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ORIGINAL RESEARCH

Transport of Kynurenic Acid by Rat Organic Anion Transporters rOAT1 and rOAT3: Species Difference between Human and Rat in OAT1

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Abstract: A tryptophan catabolite, kynurenic acid, is involved in schizophrenia and uremia; there is little information on the mechanism of its disposition. Recently, our laboratory showed that kynurenic acid is a good substrate of human organic anion transporters hOAT1 and hOAT3. In this study, we performed uptake experiment using *Xenopus laevis* oocytes to characterize the transport of kynurenic acid by rat homologs of the transporters, rOAT1, and rOAT3. These transporters stimulated the uptake of kynurenic acid into oocytes, and transport by rOAT3 was marked. The K_m values of the transport were estimated to be 8.46 μM for rOAT1 and 4.81 μM for rOAT3, and these values are comparable to their human homologs. The transport activity of kynurenic acid by rOAT1 was about one quarter of that of *p*-aminohippurate, although they were at the similar levels in hOAT1. A comparative experiment with hOAT1 was added in this study, showing that uptake amounts of kynurenic acid by hOAT1-expressing oocytes were 4 times greater than rOAT1-expressing oocytes. rOAT3 transported kynurenic acid as efficiently as estrone sulfate; this phenomenon was also observed in hOAT3. In conclusion, transport of kynurenic acid by rOAT1 and rOAT3 was shown. The characteristics of rOAT3 were similar to hOAT3, but low transport activity of kynurenic acid by rOAT1 was exhibited compared with hOAT1.

Keywords: OAT1, OAT3, transport, kynurenic acid, species difference

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Introduction

Kynurenic acid is one of the final metabolites of tryptophan and is known to antagonize both the *N*-methyl D-aspartate (NMDA) receptor and the α7 nicotinic acetylcholine receptor in the brain under physiological conditions.[1,2] Clinical studies have shown that administration of NMDA receptor antagonists causes schizophrenia-like symptoms; several groups have also shown the increased concentration of kynurenic acid in the brains of schizophrenic patients.[3–7] Taking these findings together, it is suggested that kynurenic acid is one of the key molecules in schizophrenia. In addition, it was shown that the serum concentration of kynurenic acid was elevated in patients with developed chronic renal failure.[8,9] Its accumulation was thought be related to certain uremic symptoms, including neurological disturbances, increased susceptibility to infectious disorders, lipid metabolism disorders, and anemia.[1,2,8,9] It was shown that a reduction in kynurenic acid levels enhanced vulnerability to an extotoxic insult in the rat striatum; kynurenic acid has been considered to have anticonvulsant and neuroprotective properties.[2] Therefore, the molecular mechanism of the disposition of kynurenic acid is considered to be intriguing, although it remains to be elucidated.

In epithelial cells of renal proximal tubules and brain capillary endothelial cells, several organic anion transporters mediate tubular secretion and efflux from the brain of a variety of endogenous metabolites, xenobiotics, and drugs.[10,11] Organic anion transporter 1 (OAT1) and OAT3 are expressed in the basolateral membrane of the renal proximal tubule. They are responsible for tubular uptake of organic anions from blood.[10,11] In addition, using rats, OAT3 has been shown to be expressed at the abluminal membrane of brain capillary endothelial cells and to play an important role in the transport of indoxyl sulfate, homovanillic acid, benzylpenicillin, and pravastatin from the brain to blood.[12–15] Goralski et al have shown that renal clearance of kynurenic acid is greater than that of creatinine in rats, implying that renal tubular secretion contributes to its urinary excretion.[16] In the rat brain, it has been reported that a representative inhibitor of organic anion transporter, probenecid, escalates the concentration of kynurenic acid.[17,18] These reports propose that OAT1 and/or OAT3 might be responsible for the disposition of kynurenic acid in the kidney and brain.

Recently, we examined the interaction of kynurenic acid with human OAT1 (hOAT1) and hOAT3; it was revealed that kynurenic acid is a substrate of both transporters.[19] In addition, hOAT1 and hOAT3 transported kynurenic acid as efficiently as their typical substrates, *p*-aminohippurate and estrone sulfate, respectively; it is suggested that the transport would play an important role physiologically.[19] To investigate the contribution of these transporters in the handling of kynurenic acid in the brain and kidney in detail, experiments using animals and tissues should be performed and information on transporter characteristics is also required, for the different species. In the present study, to characterize the transport of kynurenic acid by rat OAT1 (rOAT1) and rOAT3, we conducted an uptake experiment with the *Xenopus laevis* oocyte expression system. The obtained results together demonstrate species differences between human and rat in the transport of kynurenic acid by OAT1.

Materials and Methods

Materials

[^{3}H]*p*-Aminohippurate (4.53 Ci/mmol) and [^{3}H] estrone sulfate (57.3 Ci/mmol) were obtained from PerkinElmer Life Science (Boston, MA, USA). [^{3}H] Aminopterin (35.1 Ci/mmol) and [^{3}H]kynurenic acid (50 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA) and American Radiolabeled Chemicals, Inc (St. Louis, MO, USA), respectively. Unlabeled kynurenic acid was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). All other chemicals used were of the highest purity available.

Uptake experiment using *Xenopus laevis* oocytes expressing rOAT1, rOAT3, or hOAT1

pBK-CMV plasmid vectors containing cDNA of rOAT1, rOAT3 or hOAT1 were a kind gift from Professor Ken-ichi Inui (Kyoto University Hospital, Kyoto, Japan). The uptake experiment using *Xenopus laevis* oocytes was performed as previously reported.[20] Briefly, capped RNA encoding each organic anion transporter was transcribed from the appropriate restriction enzyme-linearized pBK-CMV containing cDNA of rOAT1, rOAT3 or hOAT1, with T3 RNA polymerase. After 50 nL of

water or cRNA (25 ng) was injected into defolliculated oocytes, the oocytes were maintained in modified Barth's medium (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$ and 5 mM HEPES; pH 7.4) containing 50 mg/L gentamicin at 18 °C. Two or three days after injection, the uptake reaction was initiated by incubating the oocytes in 500 µL uptake buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES; pH 7.4), with each radiolabeled compound at room temperature in the absence or presence of an unlabeled compound for the indicated periods. The uptake reaction was terminated by adding 2 mL ice-cold uptake buffer to each well; oocytes were washed three times with 2 mL ice-cold buffer. After washing, each oocyte was transferred to a scintillation counting vial and solubilized in 150 µL of 10% sodium lauryl sulfate. Two milliliters of scintillation cocktail Clear-sol II (Nacalai Tesque, Kyoto, Japan) were added to each solubilized oocyte and radioactivity was determined using a liquid scintillation counter.

Kinetic analysis

The kinetic parameters of kynurenic acid transport by rOAT1 and rOAT3 were calculated using 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 (pmol/oocyte/2 hours for rOAT1, pmol/oocyte/hourr for rOAT3), V_{max} is the maximum velocity by the saturable process (pmol/oocyte/2 hr for rOAT1, pmol/oocyte/hr for rOAT3), [S] is the concentration of kynurenic acid (µM), K_m is the Michaelis-Menten constant (µM).

Data analysis

Eight to ten oocytes were used in each condition in one uptake experiment; the same experiments were performed three times with different frogs. The mean ± S.E.M. was estimated using the data from these 3 experiments. It is shown in Table and Figures.

Data were analyzed by the unpaired *t*-test or one-way analysis of variance followed by Dunnett's test using GraphPad Prism, version 5.0 (GraphPad Software, San Diego, CA, USA), and by Scheffé's test using KaleidaGraph (Synergy Software, Reading, PA, USA). Differences were considered significant at $P < 0.05$.

Results and Discussion

We investigated the transport characteristics of kynurenic acid by rOAT1 and rOAT3. Figure 1 shows the time dependency of kynurenic acid uptake by the transporters. The injection of rOAT1 cRNA stimulated the uptake of the compound into oocytes; the rOAT1-mediated transport of kynurenic acid increased linearly up to 2 hours. Time-dependent transport of kynurenic acid by rOAT3 was also observed. These findings indicate that rOAT1 and rOAT3 recognize kynurenic acid as a substrate. The uptake amounts of kynurenic acid in the oocytes injected with rOAT3 cRNA were much greater than those in the oocytes injected with rOAT1 cRNA.

Figure 2 demonstrates the dose-dependent uptake of kynurenic acid by rOAT1 and rOAT3. Because the transport activity of kynurenic acid by rOAT1 was not high enough, the uptake experiment of the compound was performed with an incubation time of 2 hours for the transporter. However, an hour-long incubation was conducted for rOAT3 due to the linearity of kynurenic acid transport by rOAT3 (Fig. 1). The uptake of kynurenic acid by rOAT1 and rOAT3 elevated concentration-dependently; saturation was observed in both transporters. The K_m values of the transports were calculated to be

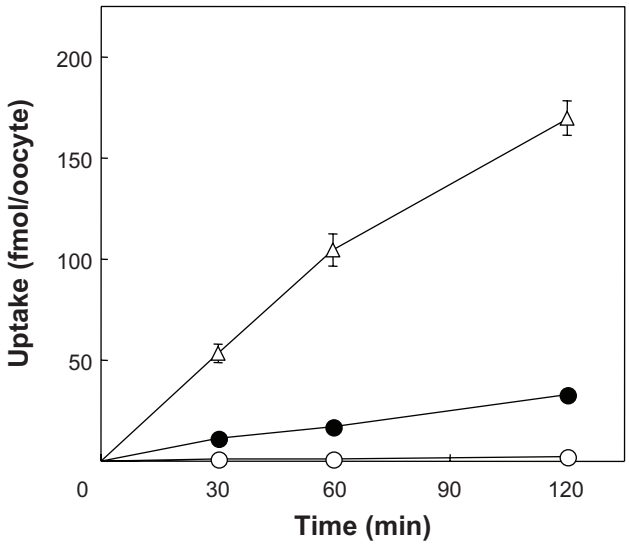

Figure 1. Time-dependent uptake of kynurenic acid by rOAT1 and rOAT3.
Notes: Oocytes injected with water (open circle), rOAT1 cRNA (closed circle), or rOAT3 cRNA (open triangle) were incubated with 20 nM [^{3}H] kynurenic acid for the indicated periods. The uptake amounts of [^{3}H] kynurenic acid in each oocyte were determined. Each point represents the mean ± S.E.M. of 29 to 30 oocytes from 3 experiments. When an error bar is not shown, it is smaller than the symbol.

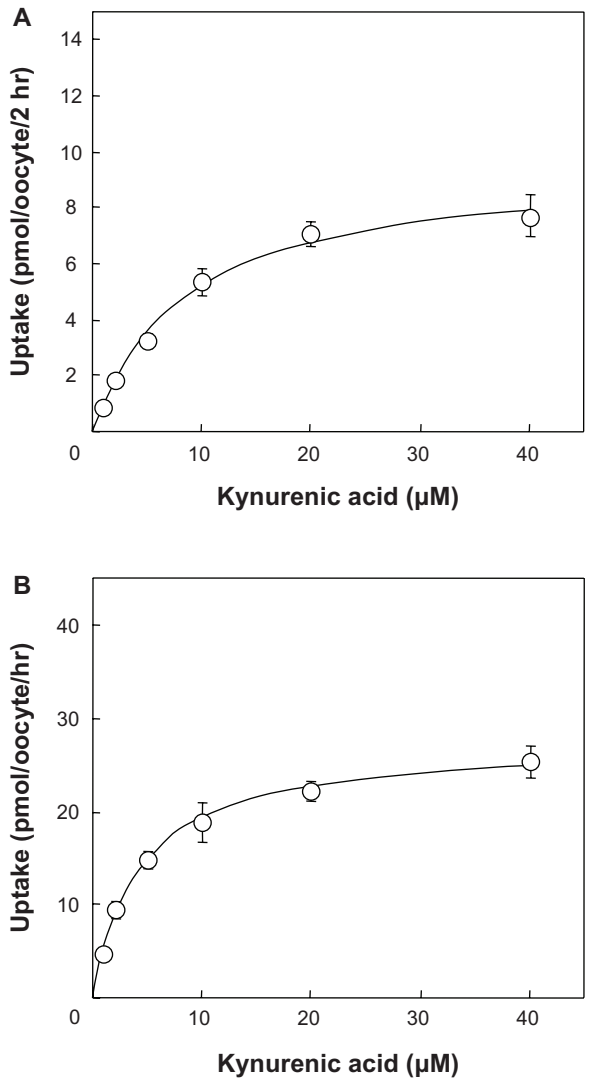

Figure 2. Concentration-dependent uptake of kynurenic acid by rOAT1 (**A**) and rOAT3 (**B**). (**A**) Oocytes injected with rOAT1 cRNA were incubated with [^{3}H]kynurenic acid at various concentrations for 2 hours. rOAT1-mediated uptake of [^{3}H]kynurenic acid was determined by subtracting its uptake amount in water-injected oocytes from that in oocytes injected with rOAT1 cRNA. (**B**) Oocytes injected with rOAT3 cRNA were incubated with [^{3}H]kynurenic acid at various concentrations for 1 hour.
Notes: rOAT3-mediated uptake of [^{3}H]kynurenic acid was determined by subtracting its uptake amount in water-injected oocytes from that in oocytes injected with rOAT3 cRNA. Each point represents the mean ± S.E.M. of 27 to 30 oocytes from 3 experiments. When an error bar is not shown, it is smaller than the symbol.

8.46 ± 0.30 μM for rOAT1 and 4.81 ± 1.15 μM for rOAT3 (mean ± S.E.M. from 3 experiments). The V_{max} values were estimated to be 9.67 ± 1.22 pmol/oocyte/2 hours for rOAT1 and 28.0 ± 0.7 pmol/oocyte/hour for rOAT3.

Table 1 shows the transport activities of kynurenic acid by rOAT1 and rOAT3, in addition to those of their typical substrates, *p*-aminohippurate and estrone sulfate, respectively. The uptake of kynurenic acid in

Table 1. Uptake of *p*-aminohippurate, estrone sulfate and kynurenic acid by rOAT1 and rOAT3

	Control	Uptake (nL/oocyte/hr)	
		rOAT1	rOAT3
p-aminohippurate	105 ± 6	2961 ± 190***	N.D.
Estrone sulfate	102 ± 6	N.D.	3816 ± 403***
Kynurenic acid	71.0 ± 3.6	848 ± 59***	4486 ± 216***

Notes: Oocytes injected with water (control), rOAT1 cRNA, or rOAT3 cRNA were incubated with 221 nM [^{3}H]*p*-aminohippurate, 17.5 nM [^{3}H]estrone sulfate or 20 nM [^{3}H]kynurenic acid for 1 hour. The uptake amounts of the radiolabeled compounds in each oocyte were determined and divided by their concentrations in uptake buffer. Each value is the mean ± S.E.M. of 28 to 30 oocytes from 3 experiments. ***$P < 0.001$, significantly different from the control values.
Abbreviation: N.D., not determined.

rOAT1 cRNA-injected oocytes was about one quarter that of *p*-aminohippurate. This finding is thought to be the most impressive in this study when it is compared with the results on the human homolog of OAT1. The transport activity of kynurenic acid by hOAT1 was comparable to that of *p*-aminohippurate,[19] indicating a species difference in kynurenic acid transport by OAT1. The uptake of kynurenic acid by rOAT3 was matched to that of estrone sulfate, implying that kynurenic acid was transported by rOAT3 as efficiently as estrone sulfate. Equivalence to the transport of estrone sulfate and kynurenic acid by hOAT3 was also observed.[19]

To superimpose data representing the transport activity of *p*-aminohippurate and kynurenic acid by hOAT1 and rOAT1, we measured their uptake amounts in oocytes injected with water or cRNA of hOAT1 and rOAT1. Furthermore, transport of antifolate aminopterin was added. Aminopterin is a compound structurally related with methotrexate; our previous report showed that hOAT1 preferred aminopterin to methotrexate.[20] As illustrated in Figure 3, uptake of all compounds was stimulated by hOAT1 and rOAT1. No difference was recognized between hOAT1 and rOAT1 in the uptake of *p*-aminohippurate. hOAT1 mediated aminopterin uptake into oocytes as efficiently as rOAT1. However, the uptake amount of kynurenic acid by rOAT1 was nearly one quarter of that by hOAT1. These findings clearly show that the transport activity of kynurenic acid by rOAT1 is

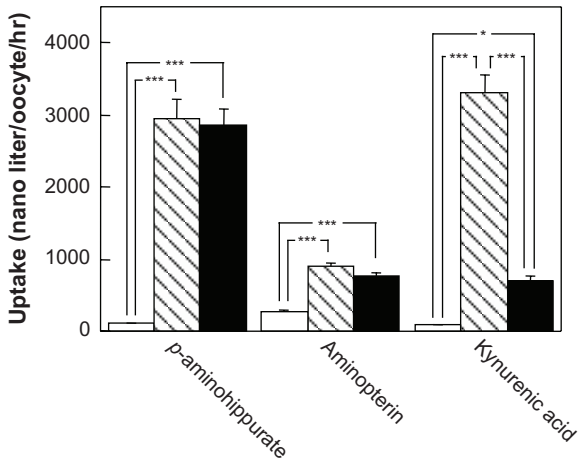

Figure 3. Uptake of *p*-aminohippurate, aminopterin and kynurenic acid by hOAT1 and rOAT1.
Notes: Oocytes injected with water (open column), hOAT1 cRNA (hatched column) or rOAT1 cRNA (closed column) were incubated with 221 nM [^{3}H]*p*-aminohippurate, 28.5 nM [^{3}H]aminopterin or 20 nM [^{3}H] kynurenic acid for 1 hr. The uptake amounts of the radiolabeled compounds in each oocyte were determined and divided by their concentrations in uptake buffer. Each column represents the mean ± S.E.M. of 26 to 30 oocytes from 3 experiments. ***$P < 0.001$, significantly different; *$P < 0.05$, significantly different.

lower than hOAT1. As described above, this study estimated the K_m value of rOAT1-mediated transport of kynurenic acid to be 8.46 μM. The value of hOAT1 was reported to be 5.06 μM,[19] implying no difference in the affinity with kynurenic acid. Accordingly, from a kinetic aspect, the low maximum velocity of its transport is speculated to be the main factor for the poor transport of kynurenic acid by rOAT1.

Meanwhile, the K_m value of the kynurenic acid transport by hOAT3 was calculated to be 4.86 μM in the previous study;[19] a value compatible to that of rOAT3. Including the comparison with the uptake activity of estrone sulfate, the transport characteristics of kynurenic acid by rOAT3 are identical to those of hOAT3.

Thus far, from the species differences perspective, transport characteristics of substances by OAT1 and OAT3 have not been investigated well. Because the amino acid sequence of rOAT1 exhibits 87.8% identity to that of hOAT1,[21] few researchers might suppose any existence of a species difference in their function. To our knowledge, kynurenic acid is the first compound that shows species difference between humans and rats in transport by OAT1.

Kynurenic acid is attracting attention because its increased content in the brain has been linked to schizophrenia.[5–7] Additionally, kynurenic acid is accepted as an uremic toxin.[22] Accordingly, elucidation of its elimination route is necessary to deepen understanding of the molecular mechanism of kynurenic acid-involved pathology; the contribution of OAT1 and OAT3 is therefore interesting. The results of our study suggest that consideration should be given to whether rOAT3 might play an important role in the elimination of kynurenic acid in the brains and the tubular secretions of rats. To assess the role of rOAT3 in the brain and kidney, experiments with the brain efflux index method and renal slices are required, respectively.[12,23] The final purpose is to assess the work of hOAT1 and hOAT3 in the disposition of kynurenic acid in humans, as well as its relationship with the pathology. Because the present study shows the high transport activity of kynurenic acid by hOAT1 in comparison with rOAT1, hOAT1 should not be ignored.

Conclusion

In conclusion, we investigated the transport of kynurenic acid by rOAT1 and rOAT3; the findings obtained show that kynurenic acid is their substrate. The transport characteristics of kynurenic acid by rOAT3, including the K_m value and the equivalence in the transport efficiencies of kynurenic acid and estrone sulfate, corresponded to those of hOAT3. On the other hand, the transport activity of kynurenic acid by rOAT1 was lower than hOAT1, and a species difference was demonstrated. This is a rare example exhibiting species differences in the transport of a compound by OAT1. The obtained findings provide useful information in the consideration of the role of OAT1 and OAT3 in the disposition of kynurenic acid in rats.

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Author Contributions

Conceived and designed the experiments: YU, HH. Analysed the data: YU, HH. Wrote the first draft of the manuscript: YU. Contributed to the writing of the manuscript: YU, KI. Agree with manuscript results and conclusions: YU, HH, KI. Jointly developed the

structure and arguments for the paper: YU, KI. Made critical revisions and approved final version: YU, HH, KI. All authors reviewed and approved of the final manuscript.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

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