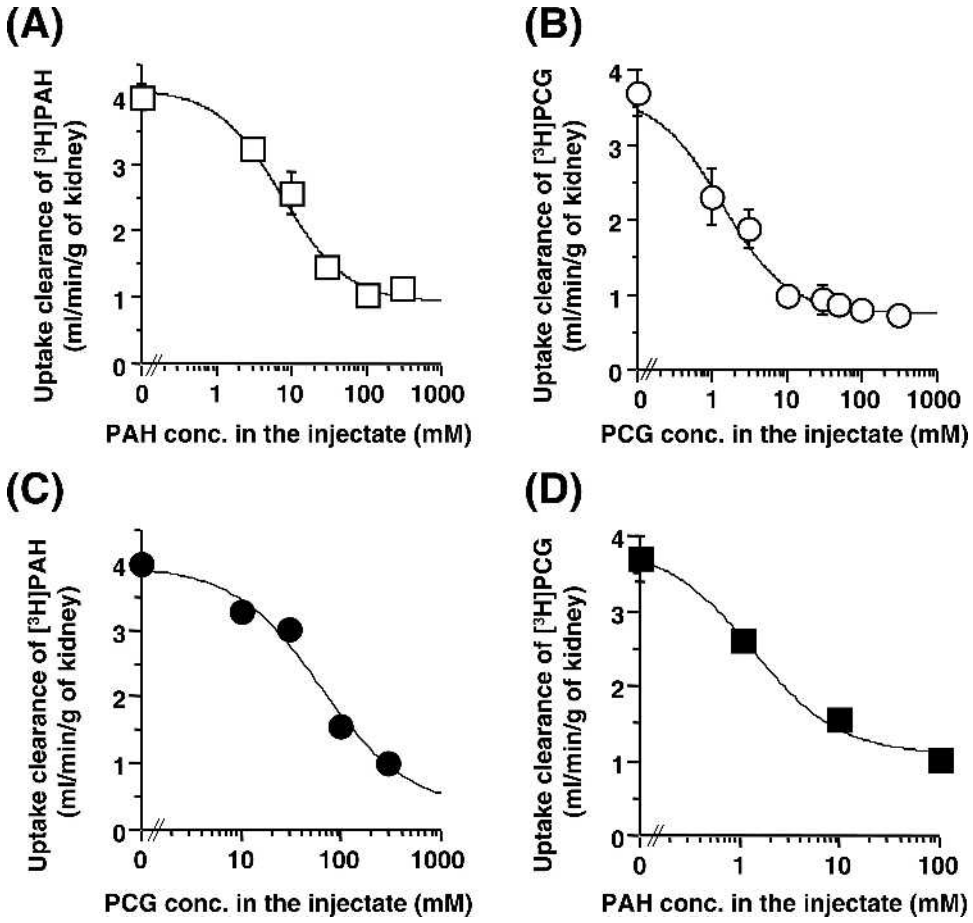

Fig. 1. Concentration-dependence of the renal uptake of PAH and PCG and their mutual inhibitory effects. (A and D) Concentration-dependent renal uptake of [^{3}H]PAH (A) and the effect of unlabeled PAH on the renal uptake of [^{3}H]PCG (D). A mixture of [^{3}H]PAH (0.3 μCi/rat) (A) or [^{3}H]PCG (0.3 μCi/rat) (D) and [^{14}C]butanol (20 nCi/rat) dissolved in 300 μl of buffered Ringer's solution was injected into abdominal aorta in the presence of 0 to 300 mM unlabeled PAH in the injectate. (B and C) Concentration-dependent renal uptake of [^{3}H]PCG (B) and the effect of unlabeled PCG on the renal uptake of [^{3}H]PAH (C). A mixture of [^{3}H]PCG (0.3 μCi/rat) (B) or [^{3}H]PAH (0.3 μCi/rat) (C) and [^{14}C]butanol (20 nCi/rat) dissolved in 300 μl of buffered Ringer's solution was injected into abdominal aorta with 0 to 300 mM unlabeled PCG in the injectate. Solid lines represent the fitted line obtained by nonlinear regression analysis. Each point represents the mean ± SE (n = 3 to 6). When the SE was small, it was included in the symbol. Abbreviations are HA, hippurate; IA, indoleacetate; IS, indoxyl sulfate; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate.

kidney, while 100 mM PCG in the injectate completely inhibited the uptake of CMPF. The renal uptake of HA, IA and IS was partially inhibited by PCG. The degree to which 10 mM PCG inhibited saturable uptake of HA, IA, IS and CMPF by the kidney was approximately 10%, 10%, 45%, and 65%, respectively.

DISCUSSION

In the current study, we used the KUI method to perform *in vivo* examination of the mechanisms by which uremic toxins are transported into the kidney. The following assumptions were used in the current methodology: 1) minimal mixing of the injection bolus with the circulating rat serum during the 5-s circulation period after injection; 2) minimal recirculation of radioactivity during the 5-s circulation period; and 3) minimal efflux of radioactivity from tissue back to the capillary compartment during the 5-s circulation period. In previous studies, KUI values of [^{14}C]PAH relative to [^{3}H]water exhibited linearity from 5 to 30 s after bolus injection, and amounts of the renal uptake of [^{3}H]PAH were not altered for up to 30 s (12,16). The above assumptions 2 and 3 are consistent with these findings. However, the potential for bolus mixing after bolus injection has not been quantified.

At concentrations of rat serum in the injection solution ranging from 5% to 90%, when the concentration of rat serum was increased, extraction of ES significantly decreased but extraction of PAH did not significantly change (Table I). In a previous study, no competitive inhibition of the extraction of [^{3}H]ES was observed in the presence of 200 μM unlabeled ES (16). Also, the unbound concentration of endogenous organic anions, such as anionic metabolites (HA, 2.86 μM; IA, 241 nM; IS, 129 nM; CMPF, 70.8 nM) (2), is relatively low in normal rat plasma compared to the K_m value of

Table III. K_m and IC_{50} Values for the Uptake of PAH and PCG into the Kidney

	[^{3}H]PAH	[^{3}H]PCG
PAH	K_m = 7.44 ± 2.94 mM (408 ± 142 μM)	IC_{50} = 1.35 ± 0.54 mM (512 ± 245 μM)
PCG	IC_{50} = 62.7 ± 28.5 mM (47.3 ± 24.1 mM)	K_m = 1.37 ± 0.33 mM (346 ± 93 μM)

The effects of unlabeled PAH or PCG on the renal uptake of [^{3}H]PAH and [^{3}H]PCG were examined. Data shown in Fig. 1 was used to determined the kinetic parameters by nonlinear regression analysis. Units of kinetic parameters are mM in the injection solution. Values corrected by the mean capillary concentration of each substance are given in parentheses. Each value represents the mean ± SD (n = 3 to 6).

PAH (408 μM, Table III), suggesting that mixing with endogenous organic anions is unlikely to inhibit renal uptake. The magnitude of the unbound fraction of ES and PAH (1.73 ± 0.14 and 80.3 ± 1.3% in rat serum, data not shown) indicates that the decrease in extraction is caused by the binding of test compounds to rat serum proteins. Thus, the effects of serum dilutions on the renal uptake of ES were used to quantify the mixing of the bolus (i.e., X values) with circulating rat serum. The mixing fractions (X values) were computed (Table II) for hypothetical values of E_0 (i.e., the true maximal extraction in the absence of bolus dilution). Even if ES clearance by the kidney, in the absence of binding proteins, is in the flow-limited range (i.e., E_0 = 90% to 100%), maximum mixing is 5.8% in the anesthetized rats. In a perfusion study using isolated rat kidney, renal extraction of unbound ES was reported to be about 55% (21). Accordingly, we hypothesized that bolus mixing with circulating rat serum would be minimal during the 5-s circulation period after injection. It has been reported that in the brain uptake index (BUI) technique, the mixing effect is less than 5% (20). Thus, the maximum mixing effect obtained in the current KUI experiment is close to that observed in experiments using the BUI technique.

Using the present method to study the unidirectional transport of uremic toxins from circulation into the kidney, the contributions of rOat1 and rOat3 to the renal uptake of uremic toxins were evaluated by examining the effects of inhibitors for rOat1 and rOat3. Previous transport studies using expression systems and rat kidney slices have revealed that the renal uptake of PAH and PCG is predominantly mediated by rOat1 and rOat3, respectively (22,23). Also, studies have shown a great difference between PAH and PCG in their K_m and K_i values for rOat1 and rOat3 (11,22,24). In the current study, the K_m values of the intracellular uptake of PAH and PCG by the kidney were 408 and 346 μM, respectively (Table III). The renal uptake of PCG and PAH was inhibited in a dose-dependent manner by PAH and PCG, respectively, with IC_{50} values of 512 μM and 47.3 mM, respectively. The IC_{50} value of PCG for PAH transport was about 137-fold greater than its K_m value (Table III), suggesting that different transporters are responsible for their uptake in the kidney. The K_m value of PAH was comparable to the IC_{50} of PAH for PCG transport, indicating that PAH affects PCG transport. Although PAH is reported to be a selective inhibitor of rOat1, the current results suggest that PAH exhibits an inhibitory effect on the renal uptake of PCG mediated by rOat3. There is no clear explanation for this difference in observed inhibitory effects of PAH. However, in transport studies using *X. laevis* oocytes, PAH has been shown to be a substrate or inhibitor of rOat3 (K_m = 65 μM) (25–27), suggesting that this discrepancy is due to differences in experimental conditions such as the environment in which rOat3 is expressed. Another issue, previous *in vivo* perfusion study using isolated rat kidney has demonstrated that K_m value of PAH was 89 μM in the renal extraction process (28), and it was comparable with those of the uptake by rOat1-expressing cells (K_m = 47 to 85 μM) (22,24,29). However, in the current KUI method, values of kinetic parameters (K_m and IC_{50} values) were several times greater than in studies using kidney slices or rOat1- and rOat3-expressing cells. The differences in kinetic parameters between *in vivo* KUI method and *in vitro* studies may be partly the result of overestimation of mean capillary concentration of substrates and binding to extracellular protein derived from minimal mixing. Considering that PCG reportedly exhibits low affinity for PAH transport by rOat1, and PAH is suggested to have inhibitory potency to rOat1 and rOat3 (30), the tendency of present inhibition studies is consistent with previous reports, although there are some technical limitations, such as estimations of capillary concentration of test compounds.

It has been reported that HA, IA, IS and CMPF inhibit rOats-mediated transport in the kidney, and suggested that

Table IV. Inhibitory Effect of Uremic Toxins on the Intracellular Uptake of PAH and PCG by the Kidney

	PAH uptake			PCG uptake		
Inhibitors	Number studied	Clearance (ml min^{-1} g^{-1} of kidney)	% of control (%)	Number studied	Clearance (ml min^{-1} g^{-1} of kidney)	% of control (%)
Control	3	4.01 ± 0.21	100	3	3.69 ± 0.30	100
HA	3	1.66 ± 0.05[a]	41.5	4	1.49 ± 0.14[a]	40.4
IA	4	1.92 ± 0.15[a]	47.9	4	3.28 ± 0.10	89.0
IS	6	1.78 ± 0.09[a]	44.3	3	2.63 ± 0.03[a]	71.2
CMPF	3	2.90 ± 0.13[a]	72.3	4	1.30 ± 0.14[a]	35.3

The effects of unlabeled uremic toxins on the renal uptake of [^{3}H]PAH and [^{3}H]PCG were examined. A solution containing both test compounds ([^{3}H]PAH or [^{3}H]PCG) and inhibitors was injected via the abdominal aorta. Concentration of inhibitors was 10 mM in the injection solution. Each value represents the mean ± SE.
HA, hippurate; IA, indoleacetate; IS, indoxyl sulfate; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate.
[a] $p < 0.01$, significantly different from corresponding control value.

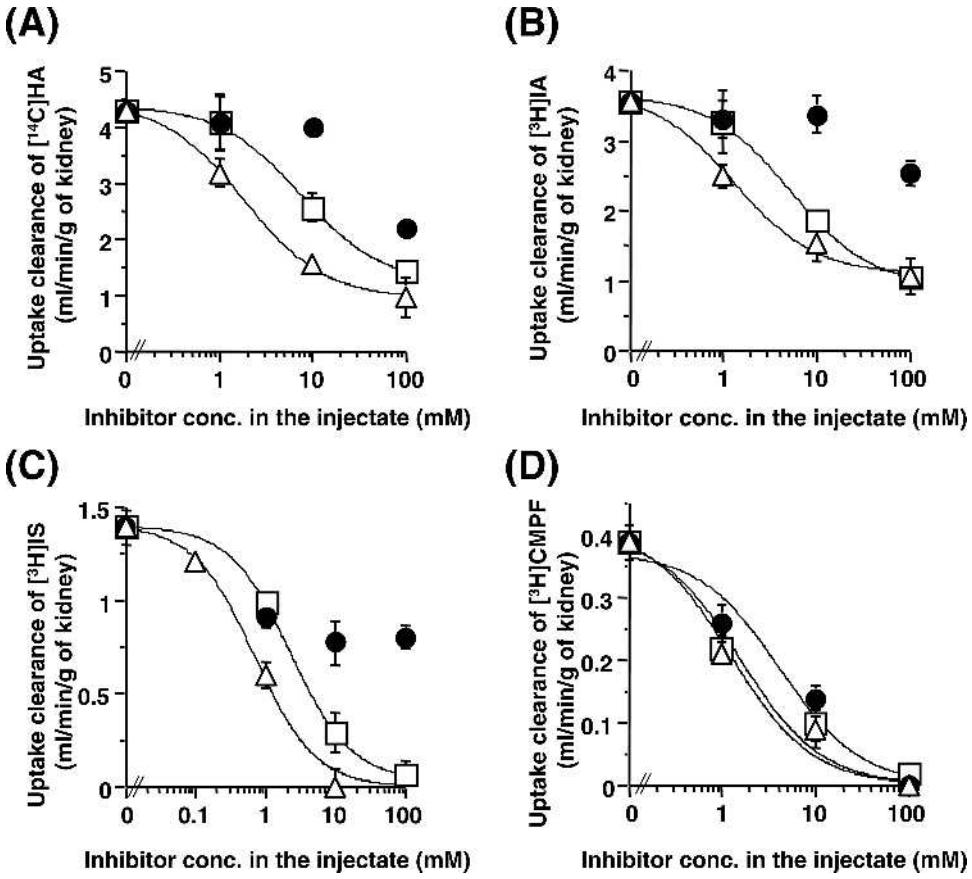

Fig. 2. Inhibitory effects of PAH, PCG, and probenecid on the intracellular uptake of uremic toxins by the kidney. Intracellular uptake of [^{14}C]HA (A), [^{3}H]IA (B), [^{3}H]IS (C), and [^{3}H]CMPF (D) by the kidney were determined in the presence and absence of unlabeled PAH (□), PCG (●), and probenecid (△) at the designated concentrations. Three hundred microliters of buffered Ringer's solution containing both test compounds ([^{14}C]HA, 60 nCi/rat; [^{3}H]IA, [^{3}H]IS, and [^{3}H]CMPF, 0.3 μCi/rat) and reference ([^{3}H]water, 0.6 μCi/rat; [^{14}C]butanol, 20 nCi/rat) was injected into abdominal aorta with 0 to 100 mM unlabeled inhibitors in the injectate. Solid line represents the fitted line obtained by nonlinear regression analysis. Each point represents the mean ± SE (n = 3 to 6). When the SE was small, it was included in the symbol. Abbreviations are: HA, hippurate; IA, indoleacetate; IS, indoxyl sulfate; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate.

these compounds are also transported by the organic anion transport system (11,13). In the current study, we demonstrated that these uremic toxins strongly inhibited the uptake of [^{3}H]PAH into the kidney (Table IV). And, HA, IS and CMPF significantly inhibited PCG uptake, while IA did not. Therefore, it is suggested that these uremic toxins would share transport systems as PAH or PCG in the kidney, although the transport system for IA would be different from that for PCG. These differential inhibitory effects agreed with the *in vitro* results obtained by the rOats-expressing cells (11,13), supporting the involvement of rOats in uremic toxins uptake in the kidney.

Table V. Inhibitory Effects of Organic Anions on the Intracellular Uptake of Uremic Toxins by the Kidney

	IC_{50} (mM) or % inhibition at 100 mM			
	[^{14}C]HA	[^{3}H]IA	[^{3}H]IS	[^{3}H]CMPF
Inhibitors				
Probenecid	1.85 ± 0.31 mM	1.31 ± 0.59 mM	0.692 ± 0.150 mM	1.23 ± 0.53 mM
PAH	7.44 ± 0.79 mM	5.24 ± 0.53 mM	2.26 ± 0.02 mM	1.54 ± 0.75 mM
PCG	62.6 ± 0.8%^{b}	41.0 ± 2.9%^{a}	64.6 ± 4.3%^{a}	4.12 ± 2.21 mM

Units of kinetic parameters are mM in the injection solution. Data are means ± SD (n = 3 to 6). HA, hippurate; IA, indoleacetate; IS, indoxyl sulfate; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionate.

^{a} $p < 0.05$, ^{b} $p < 0.01$, significantly different from corresponding control uptake.

In order to evaluate the contribution of each transporter to the renal uptake of uremic toxins, we conducted inhibition studies using PAH and PCG. Probenecid, a nonspecific inhibitor of organic anion transporters, markedly inhibited the intracellular uptake of uremic toxins by the kidney, indicating that saturable transport was involved in their uptake by the kidney. The renal uptake of HA and IA was preferentially inhibited by PAH, with IC_{50} values similar to the apparent K_m value of PAH (Fig. 2; Tables III and V), suggesting that rOat1 is the primary mediator of their uptake in the kidney. In contrast, 100 mM PCG in the injectate completely inhibited the uptake of CMPF, suggesting that rOat3 mediates uptake of CMPF. The kinetic parameters of PCG (Table III) indicate that 10 mM PCG in the injectate will saturate PCG-sensitive transport, but will have only a minimal effect on PAH-sensitive uptake. Therefore, the degree of inhibition of saturable uptake by 10 mM PCG represents the contribution of rOat3 to the renal uptake of uremic toxins (HA, 10%; IA, 10%; IS, 45%; CMPF, 65% inhibition of the total saturable component). On the other hand, 100 mM PAH in the injectate will saturate both PAH- and PCG-sensitive transport, suggesting that the inhibitory effects of 100 mM PAH (HA, 85%; IA, 100%; IS, 97%; CMPF, 100% inhibition of the total saturable component) represent the contribution of rOat1 and rOat3. Consequently, the contribution of rOat1 can be estimated to be about 75%, 90%, 55%, and 35% of the renal uptake of HA, IA, IS, and CMPF, respectively. We found that 15% of the saturable uptake of HA remained even in the presence of 100 mM PAH in the injection solution (Fig. 2A); this remaining fraction cannot be accounted for by PAH- or PCG-sensitive transport. The contribution of rOat1 and rOat3 to the renal uptake of uremic toxins determined in the present KUI experiment was in good agreement with estimates obtained in studies using kidney slices or stable transfectants of rOat1 and rOat3. That is, rOat1 was found to be the primary mediator of HA and IA uptake, whereas rOat3 was found to be the primary mediator of CMPF uptake. rOat1 and rOat3 contributed equally to the renal uptake of IS. Thus, the KUI method is a useful technique for estimating the contributions made by individual transporters to the *in vivo* uptake process. By using the KUI method, further studies are necessary to investigate whether the contribution of rOats is altered when the function and/or expression levels of rOats are modulated by various conditions.

Recent findings suggest that uremic toxins promote the progression of renal failure by damaging tubular cells (4,5,7). Consequently, increased concentrations of uremic toxins in the renal tubules would exacerbate the deterioration of renal function in chronic renal failure (8,9). Very recently, we found that rOat1/hOAT1 and rOat3/hOAT3 are involved in the renal uptake of uremic toxins, suggesting that OATs-mediated uptake of these toxins leads to further loss of nephrons (11,13). In the current study, intracellular uptake of uremic toxins by the kidney was found to be mediated by PAH- and PCG-sensitive transport, suggesting that OATs-mediated transport plays an important role in progressive renal injury by uremic toxins under *in vivo* physiologic conditions. There are two likely general mechanisms for accumulation of uremic toxins in the renal tubular cells. In the first, basolateral organic anion transporters mediate the accumulation of uremic toxins. In the second, inhibition or malfunction of excretion across the brush-border membrane causes cellular accumulation of uremic toxins. Further studies of renal physiology are needed to identify transporters responsible for luminal secretion of uremic toxins and to quantify the expression of these transporters in chronic renal failure patients.

In conclusion, we have demonstrated that rOat1 and rOat3 contribute to the renal uptake of uremic toxins under *in vivo* conditions. The present findings will be very important not only in helping us further understand the development of nephrotoxicity, but also in improving the way we treat progressive renal failure caused by uremic toxins.

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