Expression of the Na⁺ Dependent Uridine Transport System of Rabbit Small Intestine: Studies with mRNA-Injected *Xenopus laevis* Oocytes

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Xenopus laevis oocytes were used as an expression system to prove and characterize the carrier-mediated transport of uridine in the small intestine. Significant Na⁺ dependency was observed for the uptake of [³H]uridine by *Xenopus laevis* oocytes injected with poly(A)⁺RNA prepared from rabbit small intestinal mucosa. By contrast, the uptake of [³H]uridine was negligible in water-injected oocytes. There was no significant difference in the Na⁺ dependent uptake rates of [³H]uridine among oocytes expressed by using mRNA prepared by three different methods. The uptake of [³H]uridine by mRNA-injected oocytes was enhanced by increasing the culturing time after mRNA injection. Concentration dependency for uridine transport was observed with the Michaelis constant of 8.27 μM, which was comparable to that reported in the study using the brush-border membrane vesicles from rabbit small intestine (6.4 μM). Furthermore, the uptake of [³H]uridine was significantly inhibited by adenosine and thymidine, but not by adenine and uracil. Consequently, the transport system of uridine expressed in mRNA-injected oocytes is clarified to be similar to that functioning in the brush-border membrane of the small intestine.

Keywords nucleoside uptake; small intestine; expression; oocyte carrier protein; membrane transport

Introduction

In our previous studies, endogenous transport systems responsible for dipeptides,^{1–4)} monocarboxylic acids^{5–7)} and phosphoric acid^{8,9)} have been elucidated to be efficient pathways for the absorption of drugs having a structurally analogous moiety to those nutrients. Since several anticancer and anti-AIDS drugs, including 5-fluorouracil and azidodeoxythymidine (AZT), are analogous with nucleic acids, a clarification of the transport mechanism of nucleic acids may provide a clear understanding of the intestinal absorption mechanism of such drugs. The transport of nucleosides in the small intestine has been studied by using cultured rat epithelial cells,¹⁰⁾ isolated mouse intestinal cells¹¹⁾ and brush-border membrane vesicles.¹²⁾ These studies indicate that a Na⁺ dependent nucleoside transport system exists in the small intestine.

Understanding the molecular design of those drugs effective following oral administration would provide more rational insights to help clarify the nature of the transport carrier protein. Recently, mRNA-injected *Xenopus laevis* oocytes, one of expression systems of membrane protein, has been claimed as a useful technique to clarify the mechanism of the transport processes of compounds^{13–15)} and to isolate the carrier protein when used as a part of expression cloning.¹⁶⁾

The purpose of the present study is to demonstrate the expression of a uridine transporter in the small intestine by using mRNA-injected *Xenopus laevis* oocytes.

A part of this work has been published previously in a preliminary form.¹⁷⁾

Experimental

Materials [³H]Uridine (specific activity, 42.4 Ci/mmol), [³H]3-*o*-methylglucose (specific activity, 79.0 Ci/mmol), [³H]L-alanine (specific activity, 84.0 Ci/mmol) and protosol as a tissue solubilizer were purchased from New England Nuclear, Boston, MA. Collagenase (type 1A) and an mRNA purification kit were purchased from Sigma Chemical Co., St. Louis, MO., and Pharmacia LKB, Piscataway, N.J., respectively. All other

chemicals were of a reagent grade and were used without further purification.

Animals Female *Xenopus laevis* frogs (12–15 cm) and male albino rabbits (1.5–2.0 kg) were purchased from Sankyo Laboratory Co., Toyama, Japan. The rabbits were fasted overnight before use.

Isolation of Poly(A)⁺RNA from Rabbit Intestinal Mucosa After the rabbits were sacrificed by dehematization, the whole small intestine was rapidly isolated and flushed with ice-cold saline. Mucosa was collected by scraping with a glass slide. Total RNA was extracted from the scrapings by using three different methods: either the guanidinium thiocyanate extraction method followed by cesium chloride gradient centrifugation (GTC-CsCl method)¹⁸⁾; phenol/chloroform extraction (phenol/chloroform method)^{19,20)}; or the acid guanidinium thiocyanate-phenol/chloroform method (GTC-phenol/chloroform method).²¹⁾ Poly(A)⁺RNA was separated by an mRNA purification kit passing through an oligo(dT)-cellulose column, and was reconstituted in water to a concentration of 1 μg/μl.

Preparation of *Xenopus laevis* Oocytes and Injection of mRNA *Xenopus laevis* frog was anesthetized by hypothermia. After isolation of the ovarian fragments, follicular cells were removed by using forceps under a dissecting microscope following treatment with collagenase (1 mg/ml) for 2 h in modified Birn's solution (MBS, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 50 units/ml penicillin G, 50 μg/ml streptomycin and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)/NaOH, pH 7.5) at 20°C. Only healthy looking stage V–VI oocytes were used for mRNA injection. About 50 nl of mRNA (1 μg/μl) or water were injected into the oocytes, as described previously.^{22,23)} They were incubated in MBS at 20°C for 1 to 7 d until used for transport studies.

Uridine Uptake Study The uptake of uridine by mRNA- or water-injected oocytes was performed at 25°C as reported previously.²⁴⁾ Oocytes were preincubated for 30–90 min with a Na⁺-free medium (100 mM choline Cl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES–Tris (2-amino-2-hydroxymethyl-1,3-propanediol), pH 7.5) before the initiation of the uptake experiments. The reaction was initiated by adding 100 μl aliquots of the Na⁺-medium (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES–Tris, pH 7.5) containing a tracer amount of the radio labeled compound (the final concentration of uridine, 10 μCi/ml, 0.2 μM) and/or the other constituents listed in Table II to one oocyte in each well. The reaction was terminated by washing the oocyte three times with 30 ml of an ice-cold Na⁺-free medium. Each oocyte was dissolved in 1 ml of protosol at 60°C for an hour. After neutralization with 43 μl of glacial acetic acid, each solution was put into a glass scintillation vial containing 10 ml of scintillation cocktail, Clear-sol I (Nacalai Tesque Inc., Kyoto, Japan). The radioactivity of each sample was measured in a liquid scintillation counter, LSC-700 (Aloka Co., Ltd.,

Tokyo, Japan). The uptake rate was expressed as the amount of uridine taken up per unit time per oocyte, divided by the uridine concentration in the uptake medium, *i.e.*, $\mu\text{l}/\text{min}/\text{oocyte}$, except in the study of concentration dependency. It was considered convenient to use these uptake rates normalized by the medium concentration.

Results and Discussion

The effect of culturing time on the uptake rate of uridine by oocytes is shown in Fig. 1. The $[^3\text{H}]$ uridine uptake rate in the mRNA-injected oocytes was significantly higher after 3 d of injection than that of the water-injected oocytes. Furthermore, the uptake rate in the mRNA-injected oocytes markedly increased from 3 to 5 d and reached a plateau at 6–7 d, indicating that the transport protein synthesized from the mRNA developed with the culturing. By contrast, no culturing time-dependent change of uptake was observed in the water-injected oocytes, suggesting that the transport system of uridine is acquired not in water-injected oocytes but in the mRNA-injected oocytes, regardless of the time tested.

The expression of the $[^3\text{H}]$ uridine transport system was examined by mRNAs prepared by three different extraction methods, since it has been reported that different preparation methods of mRNA resulted in different results.¹⁵⁾ Table I summarizes the comparison among the uptakes of $[^3\text{H}]$ uridine in the presence or absence of Na^+ by oocytes injected with mRNA prepared using three different methods, *i.e.*, i) the GTC-CsCl method, ii) the

phenol/chloroform method or iii) the GTC-phenol/chloroform method. In all of these preparation methods, a clear Na^+ -dependent uptake of uridine was observed. No significant difference in the Na^+ -dependent uptake rate of $[^3\text{H}]$ uridine among these methods was observed, indicating the same transport system could be expressed. Therefore, in the following studies, the GTC-CsCl method was utilized to prepare mRNA.

The incubation time course of $[^3\text{H}]$ uridine uptake by mRNA- or water-injected oocytes is illustrated in Fig. 2. The uptake increased linearly for up to 4 h of incubation in the mRNA-injected oocytes, while little or no time dependency was observed for the uptake by the water-injected oocytes. Thus, the uptake time was set up at 0.5 h to measure the initial uptake rate in the following studies.

Figure 3 illustrates the effect of the Na^+ ion in the extracellular medium, where mRNA- or water-injected oocytes were incubated during the uptake experiment, on the uptake rate of $[^3\text{H}]$ uridine. The uptake rate of uridine in the presence of Na^+ was $0.720 \pm 0.131 \mu\text{l}/\text{h}/\text{oocyte}$ (mean $\pm$ S.E.M., $n=5$), which is 28-fold higher than that in the absence of Na^+ (0.0259 ± 0.00326 , $n=3$). The uptake rate of uridine in water-injected oocytes was 0.0323 ± 0.00729 ($n=4$). No significant Na^+ dependency was observed for water injected oocytes. The result indicates that the oocytes

Fig. 1. Effect of Culturing Time on the Expression of $[^3\text{H}]$ Uridine Uptake by mRNA or Water Injected *Xenopus laevis* Oocytes

Oocytes were injected with either the mRNA solution (open circles) or water (closed circles) and cultured for two to seven days. Uridine uptake was assayed at the indicated day after injection as described under Experimental. Each point represents the mean $\pm$ S.E.M. of four to seven experiments. *a)* Significantly different ($p < 0.001$) from the uptake in water injected oocytes. *b)* Significantly different ($p < 0.005$) from the uptake in water injected oocytes.

TABLE I. Comparison of $[^3\text{H}]$ Uridine Uptake by *Xenopus laevis* Oocytes Injected with mRNA Prepared by the Three Different Methods

Method	Uptake rate of $[^3\text{H}]$ uridine ^{a)} ($\mu\text{l}/0.5 \text{ h}/\text{oocyte}$)	
	Presence of Na^+	Absence of Na^+
GTC-CsCl	0.187 ± 0.065^b	0.0308 ± 0.0049
Phenol/chloroform	0.260 ± 0.039^b	0.0247 ± 0.0031
GTC-phenol/chloroform	0.187 ± 0.023^b	0.0231 ± 0.0034

a) Oocytes were injected with mRNA prepared by three different methods and cultured for 3 d. Uptake of uridine was measured by incubating the oocytes for 0.5 h in the presence or absence of Na^+ . The detailed methods are in Experimental. Each value represents the mean $\pm$ S.E.M. of six or seven experiments. *b)* Significant difference from that in the absence of Na^+ ($p < 0.05$).

Fig. 2. Time Courses of $[^3\text{H}]$ Uridine Uptake by mRNA or Water Injected *Xenopus laevis* Oocytes

Oocytes were injected with mRNA (open circles) or water (closed circles) and cultured for 3 d. Uridine uptake was measured by oocytes incubating for the indicated period with $0.2 \mu\text{M}$ uridine in a Na^+ -medium. Each point represents the mean $\pm$ S.E.M. of four to seven experiments.

Fig. 3. Expression of Rabbit Intestinal Uridine Transporter in *Xenopus laevis* Oocytes

Oocytes were injected with either the mRNA solution or water, and cultured for 3 d. Uridine uptake was measured by the oocytes incubating for 1 h in the presence or absence of 100 mM Na^+ . Assay was performed as described under Experimental. Each column represents the mean $\pm$ S.E.M. of three to five experiments. *a)* Significantly different ($p < 0.01$) between the indicated pair uptakes.

Fig. 4. Concentration Dependency of Uridine Uptake by mRNA Injected *Xenopus laevis* Oocytes

Uptake rate of uridine was measured at various concentrations of uridine (0.2–100 μM). Oocytes were incubated for 0.5 h in the Na^+ -medium described under Experimental. Each point represents the mean $\pm$ S.E.M. of seven experiments.

TABLE II. Effects of Various Compounds on the Uptake of [^3H]Uridine by mRNA-Injected *Xenopus laevis* Oocytes

Inhibitor	Concentration (mM)	Uptake rate ^{a)} ($\mu\text{l}/0.5\text{ h/oocyte}$)
Control		0.174 ± 0.043
Adenosine	0.1	0.0367 ± 0.0055^b
	1.0	0.0342 ± 0.0042^b
Thymidine	0.1	0.0593 ± 0.013^b
	1.0	0.0441 ± 0.0035^b
Inosine	0.1	0.143 ± 0.024
	1.0	0.118 ± 0.017
Ara-C	0.1	0.121 ± 0.033
	1.0	0.142 ± 0.035
AZT	0.1	0.150 ± 0.039
	1.0	0.0585 ± 0.0095^b
Adenine	0.1	0.175 ± 0.063
	1.0	0.174 ± 0.019
Uracil	0.1	0.190 ± 0.033
	1.0	0.221 ± 0.061
D-Glucose	0.1	0.211 ± 0.043
	1.0	0.176 ± 0.031

a) Oocytes were injected with mRNA and cultured for 3 d. Uridine uptake was measured for 0.5 h in a Na^+ -medium containing each inhibitor, and assayed as described under Experimental. Each value represents the mean $\pm$ S.E.M. of four to seven experiments. b) Significant difference ($p < 0.05$).

possess only a small or no uridine transport system in itself, while a Na^+ -dependent uridine transport system was expressed in oocytes injected with mRNA.

The initial uptake rate of uridine in mRNA injected oocytes was determined by increasing the concentration of uridine from 0.2 to 100 μM . As demonstrated in Fig. 4, a saturable uptake was observed. The concentration dependent uptake rates were analyzed according to the following equation:

$$J = \frac{J_{\max} \times S}{K_t + S} + k_d \times S$$

where, J and S are the uptake rate and uridine concentration, respectively. $J_{\max}$, K_t and k_d represent the maximum uptake rate, Michaelis constant and first-order uptake rate constant, respectively. The kinetic parameters were estimated by using a nonlinear-least squares analysis program, MULTI.²⁴⁾ The kinetic parameters obtained (mean $\pm$ S.D.) were as follows; $J_{\max}$ was 1.06 ± 0.355 pmol/0.5 h/oocyte, K_t was 8.27 ± 4.08 μM and k_d was $0.0351 \pm$

Fig. 5. Structure of Uridine, Cytosine Arabinoside (Ara-C) and Azidodeoxythymidine (AZT)

0.00465 pmol/0.5 h/oocyte/ μM . Our evaluated Michaelis constant was close to the value ($K_t = 6.4$ μM) reported in the study using the rabbit intestinal brush-border membrane vesicles,¹²⁾ suggesting the transporter expressed in oocytes injected with mRNA may be similar to that in the brush-border membrane of the small intestine.

The Na^+ dependent uridine transport system existing in the brush-border membrane of the small intestine is known to recognize several kinds of nucleosides¹²⁾ as substrates. The effect of various compounds (0.1 and 1 mM) on the initial uptake rate of [^3H]uridine is summarized in Table II. Two nucleosides, adenosine and thymidine at 0.1 mM, significantly inhibited uridine uptake by 79 and 66%, respectively, and were slightly inhibited by inosine (1 mM). By contrast, the uptake was not inhibited at all by purine or pyrimidine bases, adenine or uracil. Furthermore, there was no inhibitory effect by D-glucose, which transports the intestinal brush-border membrane *via* carrier-mediated mechanisms.²⁵⁾ It thus appears that the transporter expressed in those oocytes injected with mRNA can recognize nucleosides as substrates.

Nucleoside analogues AZT, which is efficiently absorbed from the small intestine,²⁶⁾ and cytosine arabinoside (Ara-C), were tested to determine whether they can influence this uridine uptake system. AZT inhibited uptake in a dose dependent manner while Ara-C did not. The structures of uridine, Ara-C and AZT are shown in Fig. 5. Since it is known that the Na^+ dependent nucleoside transport system is sensitive to the sugar moiety in a nucleoside molecule as shown in a study reported using the rabbit choroid plexus,²⁷⁾ the lack of inhibitory effect by AZT at a lower concentration and by Ara-C may be also ascribed to the structural difference of sugars in the molecules from endogenous nucleosides.

The results also support the idea that a specific transporter for one type of nucleoside is principally expressed in oocytes injected with mRNA extracted from intestinal mucosa. According to a previous report,²⁸⁾ there are two principal Na^+ -dependent nucleoside transporters in the renal brush-border membrane: One system, designated N1, transports purine nucleosides and uridine; whereas the other system, N2, has a substrate specificity for pyrimidine nucleoside, adenosine and adenosine analogues. Similar transport systems for nucleosides have also been reported for the mouse small intestine.¹¹⁾ But, since uridine is reported to be a substrate for the N1 and N2 transport systems,²⁸⁾ the possibility remains that the expressed uridine transport system in this oocytes study could include both types of transporters. However, as shown in Table II,

inosine, which is a substrate of the N1 transporter did not inhibit the uridine transport in the mRNA injected oocytes. The result suggests that the expressed transporter is principally the N2 system, although we could not rule out the possibility of a partial expression of the N1 system in the present study. This is in good accord with the recent report by Jarvis and Griffith,³⁰⁾ where thymidine was demonstrated to be taken up by oocytes injected with rabbit small intestinal mRNA in a sodium dependent manner. Moreover, the selective inhibitory effect of pyrimidine nucleosides on the thymidine uptake was also reported previously,³⁰⁾ which is consistent with the properties of the N2 Na⁺/nucleoside co-transport system.²⁹⁾

In conclusion, an expression of a Na⁺-dependent uridine transporter in the small intestine has been demonstrated for mRNA-injected *Xenopus laevis* oocytes. The expressed character was similar to that of previously reported using the brush-border membrane vesicles.¹²⁾ The expression system utilizing mRNA-injected *Xenopus laevis* oocytes would be a useful tool for understanding the functions of a transport carrier for drugs and nutrients in the small intestine and for characterization at the molecular level.

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