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European Journal of Pharmacology 524 (2005) 44–48
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Mouse organic anion transporter 2 and 3 (mOAT2/3[*Slc22a7/8*]) mediates the renal transport of bumetanide

Yasuna Kobayashi, Masayuki Ohbayashi, Noriko Kohyama, Toshinori Yamamoto *

Department of Clinical Pharmacy, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan

Received 14 April 2005; received in revised form 23 August 2005; accepted 27 September 2005
Available online 27 October 2005

Abstract

Multispecific organic anion transporters play an important role in the excretion and the elimination of a wide variety of endogenous and exogenous substrates. To date, five murine OAT homologs such as mouse organic anion transporters 1–3, 5, and 6 (mOAT1–3, 5 and 6) have been isolated and well characterized. With the exception of mOAT6, other mOAT isoforms are predominantly expressed in the kidney. The aim of this study was to examine whether mOAT2/3, as well as hOAT2/3, transports the diuretic bumetanide using a *Xenopus laevis* oocyte expression system. When expressed in *Xenopus* oocytes, mOAT2/3 mediated the high affinity transport of bumetanide. The apparent K_m values for the uptake of bumetanide via mOAT2 and mOAT3 were 9.12±2.42 μM and 1.01±0.27 μM, respectively. Immunohistochemical analysis revealed that mOAT2 is expressed on the luminal membrane site of the proximal tubule. Our results indicate that mOAT2 and 3, as well as human homologs, are molecules for the transport of bumetanide on the luminal membranes of kidney proximal tubules.

Keywords: OAT; Drug transport; Transporter; Diuretic; Bumetanide

1. Introduction

The kidney plays a pivotal role in the maintenance of both cellular and organismic homeostasis (Bossuyt et al., 1996; Muller and Jansen, 1997; Ullrich, 1997; Rémon et al., 2000). The proximal tubular cells of the kidney take up organic anions from the blood stream via multispecific organic anion transport pathway(s) in the basolateral membranes (Ullrich and Rumrich, 1993; Pritchard and Miller, 1993; Ullrich, 1997; Rémon et al., 2000). Several distinct kidney-predominant organic anion transporters such as organic anion transporting polypeptides (OATP1A2[*SLC21A3/SLCO1A2*], OATP4C1[*SLC21A20/SLCO4C1*]), urate transporter 1 (URAT1[*SLC22A12*]), OAT1 [*SLC22A6*], OAT3[*SLC22A8*], rat kidney-specific organic anion transporter 1 (OAT-K1[*Oatp1a3_v1*]), and rat kidney-specific organic anion transporter 2 (OAT-K2[*Oatp1a3_v2*]) have been isolated and well characterized (Saito et al., 1996; Sekine et al., 1997; Sweet et al., 1997; Masuda et al., 1999; Cha et al., 2001; Sun et al., 2001; Enomoto et al., 2002; Hagenbuch and Meier, 2004; Kobayashi et al., 2004). These transporters mediate the transport of various kinds of structurally unrelated organic compounds such as *p*-aminohippuric acid, methotrexate, estron-3-sulfate, thyroid hormones, urate, and ochratoxin A. Therefore, these multispecific organic anion transporters are believed to be the molecules responsible for renal handling of organic drugs and chemicals.

Regarding the mouse homolog of OATs, five isoforms (mOAT1–3[*Slc22a6-8*], mOAT5[*Slc22a19*], and mOAT6 [*Slc22a20*]) have been isolated to date (Lopez-Nieto et al., 1997; Brady et al., 1999; Kuze et al., 1999; Kobayashi et al., 2002b; Monte et al., 2004; Youngblood and Sweet, 2004). With the exception of mOAT6, four murine OATs are highly expressed in the kidney across species (Sekine et al., 1997; Sweet et al., 1997; Kusuhara et al., 1999; Race et al., 1999; Kobayashi et al., 2004; Monte et al., 2004; Youngblood and Sweet, 2004). These findings suggest that mOAT2 and mOAT3, as well as mOAT1 and 5, are responsible for the distribution of drugs and chemicals into the kidney.

Loop diuretics such as furosemide and bumetanide have been used for many years as therapy for hypertension, edema associated with congestive heart failure, and renal diseases

* Corresponding author. Tel.: +81 3 3784 8220; fax: +81 3 3784 3838.
E-mail address: yamagen@pharm.showa-u.ac.jp (T. Yamamoto).

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doi:10.1016/j.ejphar.2005.09.054

including nephrotic syndrome. It has been reported that there is a difference in diuretic activity between furosemide and bumetanide. For example, Olesen et al. have reported that the activity of bumetanide is about 40 times that of furosemide (Olesen et al., 1973). Furosemide and bumetanide are known to be potent inhibitors of the $Na^+/K^+/Cl^-$ cotransport system (Saier and Boyden, 1984). Although bumetanide is a competitive inhibitor of the Na^+-taurocholate cotransporter (Ntcp[*Slc10a1*]) or -independent (organic anion transporting polypeptide[*Slc21/Slco*]) transport systems (Blitzer et al., 1982; Petzinger et al., 1989), Horz et al. (1996) have demonstrated that bumetanide is not a substrate of Na^+-taurocholate cotransporter and organic anion transporting polypeptide, suggesting that bumetanide is taken up by a third organic anion transport system which is different from those of Na^+-taurocholate cotransporter and organic anion transporting polypeptide 1. In this respect, Hasannejad et al. (2004) have recently revealed that hOAT3, but not hOAT2, mediates the transport of bumetanide. However, we have recently reported that oocytes expressing hOAT2 also mediate the transport of bumetanide (Kobayashi et al., 2005). Although we have no definitive explanation for this discrepancy, we assumed that mOAT2/3, as well as hOAT2/3, could mediate the transport of bumetanide.

In the present study, therefore, we investigated whether bumetanide is transported via the mouse homologs of OAT2 and OAT3. Our results indicate that, at least partly, both organic anion transporters mediate the transport of bumetanide into the proximal tubular cells of the mouse kidney.

2. Materials and methods

2.1. Chemicals

[^{3}H]Bumetanide (5.0 Ci/mmol) was purchased from ARC, Inc. (St. Louis, MO, USA). All other chemicals not listed here were of the highest grade commercially available.

2.2. Xenopus laevis oocyte preparation, cRNA synthesis and transport experiments

Xenopus oocyte isolation was performed as previously described (Kobayashi et al., 2002b). Stage V and VI defolliculated oocytes obtained from adult female *X. laevis* were selected throughout this study. To remove the follicular layer from *Xenopus* oocytes, collagenase A (Roche Applied Sciences, Mannheim, Germany) was used at a final concentration of 2.0 mg/ml in oocyte Ringer 2 (83 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) and slowly shaken for 1 h at room temperature. The mOAT2 cDNA (GenBank accession no. AB069965) and the mOAT3 cDNA (GenBank accession no. AB079895) were linearized with *Bam*HI, and the capped cRNA was transcribed in vitro by T7 RNA polymerase. Defolliculated oocytes were microinjected with 50 ng of in vitro transcribed cRNA under a stereomicroscope using a microdispenser (Drummond Scientific, Broomall, PA, USA) and incubated for 2 days in a modified Barth's solution containing gentamicin (50 μg/ml) at 18 °C. Uptake experiments of radiolabeled bumetanide were performed in an ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) at room temperature. Oocytes were incubated in an ND96 solution containing [^{3}H]bumetanide for 1 h. For the kinetic study, concentration-dependent uptake experiments of [^{3}H] bumetanide via mOAT2 and mOAT3 were performed with each compound at a final concentration ranging from 1, 2, 5, 20, and 30 μM to 0.1, 1, 2, 8, and 10 μM, respectively. The uptake was terminated by the addition of 2 ml of an ice-cold ND96 solution, and the oocytes were washed with the same solution at least five times. The oocytes were solubilized with 250 μl 10% (w/v) sodium dodecyl sulfate (SDS), and accumulated radioactivity was determined with a liquid scintillation counter. Each experiment was repeated more than 3 times to confirm the results.

2.3. Immunohistochemical analysis

For immunohistochemical analysis, rabbits were immunized with a keyhole limpet hemocyanin-conjugated synthesized peptide and the 14 amino acids of the COOH terminus of mOAT2 (Transgenic Co. Ltd., Kumamoto, Japan). Five-micrometer wax sections of nephrectomized male mice kidneys (BioChain Institute Inc., San Leandro, CA, USA) were processed for light microscopic immunohistochemical analysis using the streptavidin–biotin–horseradish peroxidase complex technique (LSAB kit, DAKO, Carpinteria, CA, USA). Sections were dewaxed, rehydrated, and incubated with 3% H_2O_2 for 25 min to eliminate endogenous peroxidase activity. After rinsing in 0.05 M Tris-buffered saline containing 0.1% Tween-20, sections were treated with primary rabbit polyclonal antibodies at room temperature for 2 h. Thereafter, the sections were incubated with the secondary antibodies and with the biotinylated goat polyclonal antibody against rabbit immunoglobulin (DAKO, Carpinteria, CA, USA), for 30 min with horseradish peroxidase-labeled streptavidin. The sections were counterstained with hematoxylin and examined by light microscopy. For the preabsorption test, the mOAT2 peptide (200 μg/ml) was added to the mOAT2-specific antibody solution and incubated at least 15 h at 4 °C.

2.4. Statistical analysis

Statistical differences were evaluated using one-way Analysis of Variance (ANOVA). The values represent the Mean± S.E.M. ($^*P<0.05$).

3. Results and discussion

3.1. Transport of bumetanide mediated by mOAT2 and mOAT3

Oocytes expressing hOAT2 mediate the transport of bumetanide in a concentration-dependent manner (Kobayashi et al., 2005). Based upon our results, we tested whether the murine homolog of OAT2 could mediate the transport of bumetanide. Since transport activity increased linearly until 2.0 h (data not shown), radiolabeled bumetanide was incubated with mOAT2

expressing oocytes for 1.0 h. As expected, mOAT2 mediated the high transport of [^{3}H]bumetanide to about 27-fold that of the control oocytes (Fig. 1). Thus, mOAT2, as well as hOAT2, mediated the transport of bumetanide. Based on this finding, we subsequently examined the transport of bumetanide in oocytes expressing mOAT3. As expected, mOAT3 mediated the transport of bumetanide to a similar extent compared with mOAT2 (Fig. 1). Taking these facts and a previously published paper (Hasannejad et al., 2004; Kobayashi et al., 2005) into consideration, bumetanide is a substrate for human and mouse OAT2/3.

The concentration-dependent uptake of [^{3}H]bumetanide via mOAT2 and mOAT3 was subsequently examined. The mOAT2-mediated uptake of bumetanide showed saturable kinetics and could be modeled by the Michaelis–Menten equation (Fig. 2A). Nonlinear regression analysis yielded a K_m value of 9.12±2.42 μM for mOAT2-mediated uptake of bumetanide. Likewise, oocytes expressing mOAT3 mediated the concentration-dependent transport of bumetanide with high affinity (K_m=1.01±0.27 μM) (Fig. 2B). Our results indicate that mOAT2 and 3, as well as hOAT2 and 3, may, at least partly, be responsible for the renal uptake of bumetanide.

3.2. Membrane localization of mOAT2

Enomoto et al. (2002) have revealed that hOAT2 is localized to the basolateral side of the proximal tubule in the kidney, whereas rOAT2 is detected in the apical surface of the tubules in the medullary thick ascending limb of Henle's loop and cortical and medullary collecting ducts (Kojima et al., 2002). However, there is no publication concerning the localization of mOAT2 in the mouse kidney. As shown in Fig. 3B, light microscopy of 5-μm wax sections demonstrated that there was specific immunostaining of mOAT2 in the luminal surface of proximal tubular cells. There was no staining of mOAT2 in glomerular cells or Bowman's capsule. By incubating with mOAT2 oligopeptide instead of primary antibody, the immunoreactivity

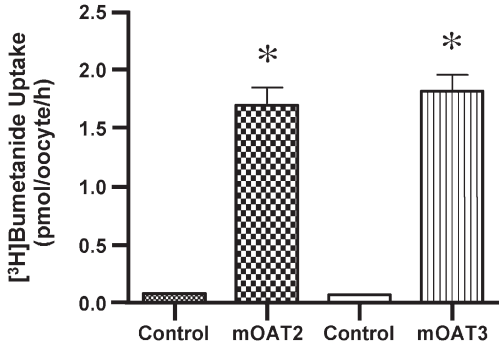

Fig. 1. Transport of [^{3}H]bumetanide mediated by mOAT2 and mOAT3. The uptake rates of [^{3}H]bumetanide (2 μM) by the control, mOAT2, or mOAT3 expressed oocytes were measured for 1 h. Data are means±SEM of 8–17 oocyte determinations. Other experimental conditions and details are described in Materials and methods. The significance between the control (non-injected), mOAT2, or mOAT3-cRNA-injected oocytes was determined by the unpaired *t*-test (*P<0.05). mOAT2, mouse organic anion transporter 2; mOAT3, mouse organic anion transporter 3.

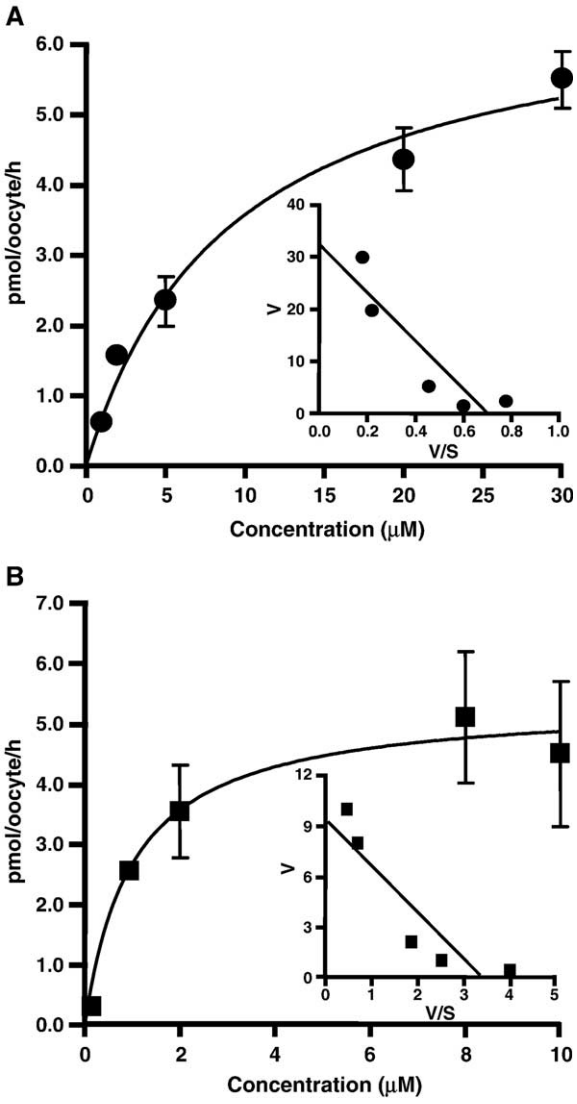

Fig. 2. Concentration dependent uptake of [^{3}H]bumetanide mediated by mOAT2 (A) and mOAT3 (B). The uptake experiments of bumetanide via mOAT2 and mOAT3 were performed at final concentrations ranging from 1, 2, 5, 20, and 30 μM to 0.1, 1, 2, 8, and 10 μM, respectively. The uptake rates of bumetanide by the control (non-injected), mOAT2- or mOAT3-expressing oocytes for 1 h were measured at variable concentrations. The uptake was saturable with K_m values of 9.12±2.42 μM (A) and 1.01±0.27 μM (B) for bumetanide uptake via mOAT2 and mOAT3, respectively, and fit to the Michaelis–Menten curve. Values are means±S.E.M. of 9–21 oocyte determinations. The mOAT2-mediated and the mOAT3-mediated transport were determined by subtracting the transport velocity in the control (non-injected) oocytes from that in the mOAT2- and the mOAT3-expressing oocytes, respectively. Other experimental conditions and details are described under Materials and methods.

was not completely detected (Fig. 3A). Our findings indicate that mOAT2 is localized on the luminal site of the kidney proximal tubules (Fig. 3C and D).

It has been reported that there is an interspecies difference in the transport of organic compounds via OATs (Sekine et al., 1998; Morita et al., 2002). Concerning the possibility of transporting bumetanide via other OAT isoforms, Sekine et al. (1998) and Kusuhara et al. (1999) have suggested that bumetanide is a candidate for the substrate of rat OAT2 (rOAT2) and rat OAT3 (rOAT3). Moreover, Uwai et al. (2000) reported

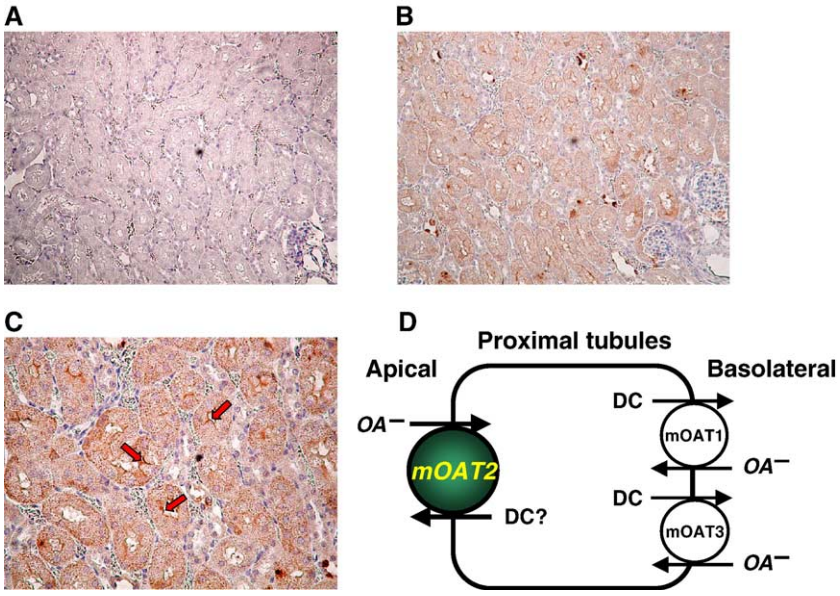

Fig. 3. Immunohistochemical analysis of mOAT2 in male mouse kidney. Five-micrometer sections were incubated with polyclonal mOAT2 antibody. The luminal membrane of proximal tubules was stained (B, × 100; C, × 200). Diagram of kidney proximal tubular cells and membrane localization of mouse OATs (D). Immunoreactivity was completely abolished by pretreatment of antibody with mOAT2 oligopeptide (A). OA^-, organic anions; DC, dicarboxylates.

direct evidence that rat OAT1 (rOAT1) is a molecule responsible for the uptake of bumetanide. Therefore, it would be interesting to elucidate whether bumetanide is a substrate of rOAT2 and rat rOAT3.

The expressions of r/mOAT2 and r/mOAT3 are exhibited in a sex-dependent manner (Buist et al., 2002; Kobayashi et al., 2002a; Kobayashi et al., 2002b; Buist and Klaassen, 2004). rOAT2 in the liver is expressed at a higher level in male rats than in females, whereas the *rOAT2* gene in the kidney is detected predominantly in females (Kobayashi et al., 2002b; Buist et al., 2002). Recently, Buist and Klaassen (2004) have revealed that there is no sex-associated differential gene expression of mOAT2 in the kidney using branched DNA (bDNA) analysis. Accordingly, it would be of interest to elucidate whether there is a pharmacokinetic difference between male and female mice in bumetanide clearance. In this respect, further studies are required.

Regarding the other transporters responsible for the uptake of bumetanide, Honscha et al. (2000) have revealed that bumetanide is transported via rat liver methotrexate carrier 1/2. However, the uptake rate of bumetanide via rat liver methotrexate carrier 1/2 exhibited only a few fold that of the control oocytes. In the present study, the transport of bumetanide mediated by mOAT2 and mOAT3 exhibited approximately 30 times higher that of the control oocytes. Our results, therefore, suggest that mOAT2/3 may be the main molecule for bumetanide uptake.

In conclusion, the present study demonstrated that bumetanide is a substrate of mOAT2 and mOAT3. These transporters may be essentially related to the transport of bumetanide on the luminal and basolateral membrane site of the mouse kidney proximal tubular cells. Moreover, we found that there was no interspecies difference in the transport of bumetanide between the human and mouse homologs of OAT2 and 3. Whether other members of the OAT family (*Slc22a*) and the OATP/oatp family (*Slc21/Slco*) are able to mediate the transport of bumetanide remains to be determined.

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