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Report

Inhibitory Effect of Anionic Uremic Toxins and Creatinine on the Renal Transport of Methotrexate in Rats

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Various substances called uremic toxins (UTs) accumulate in the blood of patients with chronic kidney disease (CKD) and induce unfavorable effects on the body. It has been reported that some kinds of UTs are excreted extensively in the urine *via* renal transporters. This characteristic of UTs often becomes a factor for influencing pharmacokinetics of drugs in CKD patients. Even now, however, information on the interactions between UTs and drugs in the process of renal excretion remains limited. Methotrexate (MTX) is widely used for the treatment of rheumatoid and leukemia. It is known that MTX is predominantly excreted in the urine and that this process is mediated by organic anion transporters (OATs). In this study, we investigated the inhibitory effects of two anionic UTs, indoxyl sulfate (IS) and indoleacetic acid (IA), as well as creatinine (Cr) on the renal transport of methotrexate (MTX) using rat renal cortical slices. IS and IA, both substrates for OATs, significantly inhibited the uptake of 50 μM MTX in a concentration-dependent manner at 0.1 mM and 1 mM. In the presence of 1 mM Cr, a cationic guanidino compound, the uptake of MTX was significantly decreased, indicating that Cr is capable of interfering with OATs. In conclusion, it was suggested that the urinary excretion of MTX is extensively suppressed through interactions *via* OATs when IS, IA, and Cr exist a high concentrations in the blood of CKD patients.

Key words chronic kidney disease, uremic toxins, organic anion transporter, interaction, methotrexate, rat renal cortical slices

INTRODUCTION

Various transporters expressed in tissues such as the intestine, liver, brain, and kidney play important roles in the disposition of both essential and inessential substances. With regard to renal tubular secretion, influx from the blood to the renal tubular cells and efflux from the renal tubular cells to the renal tubular lumen are mediated by transporters. Organic anion transporters (OATs) and organic cation transporters (OCTs) are typically involved in the transport of substrates across the basolateral membranes of renal tubular cells. Several subtypes of these transporters, OAT1, OAT3 and OCT2, are predominantly expressed on the membranes in humans.[1-3] Similarly, Oat1, Oat3 and Oct2 are expressed on the basolateral membrane of renal tubular cells in rats, and the substrate specificity of these transporters are almost the same for human and rats.[1,4] Although Oct1 is also present in the rat kidney, its expression is lower than that of Oct2.[4]

Currently, approximately 150 compounds are designated as uremic toxins (UTs).[5,6] When renal function is normal, a large part of the UTs is excreted in the urine. To date, several papers have provided evidence that the renal excretion of anionic UTs, such as indoxyl sulfate (IS) and indoleacetic acid (IA), occurs *via* OATs.[7-9] Therefore, when there is a serious decrease in renal function as in patients with advanced chronic kidney disease (CKD), IS and IA levels in the blood are markedly elevated, often leading to unfavorable systemic effects.[10,11] Most recently, we reported that IS accumulation in the serum is closely related to dementia in patients with early stage CKD.[12] On the other hand, IS and IA trigger interactions with clinically relevant drugs due to the inhibition of renal transport *via* OATs.[13] Moreover, the presence of high levels of IS and IA in the blood interfere with transporters in tissues other than the kidney.[14] However, knowledge regarding the interactions between clinically relevant drugs and UTs such as IS and IA remains insufficient to meet the needs of clinicians.

Creatinine (Cr) has been utilized as an index of the renal function as it is known to accumulate with decreased renal function. Cr, a guanidino compound, is regarded as a cationic UT and has been shown to be transported as an OCT substrate.[15] On the other hand, Shen *et al.* reported that Cr uptake increased in human embryonic kidney cells expressing OAT2 and that its uptake was suppressed in the presence of OAT2 inhibitors such as cimetidine and indomethacin.[16] According to these previous studies, the transport of Cr across the basolateral membranes of the renal tubular cells is considered to be mediated by both OCTs and OATs. Therefore, it is likely that Cr affects the renal tubular secretion of anionic drugs which are substrates for OATs when the serum Cr concentration is elevated in advanced CKD patients. However, the interaction between OAT substrates and Cr in the transport across

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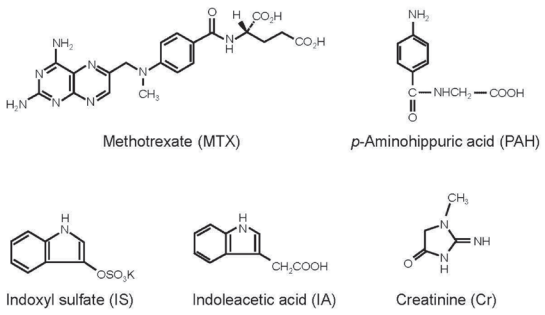

Fig. 1. Chemical Structures of MTX, PAH, IS, IA and Cr

the basolateral membrane of renal tubular cells has not been well addressed.

Methotrexate (MTX) was developed as a chemotherapeutic agent more than 50 years ago and there is now an increasing need for the treatment of rheumatoid arthritis.[17,18] It is well known that various transporters are involved in the pharmacokinetics of MTX and that OAT3 is a key transporter involved in the renal tubular secretion of MTX.[19] Therefore, there is a possibility that IS, IA, and Cr are capable of modulating the renal transport of MTX by competitively inhibiting OAT3.[20,21] Rat renal cortical slices are often utilized for experiments on drug transport across the basolateral membranes of renal tubular cells.[22-24] Through the use of this experimental model, we investigated the inhibitory effects of IS, IA and Cr on the renal transport of MTX.

MATERIALS AND METHODS

Materials MTX, *p*-aminohippuric acid (PAH), IA and Cr were purchased from Wako Pure Chem. Ind. (Osaka, Japan). IS was purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). All other reagents were of the highest grade available. The chemical structures of MTX, IS, IA and Cr are shown in Fig. 1.

Animals Male Sprague-Dawley rats (body weight: 200-500 g) were used in this study. Animal experimentation was performed under the approval of the Committee of Health Sciences University of Hokkaido (Approval No. 19-065) and in accordance with the Regulations for the Care and Use of Laboratory Animals in Health Sciences University of Hokkaido.

Preparation of Drug Solutions Drugs were dissolved in an incubation buffer (84.8 mM NaCl, 40 mM KCl, 0.74 mM $MgCl_2 \cdot 6H_2O$, 25.2 mM $NaHCO_3$, pH 7.4). Mixed gas (95% O_2:5% CO_2) was introduced into the incubation buffer for 30 min before use. MTX was used at a concentration of 50 μM. The pH of these solutions was adjusted to 7.4 just prior to experiments.

Stability Test using Homogenate of Rat Renal Cortical Slices Rats were anesthetized by intraperitoneal administration of pentobarbital sodium (40 mg/kg) or inhalation of isoflurane, and the kidneys were promptly removed, decapsulated and placed in ice-cold buffer (20 mM KCl, 130 mM NaCl). Renal cortical slices (*ca.* 50 mg/slice) were prepared using a Stadie-Riggs microtome.[25] The slice was then homogenized in 1 mL of incubation buffer using a polytron® homogenizer (Kiematica, Luzern, Switzerland). The resultant homogenate was centrifuged at 5,400*g* for 10 min at 5°C. 100 μL of the supernatant fluid was mixed with an equal volume of incubation buffer containing MTX (100 μM) and incubated at 37°C for 5 min, 10 min, 15 min or 30 min. After incubation, 200 μL of methanol was added to sample solutions. The mixture was then let stand on ice for 10 min and centrifuged (5,400g, 10 min, 5°C), and then the drug concentration in the supernatant fluid was assayed.

Uptake Experiment using Rat Renal Cortical Slices Renal cortical slice was prepared as mentioned above. It was placed in 3 mL of incubation buffer containing the drugs and incubated at 37°C for 15 min. After incubation, each slice was taken from the incubation buffer, washed in ice-cold buffer, and blotted on filter paper. The slice was then homogenized in 1 mL of incubation buffer using a polytron® homogenizer. The resultant homogenate was centrifuged at 5,400 *g* for 10 min at 5°C. An aliquot (100 μL) of the supernatant fluid was mixed with an equal volume of incubation buffer and 200 μL of methanol. The mixture was then let stand on ice for 10 min and centrifuged (5,400 *g*, 10 min, 5°C). After centrifugation, the drug concentration in the supernatant fluid was assayed.

HPLC Assay MTX was assayed using a HPLC system ($LC\text{-}10AT_{VP}$, Shimadzu, Kyoto, Japan) equipped with a UV detector ($SPD\text{-}10A_{VP}$). A Cosmosil $5C_{18}AR\text{-}II$ column (5 μm, 4.6 mm i.d. × 150 mm, Nacalai Tesque, Kyoto, Japan) was used at column temperature of 35°C. The mobile phase used was 0.05 M KH_2PO_4/CH_3CN (90:10), assay wave length was 300 nm, and flow rate was 1.0 mL/min. Under these HLPC conditions, MTX could be reproducibly determined with less than a 5% coefficient of variance.

Statistical Analysis Results are shown as the mean of the percentage against the control with standard error (S.E.). Difference of dispersibility between two groups was tested by *F*-test. Student's *t*-test, Mann-Whitney *U*-test or Dunnett test was used for evaluating the significance of the differences between two groups. A p value less than 0.05 was considered statistically significant.

RESULTS

Stability of MTX in Homogenate of Rat Renal Cortical Slices For setting incubation time for uptake experiment of MTX, the stability of MTX was evaluated in the homogenate of rat renal cortical slices in advance. As shown in Fig. 2, while the residual ratio of MTX was kept more than 85% at 15 min when incubated with the homogenate, it was decreased extensively to 39.0% after 30 min. In this study, therefore, the uptake experiment of MTX was conducted for 15 min of incubation time.

Inhibitory Effect of PAH on MTX Uptake into Rat Renal Cortical Slices It was necessary to confirm the suitability of the present uptake experiment for assessing the effect of IS, IA, and Cr on the renal transport of MTX. So, we first evaluated MTX uptake in the presence of PAH, a well-known OAT substrate. As shown in Fig. 3, MTX uptake was significantly decreased to 70.5 ± 12.2% of the control (MTX alone) in the presence of 1 mM PAH (Fig. 3). This result confirmed that Oat-mediated MTX transport is measurable by using rat renal cortical slices.

Inhibitory Effects of IS and IA on MTX Uptake into Rat Renal Cortical Slices Next, to assess the interaction between

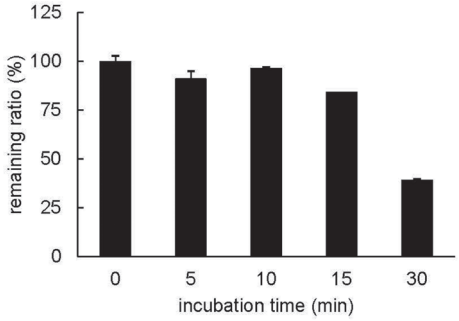

Fig. 2. Stability of MTX in Supernatant Fluid Obtained from Rat Renal Homogenate

Supernatant fluid that obtained by centrifuging rat renal cortical slice homogenate was mixed with incubation buffer containing MTX (100 μM) and incubated at 37°C. Final concentration of MTX was 50 μM. Each column represents the mean or mean with S.E. (0, 5, 10 and 30 min: n = 3, 15 min: n = 2).

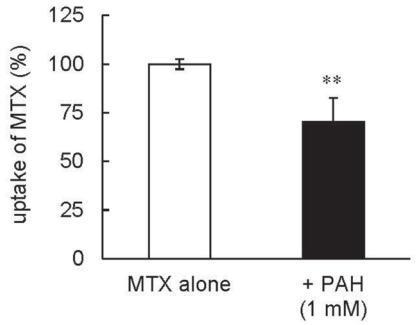

Fig. 3. Effect of PAH on MTX Uptake into Rat Renal Cortical Slices

Rat renal cortical slices (*ca.* 50 mg) were incubated in a 50 μM MTX solution in the presence or absence of 1 mM PAH for 15 min at 37ºC. Each column represents the mean with S.E. (MTX alone: n = 34, MTX + PAH: n = 3). ** $p < 0.01$, significantly different from MTX alone.

two anionic UTs (IS and IA) and MTX in the renal transport, MTX uptake into rat renal cortical slices was studied in the presence of IS and IA. MTX uptake was significantly decreased to 66.4 ± 3.2% and 52.5 ± 2.7% of the control (MTX alone) in the presence of 0.1 mM or 1 mM IS, respectively (Fig. 4). On the other hand, in the presence of 0.1 mM or 1 mM IA, MTX uptake was significantly decreased to 74.0 ± 5.5% and 58.0 ± 7.7% of the control, respectively (Fig. 4).

Inhibitory Effect of Cr on MTX Uptake into Rat Renal Cortical Slices To know whether cationic Cr has a capability to interfere with renal transport of MTX, MTX uptake into rat renal cortical slices was compared in the presence or absence of Cr. The addition of 1 mM Cr significantly decreased MTX uptake to 56.1 ± 8.3% of the control (MTX alone) (Fig. 5).

DISCUSSION

In this study, as an approach to clarify the effect of two anionic UTs (IS and IA) and Cr on the renal transport of OAT substrate, their inhibitory effects on MTX uptake into rat renal cortical slices were compared. As an initial experimental design, it was supposed to investigate their effect on the time course of MTX uptake at least for 30 min. However, the results shown in Fig. 2 implied that not a small amount of MTX, which accumulated in the renal cortical slices, disappeared possibly due to degradation during 30 min incubation. Therefore, this study focused on the inhibitory effect of IS, IA and Cr on the 15 min uptake of MTX. The significant inhibition by PAH on MTX uptake (Fig. 3) suggested that Oat3-mediated MTX transport is measurable under the present experimental condition.

As shown in Fig. 4, both IS and IA significantly and concentration-dependently decreased MTX uptake, indicating that these two anionic UTs are capable of interfering with Oat3-mediated MTX transport. According to the result of PAH (Fig. 3), it is reasonable to consider that the inhibition by IS and IA is a competitive manner. It is known that MTX is transported to renal proximal tubular epithelial cells by reduced folate carrier 1 (RFC1) in addition to OAT3.[26] Based on the results shown in Fig. 4, it can be said that Oat3 contributes approximately 50% of the overall transport of MTX.

We previously investigated plasma levels of IS and IA in hemodialysis outpatients.[27] It was shown that plasma IS levels before hemodialysis and immediately after hemodialysis were 157.9 ± 19.9 μM and 103.8 ± 13.3 μM, respectively, and that the maximum plasma IS level was 358.4 μM. We also reported that the plasma protein binding ratio of IS was *ca.* 64.8%.[27] Therefore, it can be assumed that the unbound maximum plasma IS level in hemodialysis patients was roughly 126.2 μM. Accordingly, it is likely that IS interacts with OAT3 substrates including MTX in the body of patients with end-stage renal disease. At concentrations of 0.1 mM and 1 mM, the inhibitory potency of IA against MTX uptake was almost identical to that

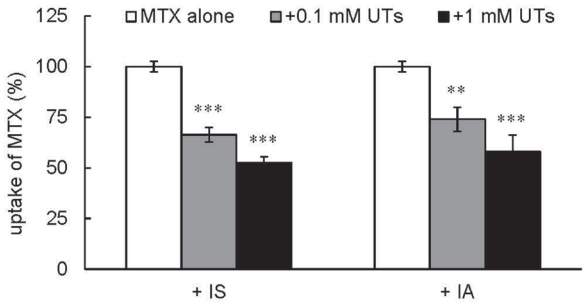

Fig. 4. Effect of IS and IA on MTX Uptake into Rat Renal Cortical Slices

Rat renal cortical slices (*ca.* 50 mg) were incubated in a 50 μM MTX solution in the presence or absence of 0.1 mM or 1 mM IS or IA for 15 min at 37ºC. Each column represents the mean with S.E. (MTX alone: n = 34, MTX + IS: n = 5, MTX + IA: n = 7). ** $p < 0.01$, *** $p < 0.001$, significantly different from MTX alone.

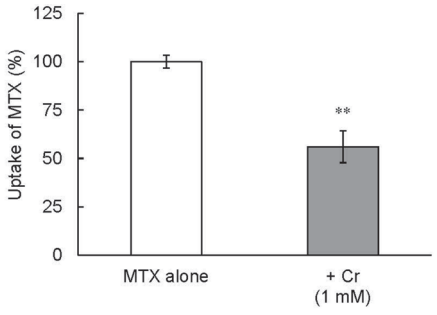

Fig. 5. Effect of Cr on MTX Uptake into Rat Renal Cortical Slices

Rat renal cortical slices (*ca.* 50 mg) were incubated in a 50 μM MTX solution in the presence or absence of 1 mM Cr for 15 min at 37ºC. Each column represents the mean with S.E. (MTX alone: n = 6, MTX + Cr: n = 5). ** $p < 0.01$, significantly different from MTX alone.

of IS (Fig. 4). However, according to our previous study,[27] the maximum plasma IA concentration in hemodialysis outpatients was 10.1 μM at most. Thus, it is less likely that IA interferes with renal transport of MTX at clinical concentrations.

At the basolateral membranes of renal tubular cells, cationic Cr is transported multi-specifically by Oat1, Oat2, Oat3, Oct2, and Oct3.[28,29] However, there are few reports describing the effect of Cr on the pharmacokinetics of OAT substrates. As shown in Fig. 5, the co-existence of 1 mM Cr lowered MTX uptake significantly, implying that Cr extensively interferes with Oat3-mediated MTX transport at the basolateral membranes of rat renal tubular cells. As MTX is also a substrate of Oat2,[30] another possibility is that Cr also inhibited Oat2-mediated MTX transport at the membranes. The mean and maximum plasma Cr levels in uremic patients are known to be 13.6 mg/dL (1.2 mM) and 24.0 mg/dL (2.1 mM), respectively.[6] Therefore, in patients with higher plasma Cr levels, inhibition of the renal excretion of MTX should be noted.

In a separate study using rat renal cortical slices, we have found that both IS and Cr exert inhibitory effect on the transport of meropenem, an anionic carbapenem antibiotic, giving a possibility that these UTs widely modulate the renal transport of anionics drugs (unpublished data). In patients with seriously impaired renal function, it is necessary to reduce the dose of renally excreted drugs. To date, this is attributed to the breakdown of glomeruli and tubules. However, our results suggested that the interaction between drugs and accumulated UTs in the renal secretory process should receive greater attention as a key factor. This study, using rat renal cortical slices, focused on the transporters expressed on the basolateral membrane of renal tubular epithelial cells. Various drug transporters such as P-glycoprotein, multidrug resistance-associated protein 2, and multidrug and toxin extrusion protein are also expressed on the brush border membranes of renal tubular cells and are involved in the transport of drugs. Previously, a paper showed that IS decreased the expression of renal-specific SLCO4C1.[31] This leads to a possibility that, when UTs such as IS accumulates in the CKD patients over the long term, the function of renal transporters are influenced by UTs to varying extents directly (through competitive inhibition) and indirectly (through modulation of transporter). The effects of IS and Cr on the drug transporters present in the kidney should be investigated in more detail.

In conclusion, it was suggested that IS and Cr potently inhibit the renal transport of Oat3 substrate drugs such as MTX at elevated plasma concentrations in patients with advanced CKD. To further evaluate the significance of the inhibitory effects of IS and Cr on clinically relevant Oat substrate drugs, additional study is required.

Conflict of interest The authors declare no conflict of interest.

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