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

Masanobu Sato [a], Tomohiko Wakayama [b], Hideaki Mamada [a], Yoshiyuki Shirasaka [a], Takeo Nakanishi [a], Ikumi Tamai [a,*]

[a] Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kakuma-machi, Kanazawa, 920-1192, Japan
[b] Department of Histology and Embryology, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa, 920-8640, Japan

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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

* Corresponding author. Tel.: +81 76 234 4479; fax: +81 76 264 6284.
E-mail address: tamai@p.kanazawa-u.ac.jp (I. Tamai).

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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