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Na^+-dependent fructose transport via rNaGLT1 in rat kidney

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Abstract We found a system of Na^+-dependent uptake of fructose by rat renal brush-border membrane vesicles. It consisted of two saturable components, and was thought to involve at least two transporters. rNaGLT1, a novel glucose transporter in rat kidney, showed fructose uptake as well as α-methyl-D-glucopyranoside uptake by transfected HEK293 cells. The features of the lower affinity type of fructose transporter in the brush-border membranes, such as affinity and substrate recognition, were very comparable with those of rNaGLT1-transfected HEK293 cells. These results indicated that rNaGLT1 is a primary fructose transporter in rat renal brush-border membranes.

Key words: Rat Na^+-dependent glucose transporter 1; Fructose; Kidney; Brush-border membrane vesicle; Na^+-dependent

1. Introduction

Fructose is found as a free monosaccharide and in combination with various other sugars and complex carbohydrates. Unlike glucose, which is absorbed across the small intestine by SGLT1, fructose uptake occurs via the sodium-independent facilitative transporters GLUT5 and GLUT2 [1–4]. In the small intestine, GLUT5 is present in the brush-border membranes and it is proposed that the transepithelial transport of dietary fructose involves apical entry via GLUT5 and subsequent basolateral exit via GLUT2 [5]. Several findings have been reported about fructose uptake in the small intestine, such as regulation or genetic polymorphisms, but renal reabsorption or excretion of fructose has remained to be elucidated [6–8]. Although GLUT5 has been thought to play a primary role in renal fructose reabsorption in the brush-border membranes, it is localized only in the S3 segment, not in the S1 and S2 segments, of renal proximal tubules [9]. Because the basolateral fructose transporter GLUT2 is localized only in S1 and S2 and not in S3 of proximal tubules, the role of these transporters in the transepithelial transport of fructose remains unclear [9,10].

Recently, we have identified an Na^+-dependent glucose transporter (rNaGLT1) in rat kidney [11]. rNaGLT1 cRNA-injected oocytes transport α-methyl-D-glucopyranoside (αMeGlc) and D-glucose in an Na^+-dependent manner. rNaGLT1 mRNA is abundantly expressed in the kidney cortex, primarily in the proximal convoluted tubules. rNaGLT1 protein is expressed in the brush-border membranes of proximal tubules and is thought to reabsorb glucose from the lumen to tubular cell. Considering its localization along the nephron, affinity for glucose transport and recognition of substrates, rNaGLT1 was suggested to be a low affinity type of glucose transporter similar to SGLT2, a sodium-dependent glucose transporter [11,12]. But rNaGLT1 showed less than 22% homology in amino acid sequence with known glucose transporters, SGLTs and GLUTs, and its precise role, especially in terms of the difference from SGLT2, remains unknown. In the present study, we found an Na^+-dependent uptake system for fructose using rat renal brush-border membrane vesicles, and rNaGLT1 was suggested to be a candidate for a low affinity type transporter.

2. Materials and methods

2.1. Materials

D-[U-^{14}C]Fructose (9.18 GBq/mmol) and [1,2-^{3}H]2-deoxy-D-glucose (2-DG) (925 GBq/mmol) were purchased from Moravek Biochemicals (Brea, CA, USA). [U-^{14}C]αMeGlc (11.7 GBq/mmol) was obtained from Amersham Biosciences (Uppsala, Sweden). Unlabeled D-fructose, αMeGlc, D-galactose, 3-*O*-methylglucose and D-sucrose were purchased from Nacalai Tesque (Kyoto, Japan). Phlorizin, phloretin and 2-DG were obtained from Sigma (St. Louis, MO, USA). 2,5-Anhydromannitol was from Toronto Research Chemicals (North York, ON, Canada). All other chemicals were of the highest purity available.

2.2. Cell culture and transfection

HEK293 cells (American Type Culture Collection CRL-1573), a transformed cell line derived from human embryonic kidney, were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Sigma) with 10% fetal calf serum (Whittaker Bioproducts, Walkersville, MD, USA) in an atmosphere of 5% CO_2, 95% air at 37°C. pBK-CMV plasmid vector containing rNaGLT1 cDNA was purified using the Marligen High Purity Plasmid Purification Midiprep System (Marligen Bioscience, Ijamsville, MD, USA). The day before the transfection, HEK293 cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells/well. The cells were transfected with 0.8 μg of total plasmid DNA per well using LipofectAMINE 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. At 48 h after transfection, the cells were used for uptake experiments.

2.3. Uptake experiment in HEK293 cells

Cellular uptake of [^{14}C]fructose was measured with monolayer cultures grown on poly-D-lysine-coated 24-well plates. The composition of the incubation medium was as follows: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4). Uptake in the absence of Na^+ was measured by substituting NaCl with choline chloride. The

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Abbreviations: rNaGLT1, rat Na^+-dependent glucose transporter 1; αMeGlc, α-methyl-D-glucopyranoside; 2-DG, 2-deoxy-D-glucose

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cells were preincubated with 0.2 ml of incubation medium for 15 min at 37°C. The medium was then removed, and 0.2 ml of incubation medium containing [^{14}C]fructose, [^{14}C]αMeGlc or [^{3}H]2-DG was added. The medium was aspirated at the end of the incubation period, and the monolayers were rapidly washed twice with 1 ml of ice-cold incubation medium. The cells were solubilized in 0.5 ml of 0.5 N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting. The protein content of the solubilized cells was determined by the method of Bradford [13] using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine γ-globulin as a standard.

2.4. Preparation of renal brush-border membrane vesicles

The renal brush-border membranes were isolated from the renal cortex of 7-week-old male rats by the Mg^{2+}/EGTA precipitation method as described previously [14]. The isolated membranes were suspended in an experimental buffer to give a final protein concentration of 7–8 mg/ml. In general, the final experimental buffer consisted of either 100 mM mannitol, 100 mM NaCl or KCl and 10 mM HEPES (pH 7.5), and the pH was adjusted with tris(hydroxymethyl) aminomethane. Uptake in the absence of Na^+ was measured by substituting NaCl with choline chloride.

2.5. Uptake analysis in renal brush-border membrane vesicles

The uptake of [^{14}C]fructose by brush-border membrane vesicles was measured by a rapid filtration technique as described previously [14]. Usually, membrane vesicles suspended in an appropriate buffer were preincubated for 10 min at 25°C before the initiation of uptake. The uptake was initiated by the addition of 20 μl of a buffer containing 4 mM [^{14}C]fructose (final 2 mM) to 20 μl of membrane suspension at 25°C. At the specified periods, the incubation was terminated by diluting the reaction mixture with 1 ml of ice-cold stop solution containing 150 mM NaCl, 20 mM HEPES-Tris (pH 7.5) and 0.1 mM phlorizin. The mixture was poured immediately onto Millipore filters (HAWP, 0.45 μm, 2.5 cm diameter), and the filters were washed with 5 ml of ice-cold stop solution. The radioactivity of the [^{14}C]fructose trapped in membrane vesicles was determined in ACS II (Amersham Biosciences) by liquid scintillation counting.

2.6. Statistical analysis

Data are expressed as the mean ± S.E.M. and were analyzed statistically using Student's unpaired *t*-test. For multiple comparisons, data were analyzed statistically by one-way analysis of variance followed by Fisher's *t*-test.

3. Results

3.1. rNaGLT1 mediated fructose uptake by HEK293 cells

To characterize the transport function of rNaGLT1, the accumulation of fructose, αMeGlc and 2-DG, a substrate specific for facilitative glucose transporters, was measured with rNaGLT1-transfected HEK293 cells. The reproducible uptake of fructose was significantly increased as was that of αMeGlc, but 2-DG showed little or no uptake by rNaGLT1-transfected HEK293 cells compared with vector-transfected cells (Fig. 1A).

We measured the initial uptake of fructose by rNaGLT1-transfected HEK293 cells in the presence of Na^+, varying the concentration of fructose (0.1–20 mM). The saturation of fructose uptake by rNaGLT1-transfected HEK293 cells is demonstrated in Fig. 1B. Based on Eadie–Hofstee plot analysis, the apparent K_m and V_{max} values for fructose were calculated to be 4.55 ± 0.29 mM and 311.5 ± 38.5 pmol/mg protein/min, respectively. The uptake of D-glucose by rNaGLT1-transfected cells could not be measured owing to high activity of endogenous transporters.

3.2. Na^+-dependent uptake of fructose by renal brush-border membrane vesicles

To analyze whether the uptake of fructose in renal brush-border membranes was Na^+-dependent or -independent, brush-border membrane vesicles from rat kidney were incubated in the presence of 100 mM NaCl or KCl. In the presence of an inward Na^+ gradient, fructose uptake was apparently accelerated, with the initial uptake at 15 s four-fold higher than in the absence of an Na^+ gradient (Fig. 2A).

The initial uptake rate for fructose (15 s) was examined as a function of the fructose concentration. The specificity of the

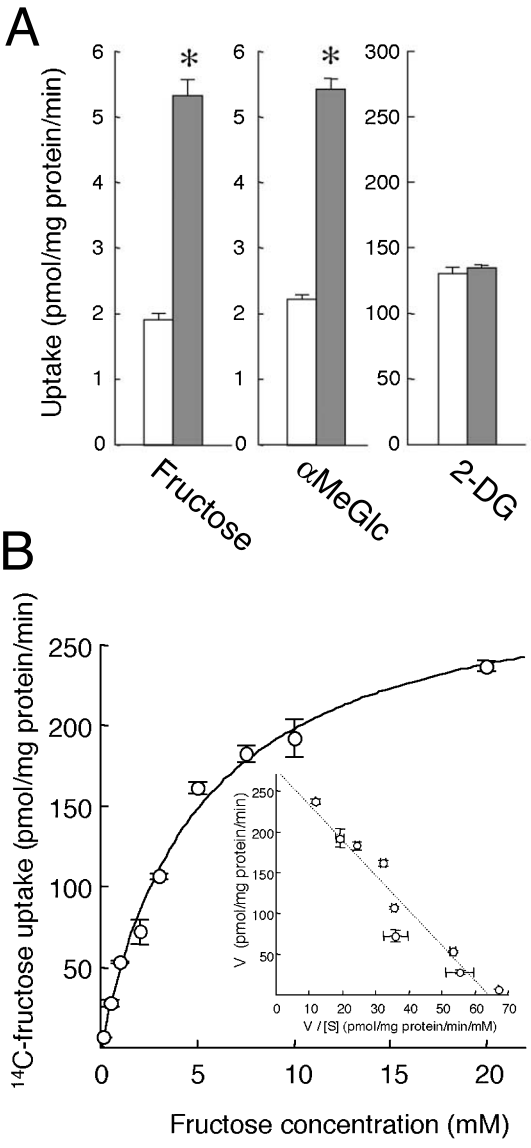

Fig. 1. rNaGLT1 mediated fructose uptake by HEK293 cells. A: Uptake assays were performed with HEK293 cells incubated at 37°C for 15 min with the buffer containing sugar analogues (0.1 mM, 37 kBq/ml) 2 days after transfection of vector (open column, 0.8 μg/well) or rNaGLT1 cDNA (closed column, 0.8 μg/well). Each column represents the mean ± S.E.M. of three monolayers. *$P < 0.05$, significantly different from vector-transfected HEK293 cells (Student's unpaired *t*-test). B: Uptake of [^{14}C]fructose by HEK293 cells transfected with rNaGLT1 cDNA (0.8 μg/well) was assayed for 15 min at 37°C in incubation buffer at various concentrations (0.1–20 mM) and the uptake measured in the vector-transfected HEK293 cells was subtracted. Inset shows an Eadie–Hofstee plot of the uptake. Each point represents the mean ± S.E.M. of three monolayers. The apparent K_m and V_{max} values were obtained from three separate experiments.

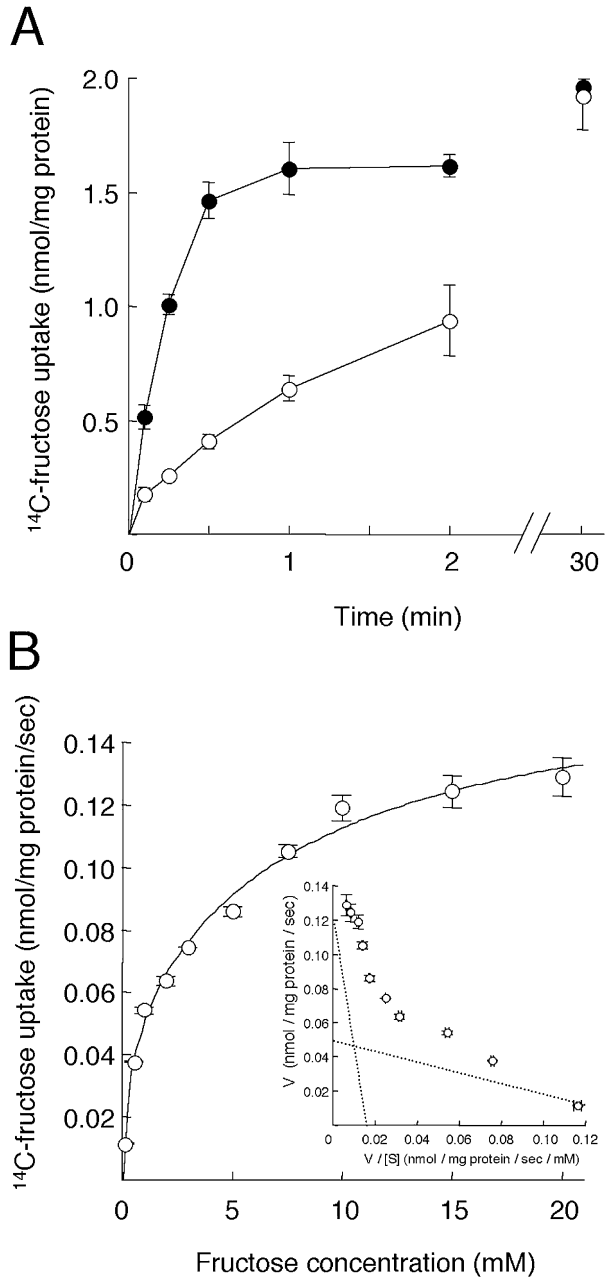

Fig. 2. Uptake of fructose by renal brush-border membrane vesicles. A: Membrane vesicles (20 μl) suspended in 100 mM mannitol and 10 mM HEPES (pH 7.5) were incubated at 25°C with a substrate mixture (20 μl) comprising 100 mM mannitol, 200 mM NaCl (closed circles) or KCl (open circles), 4 mM [^{14}C]fructose and 10 mM HEPES (pH 7.5). Each value represents the mean ± S.E.M. of three separate experiments. Each experiment was performed using the brush-border membrane vesicles isolated from five rats. B: Na^+-dependent uptake of fructose by renal brush-border membrane vesicles was assayed for 15 s at 25°C in incubation buffer at various concentrations (0.1–20 mM) in the presence of NaCl, and the uptake substituting NaCl with KCl was subtracted. Inset shows an Eadie–Hofstee plot of the uptake. Each point represents the mean ± S.E.M. of three determinations. The apparent K_m and V_{max} values were obtained from three separate experiments.

Na^+-dependent fructose uptake was calculated by subtracting the uptake in the presence of KCl from that in the presence of NaCl. Fig. 2B shows the Na^+-dependent fructose uptake concentration dependence curve. Eadie–Hofstee plot analysis indicated that the Na^+-dependent uptake consisted of two saturable components. The kinetic parameters of this uptake were calculated using a non-linear least squares regression analysis with two Michaelis–Menten equations for two transport systems. The K_m and V_{max} values for the higher affinity type of fructose uptake were 0.29 ± 0.04 mM and 30.1 ± 4.9 pmol/mg protein/s, respectively. Those for the lower affinity type were 7.84 ± 0.12 mM and 102.1 ± 14.9 pmol/mg protein/s, respectively.

3.3. *Characteristics of Na^+-dependent fructose transport in rNaGLT1-transfected HEK293 cells and renal brush-border membranes*

The substrate specificities of rNaGLT1-transfected HEK293 cells and renal brush-border membrane vesicles were tested by inhibition experiments, in which the inhibition of the uptake of [^{14}C]fructose (HEK293 cells, 0.1 mM for 15 min; brush-border membrane vesicles, 2 mM for 15 s) was determined in the presence and absence of either sugar analogues (30 mM), phlorizin (50 μM) or phloretin (50 μM) (Table 1). The uptake of [^{14}C]fructose was strongly inhibited by fructose, αMeGlc, phlorizin and the lack of an Na^+ gradient, whereas little or no inhibitory effect was exerted by sucrose and 3-*O*-methylglucose both in the rNaGLT1-transfected HEK293 cells and in the brush-border membranes. Galactose little affected the uptake by rNaGLT1-transfected HEK293 cells but slightly inhibited the uptake by renal brush-border membrane vesicles. 2,5-Anhydromannitol, an inhibitor of GLUT5 [15], and phloretin strongly inhibited the uptake by the rNaGLT1-transfected HEK293 cells, but only slightly inhibited the uptake by the brush-border membrane vesicles. 2-DG strongly inhibited the uptake by the brush-border membrane vesicles, but slightly inhibited the uptake by the rNaGLT1-transfected HEK293 cells.

4. Discussion

Fructose as well as glucose is absorbed in the small intestine and renal proximal tubules. In the basolateral membrane, fructose was transported in an Na^+-independent manner via GLUT2 [4]. The Na^+-dependence of fructose uptake by the brush-border membrane vesicles from the small intestine has remained controversial. Sigrist-Nelson and Hopfer [16], Guy and Deren [17], and Honegger and Semenza [18] reported that mucosal flux of fructose was unaffected by Na^+. In contrast, Bihler [19], Gracey et al. [20], and Macrae and Neudoerffer [21] reported an Na^+-dependent fructose transport in the small intestine. GLUT5, isolated from human small intestine, is localized to the brush-border membranes of small intestine and renal proximal tubules [2,3,22]. The expression level of GLUT5 protein, which was regulated by dietary fructose, corresponded well to the level of fructose absorption in vivo and GLUT5 is thought to be one of the major fructose transporters of the brush-border membranes in the small intestine [23]. However, the findings that fructose malabsorption disease did not result from the expression of mutant GLUT5 protein and that the stimulation of fructose absorption by sucrose was not explained by GLUT5 suggest the existence of an alternative fructose transporter in addition to GLUT5 [7,23]. Recently, we isolated rNaGLT1, a novel Na^+-dependent glucose transporter, from rat kidney [11]. In the present study, fructose as well as αMeGlc was transported by rNaGLT1 in an Na^+-

Table 1
Effects of sugar analogues, phlorizin and phloretin on [^{14}C]fructose uptake by rNaGLT1-transfected HEK293 cells or renal brush-border membrane vesicles

Addition	rNaGLT1-mediated [^{14}C]fructose uptake by HEK293 cells		Na^+-dependent [^{14}C]fructose uptake by renal brush-border membranes	
	pmol/mg protein/min	% of control	pmol/mg protein/s	% of control
Control	2.92 ± 0.04	100	49.5 ± 3.6	100
Absence of Na^+	0.16 ± 0.20*	5	−0.2 ± 1.3*	0
Fructose	0.22 ± 0.06*	8	5.4 ± 2.1*	11
αMeGlc	1.24 ± 0.13*	42	23.8 ± 2.1*	48
Galactose	2.88 ± 0.23	99	35.2 ± 3.3	71
3-OMG	2.60 ± 0.15	89	47.1 ± 4.5	95
2-DG	2.04 ± 0.19*	70	16.8 ± 3.7*	34
Sucrose	4.20 ± 0.17*	144	47.3 ± 6.0	96
2,5-AM	1.16 ± 0.16*	40	38.6 ± 2.6	78
Phlorizin	0.34 ± 0.11*	12	3.2 ± 0.8*	6
Phloretin	0.97 ± 0.09*	33	35.8 ± 3.5	72

[^{14}C]Fructose (0.1 mM, 37 kBq/ml) uptake by rNaGLT1-transfected HEK293 cells was measured for 15 min at 37°C in the absence and presence of either sugar analogues (30 mM), phlorizin (50 μM) or phloretin (50 μM), and the uptake measured in the vector-transfected HEK293 cells was subtracted. Membrane vesicles (20 μl) suspended in 100 mM mannitol and 10 mM HEPES (pH 7.5) were incubated for 15 s at 25°C with a substrate mixture (20 μl) comprising [^{14}C]fructose (final 2 mM, 74 kBq/ml) in the absence and presence of either sugar analogues (30 mM), phlorizin (50 μM) or phloretin (50 μM), and the uptake substituting NaCl with KCl was subtracted. The absence of Na^+ was the uptake measured in the condition substituting NaCl with choline chloride. Each value represents the mean ± S.E.M. of three separate experiments. *$P < 0.05$, significantly different from control values (Fisher's *t*-test). 3-OMG, 3-*O*-methylglucose; 2,5-AM, 2,5-anhydromannitol.

dependent manner (Fig. 1A, Table 1). Unlike in the small intestine, there have been few reports about fructose reabsorption by renal proximal tubules. We report here for the first time the Na^+-dependent fructose uptake by renal brush-border membrane vesicles consisting of two saturable components whose apparent K_m values were 0.3 and 7.8 mM, respectively (Fig. 2B). As the apparent K_m value for rNaGLT1-mediated fructose uptake was 4.5 mM (Fig. 1B), rNaGLT1 was suggested to be the low affinity type of fructose transporter in renal brush-border membranes.

The effects of sugar analogues and phlorizin on the uptake of fructose by renal brush-border membrane vesicles were very comparable with those by rNaGLT1-transfected HEK293 cells. We previously found that the mRNA expression level of rNaGLT1 in the kidney was higher than that of SGLTs [11]. These results indicated that rNaGLT1 plays a crucial role in the Na^+-dependent fructose uptake in renal brush-border membranes. Although the effects of galactose, phloretin, 2,5-anhydromannitol and 2-DG on the uptake of fructose by renal brush-border membrane vesicles were not in total agreement with that of rNaGLT1-transfected HEK293 cells, the differences might be due to the high affinity type of fructose transporter in renal brush-border membranes or endogenous transporter(s).

GLUT5 localizes in the S3 segment of proximal tubules in the rat kidney, but its role in the transepithelial transport of fructose remains elusive [9,10]. Mate et al. [24] reported that fructose was transported in an Na^+-independent manner in the brush-border membranes of rat kidney and GLUT5 took part in the process. However, there have been no other reports about whether renal reabsorption of fructose is Na^+-dependent or not, and the involvement of GLUT5. In addition, Na^+-independent fructose uptake by renal brush-border membranes was non-saturable up to 50 mM, and its apparent K_m value was found to be more than 50 mM (data not shown). In this study, we found that renal fructose uptake was apparently Na^+-dependent. The reason for the discrepancy between the present data and the previous report remains unknown. However, in proximal convoluted tubules, rNaGLT1 was localized in the brush-border membranes but GLUT2 in the basolateral membranes and the K_m value of Na^+-dependent fructose uptake was considerably lower than that of Na^+-independent uptake. Taken together, rNaGLT1 was suggested to play an important role in the renal reabsorption of fructose at the proximal tubules.

In summary, we found Na^+-dependent fructose uptake in rat renal brush-border membranes. The features of the low affinity type of Na^+-dependent fructose uptake by renal brush-border membrane vesicles were very comparable with those by rNaGLT1-expressing HEK293 cells. Therefore, rNaGLT1 would be responsible for renal fructose reabsorption.

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