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# Identification and functional characterization of uric acid transporter Urat1 (*Slc22a12*) in rats

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ABSTRACT

Uric acid transporter URAT1 contributes significantly to reabsorption of uric acid in humans to maintain a constant serum uric acid (SUA) level. Since alteration of SUA level is associated with various diseases, it is important to clarify the mechanism of change in SUA. However, although expression of mRNA of an ortholog of *URAT1* (*rUrat1*) in rats has been reported, functional analysis and localization have not been done. Therefore, rat rUrat1 was functionally analyzed using gene expression systems and isolated brush-border membrane vesicles (BBMVs) prepared from rat kidney, and its localization in kidney was examined immunohistochemically. Uric acid transport by rUrat1 was chloride ($Cl^-$) susceptible with a Km of 1773 μM. It was inhibited by benzbromarone and *trans*-stimulated by lactate and pyrazinecarboxylic acid (PZA). $Cl^-$ gradient-susceptible uric acid transport by BBMVs showed similar characteristics to those of uric acid transport by rUrat1. Moreover, rUrat1 was localized at the apical membrane in proximal tubular epithelial cells in rat kidney. Accordingly, rUrat1 is considered to be involved in uric acid reabsorption in rats in the same manner as URAT1 in humans. Therefore, rUrat1 may be a useful model to study issues related to the role of human URAT1.



## 1. Introduction

Uric acid is the end product of purine metabolism in humans [1]. Serum uric acid (SUA) level is maintained by the balance between synthesis, catalyzed by xanthine oxidoreductase, and excretion [2]. Uric acid is mainly produced in liver, muscles, and intestine. Approximately two thirds of the daily excretion is accounted for by urinary excretion, with the remaining one third being excreted into the gut.

A number of studies have shown that alterations of SUA level are linked to various human diseases, such as gout, hypertension, cardiovascular disease, kidney disease, multiple sclerosis, Parkinson's disease, Alzheimer's disease, and optic neuritis [3–12]. Therefore, it is suggested that SUA level should be controlled within the normal range between 120 and 380 μM, depending on gender [13]. However, various pharmaceutical drugs are known to affect SUA levels [14]. For example, some antihypertensive angiotensin II receptor blockers (ARBs) inhibit uric acid transporters for reabsorption in kidney, resulting in a decrease of SUA level. On the other hand, some ARBs, which stimulate reabsorptive transporters or inhibit secretion transporters, may cause an increase of SUA level [15–18]. But, to date, it remains unclear how other drugs influence SUA level. To better understand drug-induced alterations of SUA level, it is important to clarify the uric acid handling mechanisms in human kidney. In humans, excretion of uric acid into urine is a complex process, which has been explained in terms of the so-called four-component model, which includes glomerular filtration, reabsorption, secretion and post-secretory reabsorption [1]. Therefore, it is not easy to accurately evaluate renal handling of uric acid in humans.

Animal models would be useful to examine uric acid excretion mechanisms in detail, but species differences in renal handling of uric acid are substantial [19]. For example, swine and rabbits excrete more uric acid than is filtered through the glomerulus, and uric acid is not reabsorbed in avian kidney. Although the SUA level in rats is maintained at a lower level by uricase, which metabolizes uric acid to allantoin, uric acid is reabsorbed in kidney, as it is in humans. Furthermore, studies using isolated BBMVs of kidney [20] indicate that the rat renal transport system for uric acid is similar to that in humans. Therefore, rats are considered to be a pertinent animal model to evaluate the molecular and functional characteristics of the human uric acid handling system.

Human urate transporter 1, URAT1 (*SLC22A12*), and mouse renal specific transporter Rst (*Slc22a12*) have been identified as uric acid transporters at the apical membrane of proximal tubular cells [21,22]. Since mutations of *URAT1* in humans and deficiency of *Rst* in mice

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result in an increase of urinary excretion of uric acid [23,24], these transporters are considered to play an important role in uric acid handling in kidney. Although no functional studies have been reported, the amino acid sequence of rat Urat1 (rUrat1) indicated that this transporter might be the rat ortholog of human URAT1 [25]. Therefore, in the present study, we aimed to characterize rUrat1 function by examining the transport of uric acid in *rUrat1*-expressing cells and BBMVs prepared from rat kidney. Furthermore, immunolocalization of rUrat1 expressed in kidneys was examined using an anti-rat Urat1 polyclonal antibody raised in rabbits.

## 2. Materials and methods

### 2.1. Chemicals

[$^{14}$C]Uric acid (1.96 TBq/mol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). All other reagents were purchased from Kanto Chemicals (Tokyo, Japan), Sigma-Aldrich (St. Louis, MO), and Wako Pure Chemical Industries Ltd. (Osaka, Japan), respectively.

### 2.2. Subcloning of rUrat1 (Slc22a12)

rUrat1 cDNA (pExpress-1/rUrat1) was purchased from Open Biosystems (Huntsville, AL). The open reading frame of rUrat1 was inserted into pcDNA3.1 (Invitrogen) at the EcoRI and NotI sites and then transferred to pGEMHE [26] at the EcoRI and XbaI sites. For cRNA synthesis, rUrat1 cDNA (pGEMHE/rUrat1) was digested with NheI. The obtained cDNA sequence was verified (GenBank accession no. BC103638).

### 2.3. Transport study using Xenopus laevis oocytes

Complementary RNA (cRNA) of rUrat1 was prepared by in vitro transcription with T7 RNA polymerase in the presence of ribonuclease inhibitor and an RNA cap analog using a mMESSAGE mMACHINE kit (Ambion, Austin, TX). For transport experiments with rUrat1, defolliculated oocytes were injected with 25 ng of rUrat1 cRNA or the same volume of water and incubated in modified Barth's solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4) containing 50 μg/ml gentamycin at 18 °C for 3 days as reported previously [16]. The oocytes were transferred to a 24-well plate and preincubated in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.4) at 25 °C for 60 min. To initiate uptake of [$^{14}$C]uric acid, the oocytes were incubated with uptake buffer (96 mM Na gluconate, 2 mM K gluconate, 1 mM Mg gluconate, 1.8 mM Ca gluconate, and 5 mM HEPES, pH 7.4) containing 20 μM [$^{14}$C]uric acid at 25 °C for the designated time. In *cis*-inhibitory studies, drugs tested were added simultaneously with [$^{14}$C]uric acid. In the *trans*-stimulation study, the oocytes were microinjected with 50 nl of drug solution or water containing 1% dimethyl sulfoxide (DMSO). Immediately after the microinjection (within approximately 2 min), the oocytes were transferred to uptake buffer containing [$^{14}$C]uric acid to initiate uptake. Uptake was terminated by washing the oocytes three times with ice-cold uptake buffer. The oocytes were solubilized with 5% sodium dodecyl sulfate solution.

### 2.4. Membrane preparation

Renal BBMVs were isolated from the kidney cortex of rats using a calcium precipitation method according to the procedures reported previously [27–29]. Experiments were approved by the Committee on Care and Use of Laboratory Animals of Kanazawa University. Male Sprague–Dawley rats (7–8 weeks old) were anesthetized with diethylether. The kidneys were perfused with isotonic saline containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) via the descending aorta until cleared of blood, then removed and placed in ice-cold saline containing 0.5 mM PMSF. After removal of the capsule, they were sliced with a razor. The cortex was dissected from the medulla and homogenized in ice-cold vesicle buffer-A solution containing 50 mM mannitol and 2 mM Tris/MES (pH 7.0) with a Waring blender for 5 min at 18000 rpm. $CaCl_2$ solution (1 M) was added to the homogenate to give a final concentration of 10 mM and the suspension was stirred for 15 min at 4 °C, then centrifuged at 3000 × *g* for 15 min. The supernatant was further centrifuged at 43000 × *g* for 20 min. The resultant pellet was suspended, through a 25-gauge needle, in ice-cold vesicle buffer-B (100 mM mannitol, 120 mM NaCl and 10 mM HEPES/Tris (pH 7.4)) or buffer-C (60 mM mannitol, 125 mM K gluconate, 10 mM HEPES/Tris (pH 7.4)) for *trans*-stimulation study. The suspension was again centrifuged at 43000 × *g* for 20 mm, and the resultant pellet was resuspended in buffer-B or buffer-C through the same needle to give a protein concentration of 11.5 ± 1.5 mg of protein/ml.

The purity of the BBMVs was assessed in terms of the activity of alkaline phosphatase, which is a marker enzyme for BBM. The activity was enriched 16.5 ± 2.9-fold (mean ± S.E.M., $n = 6$) with respect to the initial homogenate. This level of enrichment of the marker enzyme activity indicates that the isolated membrane fraction had been adequately purified and was sufficiently rich in BBM.

### 2.5. Transport study using BBMVs

The uptake of [$^{14}$C]uric acid by BBMVs was performed at 25 °C using a rapid filtration technique, as described previously [27]. After preincubation of BBMVs at 25 °C for 1 min, the influx of [$^{14}$C]uric acid was initiated by adding an aliquot of 90 μL of the uptake buffer containing [$^{14}$C]uric acid to 10 μL of membrane suspension for the designated time. The uptake buffer was composed of 100 mM mannitol, 120 mM Na gluconate and 10 mM HEPES/Tris (pH 7.4). In the presence of $Cl^-$, Na gluconate was replaced with NaCl. In *cis*-inhibitory studies, tested drugs were added simultaneously with [$^{14}$C]uric acid. In the *trans*-stimulation study, BBMVs were preincubated with 10 mM Na gluconate, Na L-lactate or Na pyrazine carboxylate, 115 mM K gluconate, 60 mM mannitol and 10 mM HEPES-Tris, pH 7.4. Incubation was terminated by adding 1 ml of ice-cold stop solution containing 140 mM mannitol and 120 mM Na gluconate, 0.1 mM probenecid and 10 mM HEPES/Tris (pH 7.4) and immediately filtered through the Millipore filter (Millipore Ltd., Bedford, MA; 0.45 μm) under vacuum. The filter was washed rapidly twice with 5 ml of ice-cold stop solution.

### 2.6. Analytical method

For uptake studies, the radioactivity was measured using a liquid scintillation counter (LSC-5100, Aloka, Tokyo). Uptake was expressed as the cell-to-medium ratio (μl per mg protein or per oocyte), obtained by dividing the uptake amount by the concentration of substrate in the uptake medium. In uptake studies using oocytes, results are shown as mean ± standard error obtained from 10 oocytes. Transporter-mediated initial uptake rates were obtained after subtraction of the uptake by water-injected oocytes from that by rUrat1 cRNA-injected oocytes. In uptake studies using BBMVs, $Cl^-$ gradient-dependent uptake of uric acid was determined by subtraction of the uptake in $Cl^-$-containing buffer from that in $Cl^-$-free buffer. For the evaluation of the kinetic parameters, the rates were fitted to the following Eq. (1) by means of nonlinear least-squares regression analysis using Kaleidagraph (Synergy Software, Reading, PA).

$$v = \frac{\mathrm{Vmax} \times s}{\mathrm{Km} + s}, \tag{1}$$

where $v$, $s$, Km, and Vmax are the initial uptake rate of substrate (pmol per indicated time per mg protein or per oocyte), the substrate

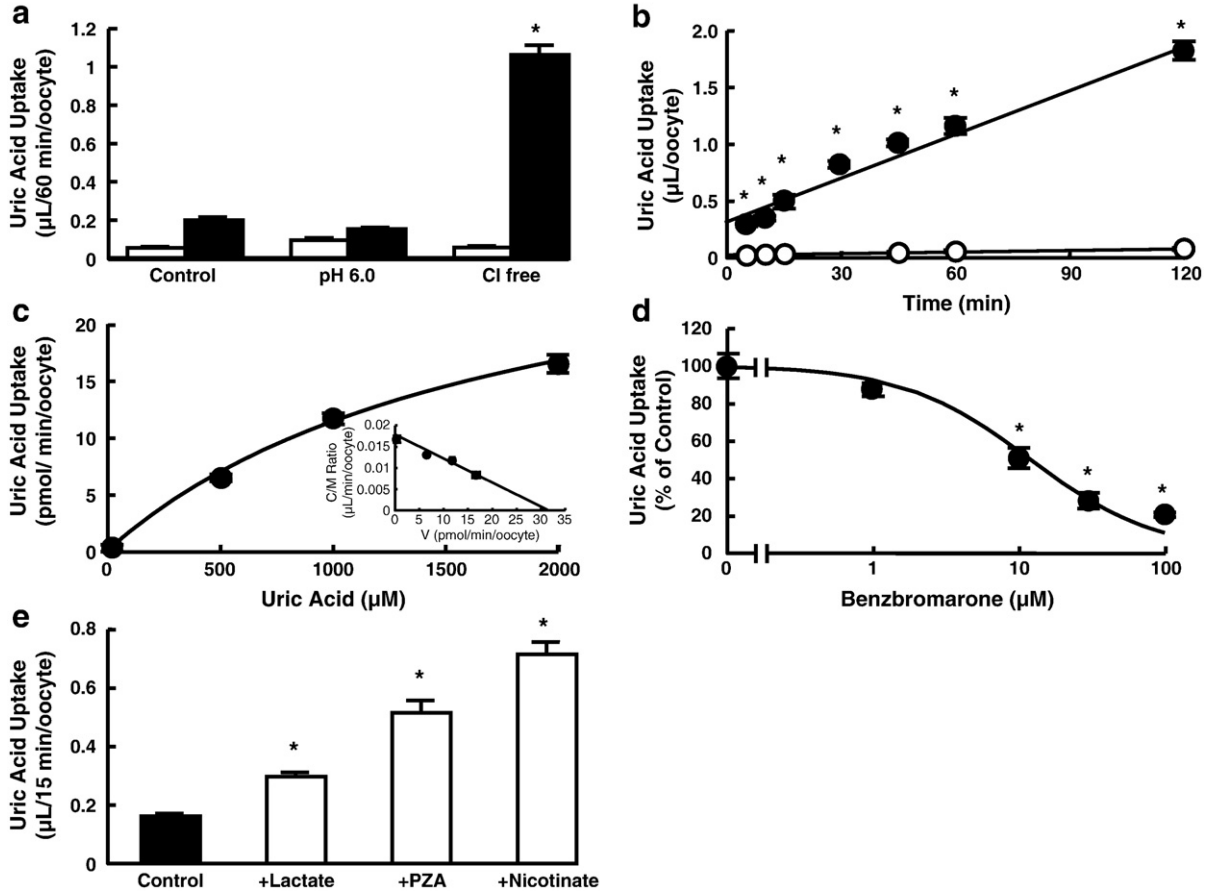


**Fig. 1.** Uric acid transport by rUrat1. (a) Effect of pH and $Cl^-$ on uric acid uptake by rUrat1. Effects of extracellular pH and $Cl^-$ on the uptake of [$^{14}$C]uric acid (20 μM) by water-injected oocytes (open column) and Urat1-cRNA-injected oocytes (closed column) were examined at room temperature for 60 min. $Cl^-$ in the ND96 buffer was replaced with gluconate. Each result represents the mean ± standard error ($n = 10$), and * indicates a significant difference from the uptake of [$^{14}$C]uric acid by rUrat1-cRNA-injected oocytes under $Cl^-$-free conditions compared with that by rUrat1-cRNA-injected oocytes in the presence of $Cl^-$ by Student's *t*-test ($p<0.05$). (b) Time course of uric acid uptake by rUrat1. Uptakes of [$^{14}$C]uric acid (20 μM) by rUrat1-cRNA-injected oocytes (closed circles) and water-injected oocytes (open circles) were measured at room temperature and pH 7.4 over 120 min. Each result represents the mean ± standard error from 8 to 10 oocytes, and * indicates a significant difference from the uptake by water-injected oocytes by Student's *t*-test ($p<0.05$). (c) Concentration dependence of uric acid uptake by rUrat1. Uptake of [$^{14}$C]uric acid (20 μM) in the uptake buffer ($Cl^-$ removal buffer) by rUrat1-cRNA-injected oocytes and water-injected oocytes were measured at room temperature and pH 7.4 for 60 min in the presence of 0.02–2 mM unlabeled uric acid. rUrat1-mediated uptake was determined by subtracting the uptake by water-injected oocytes from that by Urat1-cRNA-injected oocytes. Each point represents the mean ± standard error from 8 to 10 oocytes. (d) Concentration dependence of inhibitory effect of benzbromarone on rUrat1. Uptake of [$^{14}$C]uric acid (20 μM) by water-injected and rUrat1-cRNA-injected oocytes was measured in the presence or absence (control) of benzbromarone (1–100 μM). rUrat1-mediated uptake was determined by subtracting the uptake by water-injected oocytes from that by rUrat1-cRNA-injected oocytes, and the values obtained were divided by the control value in each assay. Each point represents the mean ± standard error from 8 to 10 oocytes. An asterisk (*) indicates a significant difference from the control by Student's *t*-test ($p<0.05$). (e) *Trans*-stimulatory effect on rUrat1-mediated uptake of uric acid. The uptake of [$^{14}$C]uric acid (20 μM) by water-injected or rUrat1-cRNA-injected oocytes was measured after preloading 50 nl of 100 mM PZA, 100 mM L-lactic acid, 100 mM nicotinate or the same volume of water (Control, closed bar). An asterisk (*) indicates a significant difference from the control by Student's *t*-test ($p<0.05$). Drugs were dissolved in water containing KOH, and adjusted to pH 7.4 with MES.

concentration in the medium (μmol/L), the apparent Michaelis–Menten constant (μmol/L), and the maximal uptake rate (pmol per indicated time per mg protein or per oocyte), respectively. The inhibitory effect was expressed as percentage of control, and the inhibitor concentration giving 50% inhibition ($IC_{50}$) was calculated by application of the following equation (2),

$$\% \text{ of control} = \frac{100 \times IC_{50}}{IC_{50} + I}, \quad (2)$$

where $I$ is the inhibitor concentration (μM).

For protein assay, cellular protein content was measured according to the method of Bradford [30] using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA) with BSA as the standard.

### 2.7. Immunohistochemistry

Male Wistar rats at 10–15 weeks of age were purchased from Nippon SLC, Inc. (Hamamatsu, Japan). They were raised under standard laboratory conditions with a 12-h light/12-h dark cycle and free access to food and water. All subsequent animal experiments approved by the Committee on Care and Use of Laboratory Animals in Kanazawa University. The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and sacrificed by bleeding from the right atrium, followed by transcardial perfusion with cold physiological saline. To make tissue sections for immunohistochemistry, the animals were fixed by perfusion with cold 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), and the kidneys were dissected out. They were further fixed by

immersion in the same fixative overnight at 4 °C, dehydrated in a graded alcohol series for 24 h, and embedded in paraffin. They were then cut into 4-μm-thick sections utilizing a routine procedure. The sections were mounted on silanized glass slides (DAKO, Glostrup, Denmark).

Polyclonal rabbit anti-rUrat1 sera were raised against synthetic peptides corresponding to 17-amino-acid sequences (321–337: RSAMQEEPNGNQAGARL and 537–553: KVTHDIAGGSVLKSARL) of rat Urat1. These peptides were conjugated to keyhole limpet hemocyanin and used as antigens to immunize rabbits (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan). When the antibody titer had increased above 62500, the whole blood was collected from each rabbit and the sera were separated.

Immunohistochemistry was performed as previously described [31]. Briefly, the prepared tissue sections of rat kidneys were treated with 0.3% $H_2O_2$ in methanol for 10 min to inhibit intrinsic peroxidase activity and with 1% bovine serum albumin for 30 min to prevent nonspecific antibody binding. Subsequently, the sections were incubated overnight at 4 °C with anti-rUrat1 antibody (1:800) and then washed with PBS. The sites of immunoreaction were visualized by incubating the sections successively with biotinylated horse anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA) for 1 h, horseradish peroxidase-conjugated streptavidin (DAKO) for 1 h and ImmPACT DAB Substrate (Vector Laboratories) for a few minutes. To confirm the specificity of the immunoreaction, the antibody was preabsorbed with the peptide antigen used for the immunization (10 μg peptide for 100 μl of diluted anti-rUrat1 antibody solution) for 1 h at room temperature prior to use.

## 3. Results

### 3.1. Uric acid uptake by rUrat1

To clarify the transport characteristics of rUrat1 proteins, uric acid uptake by *Xenopus* oocytes expressing rUrat1 was examined. When chloride was replaced with gluconate in the uptake buffer, rUrat1-mediated uric acid uptake was significantly increased by about 5-fold compared to water-injected oocytes (Fig. 1a). rUrat1-mediated uptake of uric acid was pH-dependent, showing slightly lower activity at pH 6.0 than at pH 7.4 (Fig. 1a). In the following studies, uric acid uptake by *Xenopus* oocytes expressing rUrat1 was evaluated under $Cl^-$-free conditions at pH 7.4. The uptake of uric acid by rUrat1 was linearly increased with time over 120 min and was significantly greater than that by water-injected oocytes (Fig. 1b). Uptake at 60 min was evaluated as a measure of initial uptake in the subsequent studies. The concentration dependence of uric acid uptake by rUrat1 was studied in the concentration range from 20 to 2000 μM (under this condition, solubility of uric acid is up to 2000 μM). All values are shown after subtraction of the uptake by water-injected oocytes in Fig. 1c. The uptake was saturable, and the Km and Vmax values of uric acid transport by rUrat1 were 1773 ± 334 μM (mean and standard error) and 31.6 ± 3.41 pmol/min/oocyte, respectively. Uric acid uptake by rUrat1 expressing oocytes was linearly increased over 2 mM in the presence of $Cl^-$ (data not shown), suggesting that Km is larger than 2 mM. Accordingly, since Km was 1.77 mM in the absence of $Cl^-$, it was suggested that $Cl^-$ lowers the affinity of uric acid to rUrat1. Benzbromarone inhibited rUrat1-mediated uptake of uric acid in a concentration-dependent manner. The estimated $IC_{50}$ value of benzbromarone was 11.9 ± 1.72 μM (Fig. 1d). *Trans*-stimulatory effects of lactate, pyrazinecarboxylic acid (PZA) and nicotinate on rUrat1-mediated uptake of uric acid were evaluated in oocytes injected with these compounds prior to initiation of uric acid uptake. Each of the three compounds caused a significant increase of rUrat1-mediated uptake of uric acid (Fig. 1e). *Cis*-inhibitory effects of several compounds on uric acid uptake by rUrat1 were also examined. Lactate (10 mM, 26.7 ± 1.38% of control), para-aminohippuric acid (PAH, 10 mM, 23.4 ± 5.92%), PZA (10 mM, 39.2 ± 5.34), probenecid (2 mM, 12.2 ± 1.07%), and telmisartan (10 μM, 53.5 ± 3.45%) significantly reduced the rUrat1-mediated uric acid uptake (Fig. 2), while losartan and candesartan at 10 nM and 10 μM, respectively, had no effect.

### 3.2. Uric acid uptake by isolated BBMVs of rat kidney cortex

Transport properties of uric acid by isolated renal BBMVs from rat kidney cortex were examined and compared with those of rUrat1-expressing oocytes as described above. Uric acid uptake by renal BBMVs was significantly increased to 2.6-fold in $Cl^-$-free buffer, namely, in the presence of an outwardly directed $Cl^-$ gradient, compared with chloride-containing buffer, namely in the absence of a $Cl^-$ gradient. Uric acid uptake by BBMVs at pH 6.0 exhibited a 1.4-fold increase in comparison with that at pH 7.4 (Fig. 3a). Uric acid uptake by BBMVs in the presence of an outwardly directed $Cl^-$ gradient, using a $Cl^-$-free uptake buffer, increased linearly until 30 sec, showing a transient overshoot uptake phenomenon, which was not observed in the absence of a $Cl^-$ gradient. The uptake at 120 min in $Cl^-$-free uptake buffer was lower than that at 120 s and was comparable with that observed in the presence of $Cl^-$ in the uptake buffer (Fig. 3b). Since uptake of uric acid by BBMVs was enhanced by an outward $Cl^-$ gradient, the uptake rate was evaluated by subtracting the value obtained in the presence of $Cl^-$ from that in the absence of $Cl^-$ in the uptake buffer. The uptake of uric acid by BBMVs exhibited saturation in a concentration range from 100 to 2000 μM. An Eadie-Hofstee plot indicated the involvement of a single transport system with Km of 1129 ± 242 μM and Vmax of 102.9 ± 10.5 pmol/s/mg protein (Fig. 3c). The $Cl^-$-susceptible uptake of uric acid by BBMVs was inhibited by benzbromarone with an $IC_{50}$ of 10.2 ± 1.72 μM (Fig. 3d). Uric acid uptake by BBMVs was increased by 54% and 65% by preincubation with 10 mM lactate and 10 mM PZA, respectively (Fig. 3e), suggesting that both drugs *trans*-stimulate uric acid transport.

### 3.3. Immunohistochemical localization of rUrat1 in rat kidney

To examine the intracellular localization of rUrat1, we performed immunohistochemical analysis using paraffin-embedded sections of

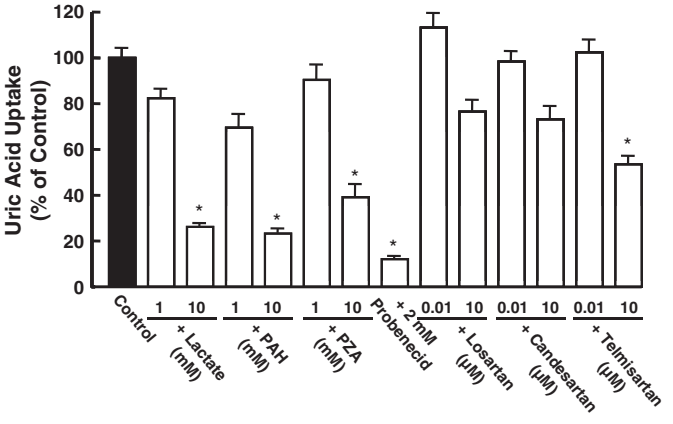


**Fig. 2.** *Cis*-inhibitory effects of various endogenous and exogenous compounds on rUrat1-mediated uptake of uric acid by rUrat1-expressing oocytes. Uptake of [$^{14}$C]uric acid (20 μM) by water-injected and rUrat1-cRNA-injected oocytes was measured in the presence and absence (control, closed bar) of L-lactate, PAH, PZA, probenecid, losartan, candesartan and telmisartan for 60 min. rUrat1-mediated uptake was determined by subtracting the uptake in water-injected oocytes from that in rUrat1-cRNA-injected oocytes, and the values obtained were divided by the control value in each assay. Each point represents the mean ± standard error from 8 to 10 oocytes. An asterisk (*) indicates a significant difference from the control by Student's *t*-test ($p<0.05$).

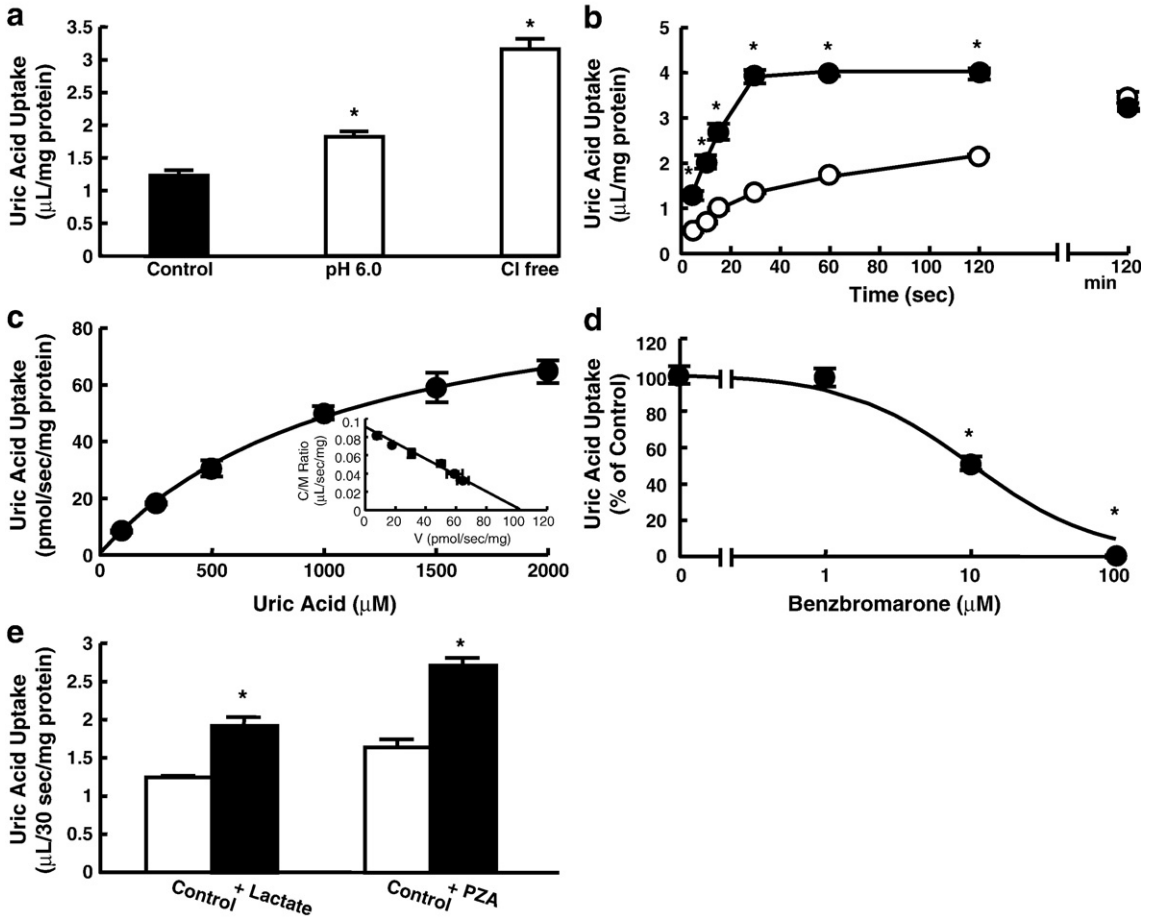


**Fig. 3.** Uric acid transport by rat renal BBMVs. (a) Effect of pH and $Cl^-$ on uric acid uptake by BBMVs. Effects of extravesicular pH and $Cl^-$ on the uptake of [$^{14}$C]uric acid (20 μM) by BBMVs for 15 s at 25 °C. $Cl^-$ was replaced in the uptake buffer with gluconate. Each result represents the mean ± standard error ($n$ = 3-4). * indicates significant difference from uptake of [$^{14}$C$^{14}$C]uric acid by BBMVs under control conditions by Student's $t$-test ($p$<0.05). (b) Time course of uric acid uptake by BBMVs. Uptake of [$^{14}$C]uric acid (60 μM) by BBMVs in the presence (open circles) or absence (closed circles) of $Cl^-$ was measured at 25 °C and pH 7.4 for designated time. $Cl^-$ was replaced in the uptake buffer with gluconate. Each result represents the mean ± S.E.M. from 3 to 4 points, and * indicates a significant difference between the uptake of [$^{14}$C]uric acid in the absence of $Cl^-$ and that in the presence of $Cl^-$ by Student's $t$-test ($p$<0.05). (c) Concentration dependence of uric acid uptake by BBMVs. Uptake of [$^{14}$C]uric acid (60 μM) by BBMVs in the presence and absence of $Cl^-$ was measured at 25 °C and pH 7.4 for 15 s in the presence of 0.4 to 2 mM unlabeled uric acid. $Cl^-$ gradient-dependent uric acid uptake was determined by subtracting the uptake in the presence of $Cl^-$ from that in the absence of $Cl^-$. The inset shows an Eadie–Hofstee plot of the $Cl^-$ gradient-dependent uptake of uric acid. Each point represents the mean ± standard error from 3 to 4 points. (d) Concentration dependence of inhibitory effect of benzbromarone on uric acid uptake by BBMVs. Uptake of [$^{14}$C]uric acid (60 μM) by rat BBMVs was measured in the presence or absence (control) of benzbromarone (1–100 μM) for 15 s. $Cl^-$ gradient-dependent uric acid uptake was determined by subtracting the uptake in the presence of the $Cl^-$ gradient from that in the absence of $Cl^-$, and the values obtained were divided by the control value in each assay. Each point represents the mean ± standard error from 3 to 4. An asterisk (*) indicates a significant difference from the control by Student's $t$-test ($p$<0.05). (e) *Trans*-stimulatory effect on uptake of uric acid by BBMVs. Effects of an outwardly directed L-lactic acid or PZA gradient on [$^{14}$C]uric acid (60 μM) uptake in BBMVs were examined at 25 °C and pH 7.4. BBMVs was preincubated with 10 mM L-lactic acid or 10 mM PZA at 25 °C and pH 7.4 for 90 min. 1 mM PZA or 1 mM L-lactic acid was added to uptake buffer-B as a control for L-lactic acid or PZA. Each point represents the mean ± standard error from 3 to 4 points. An asterisk (*) indicates a significant difference from the control by Student's $t$-test ($p$<0.05).

rat kidney (Fig. 4). Immunostaining for rUrat1 was found exclusively in the renal tubules and was not detected in the glomerulus (Fig. 4a). Immunoreactivity for rUrat1 was not evenly distributed within renal tubules, but was strong on the apical side of the proximal convoluted tubules (Fig. 4c). Moderate to weak staining was seen in the distal convoluted tubules, the loop of Henle and the collecting ducts (Fig. 4a and d). When sections with preabsorbed antibody were examined, no immunostaining was observed (Fig. 4b), suggesting that the immunoreactivity is specific to Urat1.

## 4. Discussion

rUrat1 cDNA exhibited 78.3% and 92.9% identity to hURAT1 and mRst at the nucleotide level, respectively, and its mRNA is expressed in kidney[23-25]. Therefore, rUrat1 is considered to play a role in uric acid handling in rat kidney. Nevertheless, it has remained unclear how rUrat1 functions as a uric acid transporter in proximal tubular cells and how it contributes to renal reabsorption of uric acid. The present study establishes the functional expression and immunolocalization of rUrat1 and compares rUrat1 activity with the activity of isolated BBMVs from rat kidney and with that of hURAT1.

In rats, renal uric acid reabsorption is carrier-mediated, and the transporter is suggested to be an anion exchanger, which has affinity for PAH [20,32]. Studies with rat renal BBMVs indicated that there are at least two transport systems for uric acid; one is susceptible to an outwardly directed hydroxyl ion gradient and has affinity for bicarbonate, and the other is susceptible to an outwardly directed $Cl^-$ gradient and has affinity for lactate [20,33]. Therefore, the hydroxyl ion- or $Cl^-$-sensitivity of rUrat1-mediated transport of uric acid was first examined. Uric acid uptake by rUrat1 exhibited $Cl^-$ dependence, suggesting an

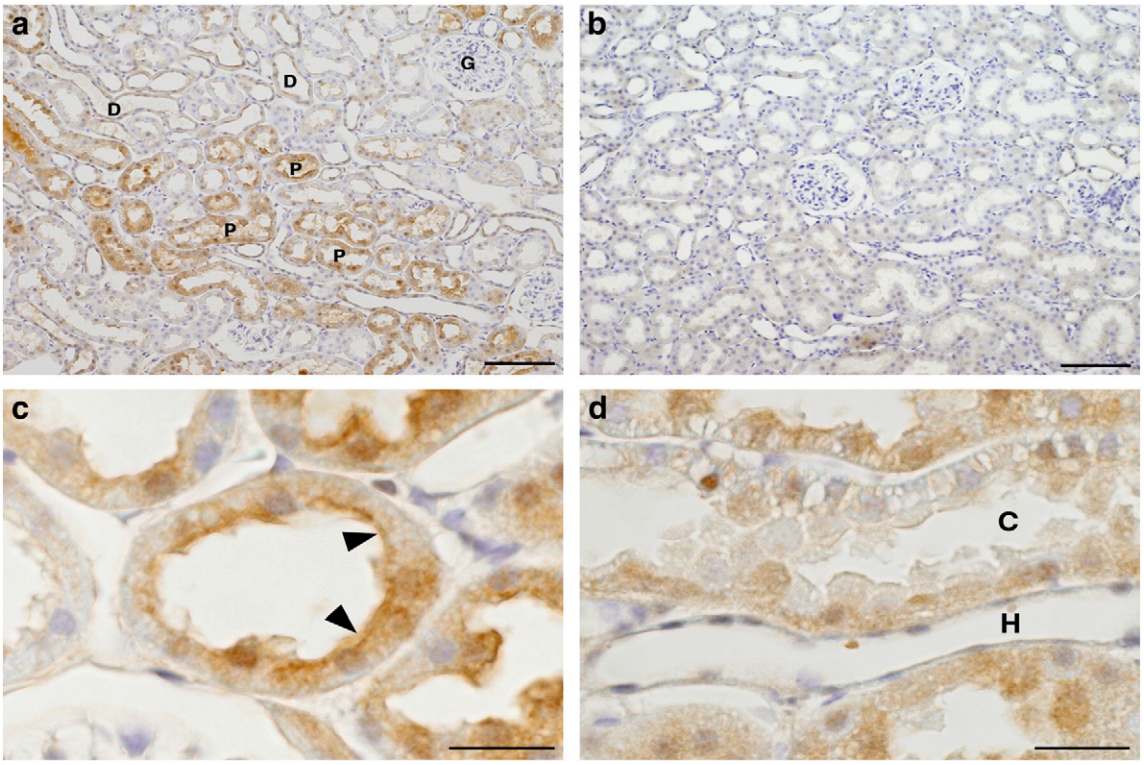


**Fig. 4.** Light microscopic immunohistochemistry showing the localization of rUrat1 in the adult rat kidney. Paraffin sections of 10–15 week kidney were immunostained with anti-rUrat1 antibody (a, c, d) or anti-rUrat1 antibody preabsorbed with the peptide antigen for rUrat1. (a) rUrat1 immunoreactivity is present in the proximal (P) and distal (D) tubules, whereas no reactivity is present in the glomerulus (G). (b) No reactivity is present in any region. (c) Strong immunoreactivity (arrowhead) is present in the apical portion of the proximal convoluted tubules. (d) Moderate to weak immunoreactivity is present in the loop of Henle (H) and the collecting duct (C). Bars = 100 μm (a, b) and 25 μm (c, d).

exchange transport of uric acid with $Cl^-$. In contrast, a negligible change of uric acid uptake was observed in the presence of outwardly directed $OH^-$ gradient (Fig. 1a). The $Cl^-$ gradient-susceptible uptakes of uric acid by rUrat1 and BBMVs were saturable with comparable Km values (1773 and 1129 μM, respectively) (Figs. 1c and 3c). The $IC_{50}$ values of benzbromarone, which exhibits a uricosuric effect by inhibiting hURAT1 in humans, for uric acid uptake by rUrat1 and rat renal BBMVs were also comparable: 11.9 and 10.2 μM, respectively (Figs. 1d and 3d). Furthermore, uptake of uric acid by rUrat1 and $Cl^-$ gradient-susceptible uptake of uric acid by BBMVs were *trans*-stimulated by preloading of lactate and PZA (Figs. 1e and 3e). In addition, our preliminary comparison of the inhibitory effects of salicylate, losartan and telmisartan on the uptake of uric acid between rUrat1 and BBMVs were comparable, showing $IC_{50}$ of salicylate, losartan and telmisartan were 352, 151 and 11 μM in rUrat1, respectively, and 217, 28 and 10 μM by BBMVs, respectively. These results suggest that the functional characteristics of rUrat1 are essentially the same as those of $Cl^-$ gradient-susceptible uric acid uptake at the apical membrane in rat kidney. We confirmed functional identification of rUrat1 on $Cl^-$ gradient-susceptible uptake of uric acid by rat renal BBMVs, although hURAT1 and mRst don't.

Micropuncture studies indicated that approximately 65% of the filtered load of uric acid is reabsorbed in the proximal convoluted tubule, and there are additional reabsorptive sites between the late proximal tubule and the early distal tubule in rats [20]. Free flow micropuncture studies also established that distal nephron segments of rat have extremely low permeability [20]. Our immunohistochemical results demonstrated that rUrat1 is mainly immunolocalized at the apical membrane in proximal tubular epithelial cells and is expressed to a lesser extent in distal convoluted tubules, the loop of Henle and the collecting ducts (Fig. 4). These observations supported the hypothesis that rUrat1 functions to reabsorb uric acid in rat kidney.

Uptake of uric acid by rUrat1 was $Cl^-$-dependent but was only slightly pH-dependent (Fig. 1a), like that by hURAT1 [21]. The Km value of uric acid uptake by rUrat1 (1773 μM) is greater than that by hURAT1 (371 ± 28 μM) [21] and is comparable to that by mRst (1213 μM) [22]. The $IC_{50}$ value of benzbromarone (12 μM) is also higher than that for hURAT1 (around 100 nM) [21] and is comparable to that in the case of mRst [22]. Although the *Ki* values of losartan and telmisartan for hURAT1 are 7.7 nM and 18.2 nM, respectively, the estimated $IC_{50}$ values of these drugs on rUrat1 are higher than 10 μM. On the other hand, the inhibitory effects of other endogenous and exogenous compounds, including lactate, PAH, and PZA, on rUrat1-mediated uptake were similar to those on hURAT1 and mRst [21,22] (Fig. 2). These observations indicate that the transport characteristics of rUrat1 are similar to those of mRst. Although there are differences in the affinities of some compounds, such as benzbromarone and ARBs, between rUrat1 and hURAT1, it is thought that the exchange transport properties of rUrat1, such as $Cl^-$ dependence and *trans*-stimulation by lactate and PZA, are essentially the same as those of hURAT1. The similarities of the significance of URAT1 for the reabsorption of uric acid between humans and rats is useful to predict the in vivo effect of drugs on human SUA level from rat studies. However, we should be careful about the difference of affinity of drugs between human and rat URAT1 for the prediction of the in vivo change in SUA level in humans by each drug.

Since missense mutations of *hURAT1* gene in humans cause an increase in urinary excretion of uric acid and the uricosuric agent benzbromarone inhibits hURAT1, hURAT1 is thought to be an essential transporter for uric acid reabsorption in humans [21,23]. Although mRst was reported to mediate uric acid efflux in in vitro experiments [34], mRst likely plays a role in uric acid reabsorption in mouse, because mRst-deficient mice show increased urinary uric acid excretion [24]. Since rUrat1 activity is accelerated by preloading of lactate, which is an anion imported into cytoplasm by lactate transporters, such as SMCT1 [35] and SMCT2 [36], and produced

by cellular metabolism [37], rUrat1-mediated uric acid reabsorption may be affected by the availability of intracellular lactate in rat kidney.

In conclusion, our results indicate that rUrat1 plays a role in uric acid transport at the apical membrane of proximal tubular epithelial cells. Furthermore, it appears that rUrat1 mediates reabsorption of uric acid in a similar manner to hURAT1. Therefore, it should be possible to extrapolate findings obtained with rUrat1 to uncover the mechanisms in humans of the alterations of SUA level and urinary excretion of uric acid by drugs and other agents.

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