Interaction and Transport of Thiazide Diuretics, Loop Diuretics, and Acetazolamide via Rat Renal Organic Anion Transporter rOAT1

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Diuretics are widely used for the clinical management of hypertension and edema. Thiazides and loop diuretics show their diuretic effect by inhibiting the Na\(^{+}\)-Cl\(^{-}\) cotransporter of the distal tubule and the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter at the loop of Henle, respectively (Haas, 1994). The facts that the binding of these diuretics to plasma protein at therapeutic levels is very high (>90%) and that their sites of action are localized at the luminal side of renal tubules indicate that the carrier-mediated mechanism of renal tubular secretion is important for their diuretic efficacy (Burg et al., 1973; Seely and Dirks, 1977). Furthermore, previous in vivo studies showed that the renal clearance of diuretics was reduced when probenecid, a potent inhibitor of renal organic anion transporters, was concomitantly administered (Hook and Williamson, 1965; Chennavasin et al., 1979; Gustafson and Benet, 1981; Bidiville and Roch-Ramel, 1986; Masereeuw et al., 1997). Taken together with these findings, it is suggested that tubular secretion of the diuretics mediated by renal organic anion transporters is related to their pharmacological effects. However, transporter molecules involved in tubular secretion of thiazides and loop diuretics have not been identified.

Carbonic anhydrase inhibitor acetazolamide has a strong diuretic effect, although it is principally given for the treatment of glaucoma (Yano et al., 1998). The binding of plasma protein to acetazolamide at a therapeutic concentration is also very high (>96%), and it is eliminated from the body via an active secretory mechanism in renal proximal tubule (Taft and Sweeney, 1995; Taft et al., 1996). According to the report by Sweeney et al. (1986), the combined use of nonsteroidal anti-inflammatory drugs and acetazolamide causes severe toxicity such as hyperchloremic metabolic acidosis, implying that they compete for protein binding and that a common transport pathway mediates the tubular secretion of acetazolamide and nonsteroidal anti-inflammatory drugs. To avoid such drug-drug interactions, it is necessary to elucidate the renal handling of acetazolamide at the molecular level.

Recently, several cDNAs encoding organic anion transporters in rat kidney were cloned and designated rOAT1, rOAT2,
and rOAT3 (Sekine et al., 1997, 1998; Sweet et al., 1997; Kusuhara et al., 1999). Although they transport a typical organic anion, p-aminohippurate (PAH), and have broad substrate specificities, only rOAT1 shows transport characteristics of PAH corresponding to that across basolateral membrane, i.e., PAH/α-ketoglutarate exchange (Sekine et al., 1997, 1998; Sweet et al., 1997; Uwai et al., 1998; Kusuhara et al., 1999). These data suggest that rOAT1 plays a main role for renal tubular uptake of organic anions, including clinically important drugs and endogenous compounds. In the present study, we examined the interactions between rOAT1 and various diuretics by using the Xenopus oocyte expression system, to clarify the involvement of rOAT1 in the tubular secretion of acetazolamide, thiazide diuretics, and loop diuretics.

Experimental Procedures

Materials. [Glycyl-14C]PAH (1.9 GBq/mmol) was purchased from DuPont-New England Nuclear Research Products (Boston, MA). Acetazolamide, chlorothiazide, hydrochlorothiazide, and furosemide were obtained from Nacalai Tesque (Kyoto, Japan). Ethoxzolamide and cyclothiazide were purchased from Aldrich Chemical Co. (Milwaukee, WI) and Tocris (Ballwin, MO), respectively. Other chemicals of the highest purity were purchased from Sigma (St. Louis, MO).

Functional Expression of rOAT1 in X. laevis Oocytes. The capped cRNA of rOAT1 was transcribed from NolI-linearized pSPORT containing rOAT1 cDNA with T7 RNA polymerase, and injected into Xenopus oocytes as described previously (Uwai et al., 1998). After 50 nl of water or rOAT1 cRNA (25 ng) was injected into each oocyte, the oocytes were maintained in modified Barth’s medium [88 mM NaCl, 1 mM KCl, 0.33 mM Ca(NO3)2, 0.4 mM CaCl2, 0.8 mM MgSO4, 2.4 mM NaHCO3, 10 mM HEPES] at 18°C.

[14C]PAH Uptake Reaction. Two or 3 days after injection of water or rOAT1 cRNA, the uptake reaction was initiated by incubating the oocytes in 500 µl of uptake buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, pH 7.4) containing [14C]PAH at 25°C in the presence or absence of an inhibitor for 1 h unless otherwise indicated. The uptake reaction was terminated by adding 2 ml of ice-cold uptake buffer to each well, and the oocytes were washed five times with 2 ml of the buffer. After washing, each oocyte was transferred to a scintillation counting vial and solubilized in 500 µl of 10% SDS. Five milliliters of ACSII (Amersham International, Buckinghamshire, UK) was added to each solubilized oocyte, and the radioactivity was determined in a liquid scintillation counter.

Efflux of [14C]PAH. To measure the efflux of [14C]PAH from oocytes, oocytes injected with rOAT1 cRNA (25 ng) were incubated in uptake buffer (500 µl) containing 50 µM [14C]PAH for 2 h at 25°C. After uptake, the oocytes were washed five times with 2 ml of ice-cold uptake buffer and transferred to a 1.5-ml tube containing 500 µl of the uptake buffer, including the compound to be tested, and incubated for 90 min at 25°C. The efflux for an oocyte was calculated as radioactivity associated with incubation medium divided by the sum of the radioactivities of the oocyte and incubation medium.

Uptake of Acetazolamide and Furosemide. Water-injected oocytes or rOAT1-expressing oocytes were placed in a 1.5-ml tube and uptake of acetazolamide (2 mM) or furosemide (500 µM) was initiated as described above. After uptake, oocytes were washed and homogenized in 100 µl of extraction solution (acetazolamide, 0.6% perchloric acid; furosemide, 10 mM sodium acetate/methanol; 50:50) per oocyte. The homogenate was centrifuged at 14,000 rpm for 20 min and the supernatant obtained was filtrated through a Milipore filter (SjGV1; 0.22 µM). Acetazolamide and furosemide taken up by oocytes were determined by high-performance liquid chromatograph LC-10AS (Shimadzu Co., Kyoto, Japan) equipped with a UV spectrophotometric detector (SPD-6A; Shimadzu Co.) and an integrator (Chromatopac C-R1A; Shimadzu Co.) under the following conditions: column, Zorbax ODS 4.6-mm inside diameter × 150 mm (DuPont, Wilmington, DE) for both drugs; mobile phase, 50 mM acetate buffer (pH 4.0) in acetonitrile at 19:1 for acetazolamide, 10 mM sodium acetate in methanol at 2:1 for furosemide; flow rate, 1.0 ml/min for acetazolamide, 0.8 ml/min for furosemide; wavelength, 254 nm for acetazolamide, 280 nm for furosemide; and temperature, 40°C for both agents.

Statistical Analysis. Data were analyzed statistically with ANOVA, followed by Fisher’s t test for multiple comparisons.

Results

To determine whether thiazide diuretics, loop diuretics, and acetazolamide interact with rOAT1, the inhibitory effect of these diuretics on [14C]PAH uptake by rOAT1-expressing oocytes was examined. As shown in Fig. 1, [14C]PAH uptake in the absence of diuretic increased almost linearly over 2 h. [14C]PAH uptake was moderately inhibited in the presence of acetazolamide (1 mM). Furthermore, hydrochlorothiazide, furosemide, and bumetanide at a concentration of 1 mM caused marked inhibition of [14C]PAH uptake by rOAT1-expressing oocytes; the latter two diuretics completely suppressed [14C]PAH uptake to the basal level of [14C]PAH uptake in the water-injected oocytes.

Figure 2 shows the effect of probenecid, a potent organic anion transport inhibitor, and various diuretics on the [14C]PAH uptake by rOAT1-expressing oocytes. PAH uptake was markedly inhibited in the presence of a thiazide diuretic such as chlorothiazide, cyclothiazide, and hydrochlorothiazide as well as in the presence of probenecid. Similarly, the loop diuretics such as bumetanide, ethacrynic acid, and furosemide strongly inhibited PAH uptake. The inhibitory effect of carbonic anhydrase inhibitors such as ethoxzolamide and methazolamide was much greater than that of acetazolamide, and was comparable with the inhibitory effect of thiazides and loop diuretics.

Next, to compare the inhibitory potency of acetazolamide, hydrochlorothiazide, furosemide, and bumetanide on rOAT1-mediated PAH transport, we examined the dose-dependent inhibition of [14C]PAH uptake by these diuretics. rOAT1-mediated PAH uptake was inhibited by bumetanide, furo-
semide, hydrochlorothiazide, and acetazolamide in the order of inhibitory potency, with bumetanide having the strongest inhibitory effect (Fig. 3). The inhibition constant (IC50) of PAH uptake was 1.9 \mu M for bumetanide, 7.4 \mu M for furosemide, 265 \mu M for hydrochlorothiazide, and 1.1 mM for acetazolamide, which was estimated by nonlinear regression analysis of the competition curves with one component.

To elucidate the inhibition mode of each diuretic on rOAT1-mediated PAH uptake, we performed Dixon plot analysis for the inhibitory effects of acetazolamide, hydrochlorothiazide, furosemide, and bumetanide. As depicted in Fig. 4, the apparent inhibition constant (Ki) values were approximately 1.1 mM for acetazolamide, 150 \mu M for hydrochlorothiazide, 9.5 \mu M for furosemide, and 5.5 \mu M for bumetanide. Furthermore, these results indicate that these diuretics inhibited PAH transport by rOAT1 in a competitive manner. Moreover, Fig. 5 revealed that the apparent Michaelis-Menten constant (Km) of PAH uptake (31 \mu M in the control) was increased in the presence of acetazolamide (58 \mu M) or furosemide (71 \mu M), whereas the maximum uptake rates were not affected. Eadie-Hofstee plots demonstrated that the inhibition of PAH uptake by acetazolamide was competitive but that the inhibition by furosemide was noncompetitive. Accordingly, it is suggested that the inhibition style of furosemide on rOAT1-mediated PAH transport could be the mixture of competitive and noncompetitive modes.

To elucidate whether rOAT1 mediates the translocation of diuretics, we examined the trans-stimulation effect of various diuretics on [14C]PAH efflux of rOAT1-expressing oocytes. As shown in Fig. 6, 5.5% of [14C]PAH was transported out of oocytes in the control condition without any compound, whereas 17.9 and 14.4% of [14C]PAH was transported out in

**Fig. 2.** Effect of various diuretics on [14C]PAH uptake by rOAT1-expressing oocytes. rOAT1-mediated [14C]PAH uptake for 1 h was determined by incubating oocytes in [14C]PAH (25 \mu M) in the absence or presence of the drug at 1 mM, except for ethoxzolamide at 100 \mu M and cyclothiazide at 200 \mu M. Control represents rOAT1 cRNA-injected oocytes incubated in the absence of diuretic. Water-injection represents water-injected oocytes. Each column represents the mean ± S.E. of [14C]PAH uptake in four to nine oocytes. *P < .05, significantly different from control.

**Fig. 3.** Dose dependence of inhibition of [14C]PAH uptake by acetazolamide, hydrochlorothiazide, furosemide, and bumetanide in rOAT1-expressing oocytes. rOAT1-expressing oocytes were incubated in [14C]PAH (25 \mu M) in the presence of various concentrations of acetazolamide (●), hydrochlorothiazide (■), furosemide (○), or bumetanide (●) for 1 h. Each point represents the mean ± S.E. of [14C]PAH uptake in 8 to 18 oocytes from 2 separate experiments.

**Fig. 4.** Dixon plots for the inhibitory effect of acetazolamide (A), hydrochlorothiazide (B), furosemide (C), and bumetanide (D) on [14C]PAH uptake. rOAT1-expressing oocytes were incubated in 12.5 \mu M (○), 25 \mu M (●), or 50 \mu M (▲) [14C]PAH in the absence or presence of the indicated drug at the indicated concentrations. The level of [14C]PAH uptake was determined. Data are expressed as 1/uptake (nmol/oocyte/h). Each point represents the mean ± S.E. of 3 to 10 oocytes.

**Fig. 5.** Dose-dependent uptake of [14C]PAH with acetazolamide or furosemide. [14C]PAH uptake at various concentrations in the incubation medium was measured for 1 h in the absence (○) or presence of 1 mM acetazolamide (●) or 10 \mu M furosemide (▲). Inset shows the Eadie-Hofstee plot of the data: V, initial uptake rate (pmol/oocyte/h); S, PAH concentration (\mu M). Each point represents the mean ± S.E. of [14C]PAH uptake in 3 to 10 oocytes.
suggesting that probenecid inhibited (control) or presence of unlabeled PAH, ethacrynic acid enhanced rOAT1-mediated [14C]PAH efflux to 19.0, 13.4, and 17.8%, respectively. Methazolamide and significantly enhanced rOAT1-mediated [14C]PAH efflux up that acetazolamide, chlorothiazide, and hydrochlorothiazide was not translocated by the transporter. It is noteworthy referred to a 1.5-ml tube containing 500 μl of uptake buffer in the absence (control) or presence of unlabeled PAH, a-ketoglutarate, probenecid, or a diuretic at the 1 mM, except for acetazolamide and methazolamide at 10 mM and hydrochlorothiazide at 2 mM. The oocytes were incubated for 90 min, and the radioactivity of an aliquot of the incubation buffer and the radioactivity of the oocytes and incubation medium were determined. Each result was expressed as the ratio of the radioactivity of the incubation buffer to the sum of the radioactivities of the incubation buffer and oocytes. Each column represents the mean ± S.E. of three to five experiments. For each experiment, 4 to 10 oocytes were used. *P < .05, significantly different from control.

the presence of unlabeled PAH and a-ketoglutarate, respectively, in the incubation medium. However, in the presence of probenecid, the efflux of [14C]PAH was reduced to 2.3%, suggesting that probenecid inhibited [14C]PAH transport, but was not translocated by the transporter. It is noteworthy that acetazolamide, chlorothiazide, and hydrochlorothiazide significantly enhanced rOAT1-mediated [14C]PAH efflux up to 19.0, 13.4, and 17.8%, respectively. Methazolamide and ethacrynic acid enhanced rOAT1-mediated [14C]PAH efflux to 9.0%. Bumetanide and furosemide showed very weak trans-stimulation effect on PAH efflux. These findings suggest that acetazolamide, chlorothiazide, and hydrochlorothiazide were translocated by rOAT1 as a substrate.

Finally, we examined directly whether rOAT1 mediates acetazolamide and furosemide uptake. As shown in Fig. 7, uptake of acetazolamide was markedly enhanced in rOAT1-expressing oocytes until 6 h compared with water-injected oocytes. The rOAT1-mediated acetazolamide uptake was significantly inhibited in the presence of 1 mM probenecid (without probenecid, 81.43 ± 5.71; with probenecid, 48.24 ± 2.97 pmol/oocyte/2 h; mean ± S.E. of four oocytes, *P < .05). Moreover, rOAT1 significantly stimulated furosemide uptake (Fig. 8).

Discussion

The major findings in the present study are that acetazolamide and furosemide were transported by rOAT1 and that chlorothiazide and hydrochlorothiazide appeared to be transported by rOAT1. Although it had been suggested that these diuretics are actively secreted into the renal tubules through a carrier-mediated transport mechanism for organic anions, the transporter protein had not been identified. Using the trans-stimulation technique, we found that acetazolamide, chlorothiazide, and hydrochlorothiazide significantly enhanced the efflux of [14C]PAH through rOAT1 (Fig. 6). These findings provide the first evidence that shows the exchange of these diuretics with PAH via rOAT1.

Acetazolamide is mainly excreted in the unchanged form into the urine. With the isolated perfused rat kidney, tubular secretion is shown to play an important role in its elimination (Taft and Sweeney, 1995; Taft et al., 1996). There are case reports describing toxic interaction between acetazolamide and salicylate, which are often coadministered for the elderly with glaucoma and arthritis (Sweeney et al., 1986). The reasons are suggested that they compete with each other at the protein binding and renal tubular secretion. Also, Yano et al. (1998) demonstrated that the large interindividual variability of acetazolamide clearance is associated with individual renal functions. Therefore, to elucidate the elimination mechanism of acetazolamide at molecular level will give useful information to the optimal dosage regimen adjustment. So far, it has not been identified which transporters mediate renal tubular secretion of acetazolamide. In this study, we demonstrated that acetazolamide is a transport substrate of rOAT1, suggesting that the tubular uptake of acetazolamide across the basolateral membrane may be mediated, at least in part, by rOAT1 in the kidney (Fig. 7). The pharmacological effect of acetazolamide is reported to be obtained at about 4 μg/ml (20 μM) in plasma concentrations (Yano et al., 1998). As shown in Fig. 4, its Kᵣ value for rOAT1 is 1.1 mM. Accordingly, acetazolamide in the therapeutic range should not show inhibitory effect on rOAT1-mediated uptake of other anions in the renal tubules.

**Fig. 6.** [14C]PAH efflux of rOAT1-expressing oocytes in the presence of unlabeled PAH, a-ketoglutarate, probenecid, or various diuretics. After rOAT1-expressing oocytes were incubated with [14C]PAH (50 μM) for 2 h, each oocyte was washed five times with ice-cold uptake buffer and transferred to a 1.5-ml tube containing 500 μl of uptake buffer in the absence (control) or presence of unlabeled PAH, a-ketoglutarate, probenecid, or a diuretic at the 1 mM, except for acetazolamide and methazolamide at 10 mM and hydrochlorothiazide at 2 mM. The oocytes were incubated for 90 min, and the radioactivity of an aliquot of the incubation buffer and the radioactivity of the oocytes and incubation medium were determined. Each result was expressed as the ratio of the radioactivity of the incubation buffer to the sum of the radioactivities of the incubation buffer and oocytes. Each column represents the mean ± S.E. of four oocytes. *P < .05, significantly different from control.

**Fig. 7.** Acetazolamide uptake by rOAT1-expressing oocytes. Water-injected (○) or rOAT1 cRNA-injected (●) oocytes were incubated with 2 mM acetazolamide for the indicated period. Each point represents the mean ± S.E. of four oocytes.

**Fig. 8.** [14C]PAH and furosemide uptake by rOAT1-expressing oocytes. Water-injected (○) or rOAT1 cRNA-injected (●) oocytes were incubated with 500 μM [14C]PAH (A) or furosemide (B) for 1 h. Each column represents the mean ± S.E. of 5 to 10 oocytes. *P < .05, significantly different from water-injected oocytes.
In humans, the diuretic effect of hydrochlorothiazide is achieved at much lower plasma concentrations than the concentration used in the isolated perfused rat kidney. The therapeutic concentration of hydrochlorothiazide is reported to be between 100 and 500 ng/ml, concentrations at which no diuretic effect was observed in the isolated perfused rat kidney (Masereeuw et al., 1997). Therefore, it has been considered that hydrochlorothiazide is transported efficiently to the site of action in the distal lumen due to efficient tubular secretion in the proximal tubules. The present finding that hydrochlorothiazide is transported by rOAT1 supports that on administration of the drug, this drug is extensively distributed and accumulates in the kidney, and that it is secreted efficiently into the proximal lumen to reach the site of action in the distal lumen.

In the present study, we showed that the inhibition style of furosemide is the mixture of competitive and noncompetitive modes (Figs. 4 and 5). Furthermore, [14C]PAH efflux mediated by rOAT1 was not trans-stimulated by furosemide but by unlabeled PAH at a concentration of 1 mM (Fig. 6). We speculate that the lack of a trans-stimulation effect of furosemide on PAH efflux is not due to the concentration of furosemide because the affinities of PAH and furosemide for rOAT1 were similar (Figs. 3–5). However, rOAT1-mediated furosemide uptake was observed (Fig. 8). These data suggested that the lack of trans-stimulation effect of furosemide on PAH efflux may be due to the noncompetitive inhibitory mechanism of furosemide. Previously, it was reported that rOAT1 transported indomethacin but that the drug did not stimulate PAH efflux via rOAT1 (Apivatthanakul et al., 1999). There might be a possibility that rOAT1 has another transport mechanism except as an exchanger.

Previous in vivo studies demonstrated that the renal excretion of furosemide is reduced when given concomitantly with probenecid, suggesting that tubular secretion via the organic anion transport system is the main route for its excretion from the body (Hook and Williamson, 1965; Chennavasin et al., 1979). As shown in Fig. 8, furosemide was a transport substrate of rOAT1, which mediates predominant PAH uptake across basolateral membrane (Sekine et al., 1997, Sweet et al., 1997, Uwai et al., 1998). Bidiville and Roch-Ramel (1986) reported that probenecid inhibited the renal secretion of furosemide by 95%, whereas PAH depressed the renal secretion of furosemide by only 44 to 66% in rabbit kidneys. Taken together with our report and our findings, there should be a transporter besides rOAT1 that plays an important role for “kidney-specific” excretion of furosemide and whose affinity for PAH was low. To date, rOAT2 and rOAT3 have been identified as members of the OAT family that mediate PAH transport, but not PAH/dicarboxylate exchange (Sekine et al., 1998; Kusuhara et al., 1999). In particular, rOAT3 has been considered to be one of the interaction sites for anionic drugs in the renal tubules. However, the findings that furosemide is immediately eliminated by renal tubular secretion, and that mRNA expression levels of rOAT2 and rOAT3 were found predominantly in the liver and moderately in the kidney suggest that the contribution of both transporters for renal tubular secretion of furosemide could be little. It is likely that rOAT1 and other unidentified organic anion transporters expressed especially in the kidney mediate the tubular secretion of furosemide.

It was reported that the renal clearance of probenecid is reduced when coadministered with PAH in humans (Meisel and Diamond, 1977). This finding suggests that probenecid and PAH share the same transporter(s). In contrast, our previous study showed that probenecid was not transported by rOAT1 (Uwai et al., 1998). As illustrated in Figs. 2 and 6, rOAT1-mediated [14C]PAH uptake was drastically reduced in the presence of probenecid, and PAH efflux was not trans-stimulated by probenecid. These findings suggest that probenecid is not accepted as a transport substrate by rOAT1, but has a potent inhibitory interaction with rOAT1. There may be a transporter that mediates the renal secretion of probenecid.

In conclusion, this study demonstrated that acetazolamide, thiazide diuretics, and loop diuretics interact with rOAT1 and that rOAT1 transports acetazolamide and furosemide and appears to mediate thiazide uptake. Accordingly, rOAT1 is suggested to be involved in the renal tubular secretion of acetazolamide, thiazides, and furosemide, thereby being responsible for their delivery to the site of action in effective amounts.

References