J Pharmacol Sci **103**, 48 – 55 (2007)

Journal of Pharmacological Sciences

Full Paper

Role of Mouse Organic Anion Transporter 3 (mOat3) as a Basolateral Prostaglandin E_2 Transport Pathway

Sirinun Nilwarangkoon[1], Naohiko Anzai[1], Katsuko Shiraya[1], Erkang Yu[1], Rafiqul Islam[1], Seok Ho Cha[1], Maristela Lika Onozato[2], Daisaku Miura[1], Promsuk Jutabha[1], Akihiro Tojo[2], Yoshikatsu Kanai[1], and Hitoshi Endou[1,*]

[1]*Department of Pharmacology and Toxicology, Kyorin University School of Medicine, 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan*
[2]*Department of Nephrology and Endocrinology, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan*

Received July 24, 2006; Accepted November 14, 2006

Abstract. Renal organic anion transporters play an important role in the handling of a number of endogenous and exogenous anionic substances in the kidney. In this study, we investigated prostaglandin E_2 (PGE_2) transport properties and intrarenal localization of mouse organic anion transporter 3 (mOat3). When expressed in *Xenopus* oocytes, mOat3 mediated the time- and concentration-dependent transport of PGE_2 (K_m: 1.48 μM). PGE_2 transport mediated by mOat3 was *trans*-stimulated by intracellular glutarate injected into the oocytes. PGE_2 efflux via mOat3 was also *trans*-stimulated by extracellular glutarate. Thus, mOat3 was shown to mediate the bidirectional transport of PGE_2, partly coupled to the dicarboxylate exchange mechanism. Immunohistochemical study revealed that mOat3 protein was localized at the basolateral membrane of renal proximal and distal tubules. Furthermore, diffuse expression of mOat3, including expression in the basolateral membrane in macula densa (MD) cells, was observed. These results indicate that mOat3 plays an important role as a basolateral transport pathway of PGE_2 in the distal nephron including MD cells that may constitute one of the indispensable steps for renin release and regulation of the tubuloglomerular feedback mechanism.

Keywords: organic anion transporter, OAT, prostaglandin E_2, glutarate, macula densa

Introduction

Prostanoids, which include prostaglandins (PGs) and thromboxanes (TXs), are cyclooxygenase (COX)-dependent metabolites of arachidonic acids and play various physiological and pathophysiological roles (1, 2). Among them, PGE_2 is the major prostanoid in the kidney and is synthesized at high rates along the nephron, particularly in the collecting duct (3). PGE_2 plays an important role in the tubular reabsorption of salt and water as well as in the control of renal vascular resistance and the maintenance of glomerular hemodynamics. In addition, PGE_2 stimulates the release of renin from the juxtaglomerular apparatus (JGA). Recently, it has been reported that intact macula densa (MD) cells synthesize and release PGE_2 when luminal salt content is reduced, and it has been suggested that this response is involved in the control of renin release and renal vascular resistance during salt deprivation (4). In these functions, PGE_2 mediates autocrine and paracrine signaling over short distances through the activation of its four receptor subtypes (EP_1, EP_2, EP_3, and EP_4) (3). Thus, to maintain the extracellular concentration of PGE_2, the termination of PGE_2 signaling requires rapid re-uptake of released PGE_2 followed by cytoplasmic oxidation (5). Since PGE_2 possesses anionic moieties at physiological pH and diffuses poorly through the lipid bilayer, it is thought that PGE_2 transport across the plasma membrane is a carrier-mediated transport process (5). However, little is known about the molecular mechanism of the release of PGE_2 in distal nephron including MD cells.

To date, several PG carriers have been characterized (5). Prostaglandin transporter PGT (Slc21a2, oatp2A1)

*Corresponding author. endouh@kyorin-u.ac.jp
Published online in J-STAGE: January 13, 2007
doi: 10.1254/jphs.FP0060816

is broadly expressed in COX-positive cells and is coordinately regulated with COX. By analogy with neurotransmitter release and re-uptake, PGT may regulate pericellular PG levels via re-uptake (6). Immunocytochemical study has revealed that PGT in rat kidneys was expressed in glomerular endothelial and mesangial cells, arteriolar endothelial and muscularis cells, principal cells of the collecting duct, medullary interstitial cells, and the medullary vasa rectae endothelia (7). In the collecting duct, PGT is expressed in subapical vesicles. These results indicate that PGT is unlikely to be involved in the basolateral transport of PGE_2 in distal nephron including MD cells. Certain PGs are actively extruded from cells by multidrug resistance-associated proteins (MRPs) (5); these may play a role in metabolic clearance of PGs. However, the expression of MRPs in MD cells is still unclear.

Organic anion transporter (OAT) family members are other PG transporters (8). OATs play important roles in the elimination of a variety of endogenous substances, xenobiotics and their metabolites, many of which are potentially toxic to the body (9 – 14). Recently, cDNAs encoding OAT family members, including OAT1, OAT2, OAT3, OAT4, URAT1 (urate transporter 1), and Oat5, have been successively cloned (9). Among these clones, OAT1(human)/Oat1(rodents) and OAT3/Oat3 were shown to be localized to the basolateral side of the proximal tubule, whereas OAT4, URAT1, and Oat5 were shown to be localized to the apical side of the proximal tubule. In contrast, the exact localization of OAT2 protein in the kidney is still controversial: Rat Oat2 was formerly identified at the apical membrane of the thick ascending limb of Henle and cortical collecting ducts (15), but recently it has been shown to be localized at the apical side of proximal straight tubules (S_3 segment) (16), whereas human OAT2 was found to be localized to the basolateral membrane of the proximal tubule (17).

Among the OATs, OAT3 protein expression was detected in nearly all of the nephron segments in the rat kidney (15). Thus, OAT3 is likely to be a transporter responsible for the basolateral transport of PGE_2 in distal nephron including MD cells. Although Oat3 knockout mice have been generated several years ago (18), information on the functional properties of mouse Oat3 (mOat3) is limited (19 – 21). In this study, we examined PGE_2 transport properties and intrarenal localization of mOat3.

Materials and Methods

Materials

The materials used in this study were purchased from the following sources: [^{14}C]*p*-aminohippurate (PAH) (1.90 GBq/mmol) was from Moravek Biochemicals (Brea, CA, USA); [^{3}H]estrone-3-sulfate (E_1S) (2.0 TBq/mmol), [^{14}C]glutarate (2.035 GBq/mmol), and [^{3}H]PGE_2 (7.429 TBq/mmol) were from PerkinElmer Life Science Products (Boston, MA, USA); and glutarate was from Wako (Osaka). All other chemicals and reagents used were of analytical grade and obtained from commercial sources.

Animals

Six-week-old male ICR mice were purchased from Saitama Experimental Animal Co., Ltd. (Saitama) and kept under routine laboratory conditions with free access to standard laboratory chow and water.

Isolation of mOat3

A nondirectional cDNA library for screening was prepared from mouse kidney poly(A)$^+$ RNA using a Superscript Choice System (Invitrogen, Carlsbad, CA, USA) and was ligated into the phage vector ZipLox EcoRI arms (Invitrogen). The library was screened by homology using full-length rOat3 cDNA labeled with [^{32}P]dCTP by random priming (T7Quick Prime Kit; Amersham Pharmacia Biotech, Uppsala, Sweden) as a probe as described previously (22). cDNA inserts in positive ZipLox phages were recovered in the plasmid pZL1 vector by in vitro excision and completely sequenced with specially synthesized oligonucleotide primers by the dye terminator method using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

cRNA synthesis and uptake experiments using Xenopus laevis oocytes

cRNA synthesis and uptake experiments were performed as described previously (23). The capped cRNA of mOat3 was synthesized in vitro by T7 RNA polymerase from a plasmid linearlized with Xba I. Defolliculated oocytes were injected with 10 ng of the capped mOat3 cRNA or water (control) and incubated in Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 10 mM HEPES) containing 50 μg/ml gentamicin at 18°C. After 2 to 3 days of incubation, uptake experiments of radiolabeled substrates, as indicated in each experiment, were performed at room temperature in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.4). Each experiment was repeated more than two times to confirm the results. Representative results are shown in the figures.

Kinetic parameter for the uptake of PGE_2 via mOat3

was estimated from the following equation: $v = (V_{max} \times S) / (K_m + S)$, where v is the rate of substrate uptake (pmol/h·oocyte), S is the substrate concentration in the medium (μM), K_m is the Michaelis-Menten constant (μM), and V_{max} is the maximum uptake rate (pmol/h·oocyte). These kinetic parameters were determined with the Eadie-Hofstee equation.

To examine the *trans*-stimulatory effects of both the uptake and efflux of radiolabeled substrates, cold glutarate (50 mM) (Fig. 3) or 50 nl of [^{3}H]PGE_2 (0.6 μM) (Fig. 4) was injected into oocytes expressing mOat3 with a fine-tipped glass micropipette as described previously (24). Then individual oocytes were washed twice with ice-cold ND96 solution, placed on ice for 5 min, then incubated with ND96 at room temperature for 1 h, and finally transferred into a medium with or without radiolabeled E_1S (Fig. 3A) or PGE_2 (Fig. 3B) or with cold glutarate (10 mM) (Fig. 4) and incubated at room temperature for 1 h. [^{3}H]PGE_2 before and after taken up by the oocytes was little degraded as Chan et al. reported previously (25). Radioactivity in both the medium and oocytes was determined after a 1-h incubation.

For the uptake and efflux measurements in the present study, 8 – 10 oocytes were used for each data point. The values are expressed as means ± S.E.M. Each experiment was repeated at least twice to confirm the results. Results from representative experiments are shown in the figures.

Immunohistochemical analysis

For immunohistochemical analysis, rabbits were immunized with a keyhole limpet hemocyanin-conjugated synthesized peptide, CKASQTIPLKTGDPS, corresponding to cysteine and the 14 amino acids of the COOH terminus of mOat3. Two-micrometer wax sections of nephrectomized mouse kidney 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 10 min to eliminate endogenous peroxidase activity. After rinsing in 0.05 M Tris-buffered saline containing 0.1% Tween-20, sections were treated with 10 μg/ml of primary rabbit polyclonal antibody (at 4°C overnight). Thereafter, the sections were incubated with the secondary antibody, biotinylated goat polyclonal antibody against rabbit immunoglobulin (DAKO), diluted 1:400 for 30 min with horseradish peroxidase-labeled streptavidin. This step was followed by incubation with diaminobenzidine and hydrogen peroxide. The sections were counterstained with hematoxylin and examined by light microscopy. For a preabsorption experiment, the mOat3 peptide (200 μg/ml) was added to the mOat3-specific antibody solution and incubated overnight at 4°C. Using this preabsorbed antibody, immunohistochemistry was performed as described above.

Statistical analysis

Data are expressed as means ± S.E.M. Statistical differences were determined using Student's *t*-test. The reproducibility of the results in the present study was confirmed using two or three separate experiments. Results from representative experiments are shown in the figures.

Results

mOat3 cDNA was isolated from the kidney. As shown in Fig. 1, mOat3 mediated the transport of [^{14}C]PAH, [^{3}H]E_1S, [^{3}H]glutarate, and [^{3}H]PGE_2. These results indicate that our mOat3 clone is functional and its transport activity is compatible with that reported previously (18 – 20).

The uptake by mOat3 cRNA-injected oocytes increased linearly for about 180 min (Fig. 2A). Accord-

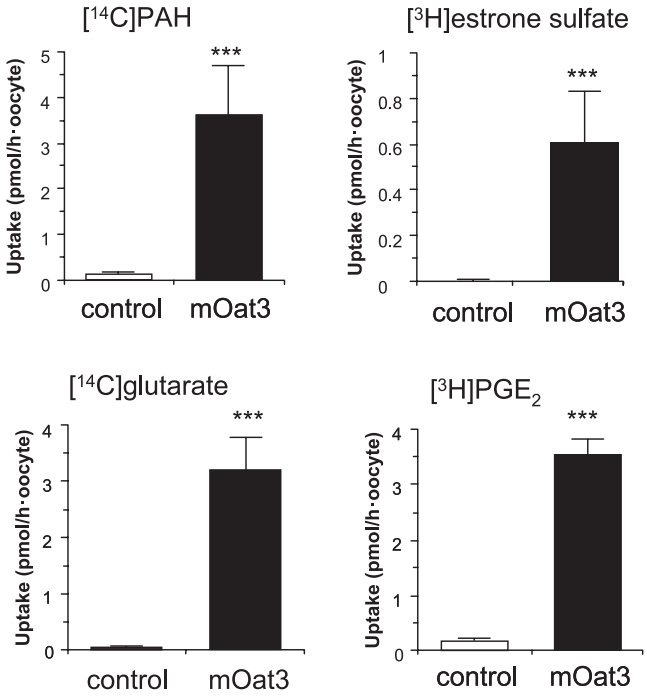

Fig. 1. Functional expression of mOat3 in *Xenopus* oocytes. mOat3 mediated the transport of several organic anions. The uptakes of radiolabeled [^{14}C]*p*-aminohippurate (PAH) (12 μM), [^{3}H]estrone sulfate (55 nM), [^{14}C]glutarate (5.5 μM), and [^{3}H]prostaglandin E_2 (PGE_2) (5 nM) by water-injected control oocytes and by mOat3-expressing oocytes were determined over a period of 1 h (mean ± S.E.M., n = 8 – 10). ***P<0.001 versus control.

ingly, analysis was performed at 60 min in the following experiments. The concentration dependence of the uptake of [^{3}H]PGE_2 via mOat3 is shown in Fig. 2B. The mOat3-mediated [^{3}H]PGE_2 uptake showed saturable kinetics and could be modeled by the Michaelis-Menten equation. Eadie-Hofstee plot analyses yielded a K_m value of 1.48 μM for PGE_2.

It is well established that OAT1 is a classical PAH /dicarboxylate exchanger (9). In addition, Sweet et al. and Bakhiya et al. reported that rat and human Oat3 /OAT3 functions as an organic anion/dicarboxylate exchanger (26, 27). Given the high sequence identity between rat Oat3 and mOat3, we assumed that their functions are very similar. However, a recent study by Ohtsuki et al. failed to demonstrate the E_1S/dicarboxylate exchange mechanism in mOat3 (19). Therefore, we next examined whether mOat3 is also organic anion /dicarboxylates exchanger or not. The uptake of [^{3}H]E_1S via mOat3 was *trans*-stimulated by the injection of cold glutarate into the oocytes (Fig. 3A). In addition, mOat3 mediated PGE_2/dicarboxylate exchange (Fig. 3B). We conclude that mOat3, as well as rat and human Oat3/OAT3, functions as an organic anion/dicarboxylate exchanger.

Then we examined the efflux of [^{3}H]PGE_2 from oocytes expressing mOat3. mOat3 exhibited significant efflux for [^{3}H]PGE_2, compared with water-injected

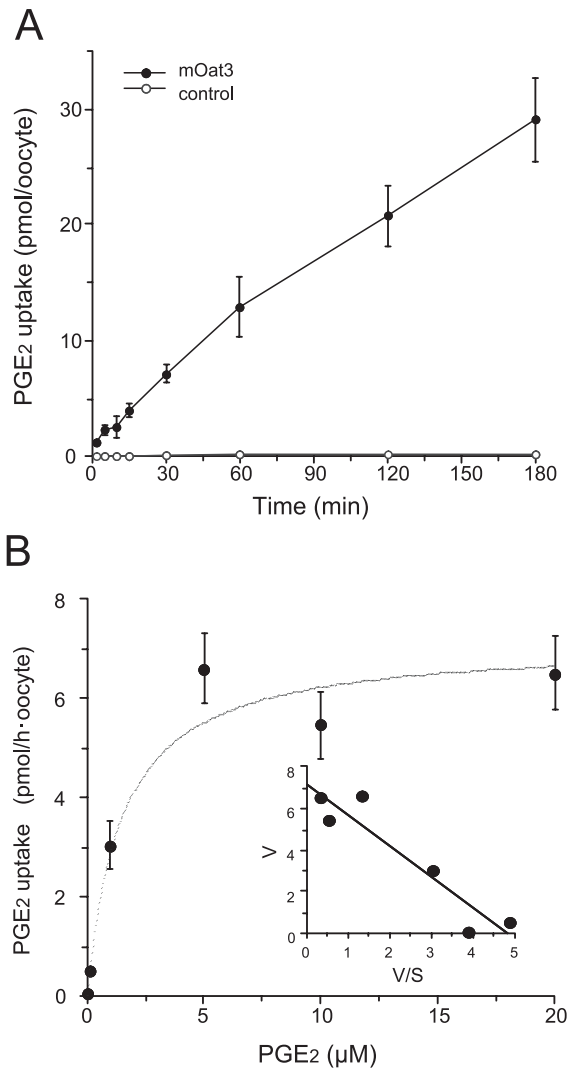

Fig. 2. Transport properties of PGE_2 via mOat3. A: Time-dependent uptake of [^{3}H]PGE_2 via mOat3. The uptake of 5 nM [^{3}H]PGE_2 was measured for 3 h in control oocytes and oocytes expressing mOat3 (mean ± S.E.M., n = 8 – 10). B: Concentration-dependence of mOat3-mediated uptake of PGE_2. The uptake rate of PGE_2 by control or mOat3-expressing oocytes for 1 h was measured at various concentrations (mean ± S.E.M., n = 8 – 10). Inset: Eadie-Hofstee plot. V, velocity; V/S, velocity per concentration of PGE_2.

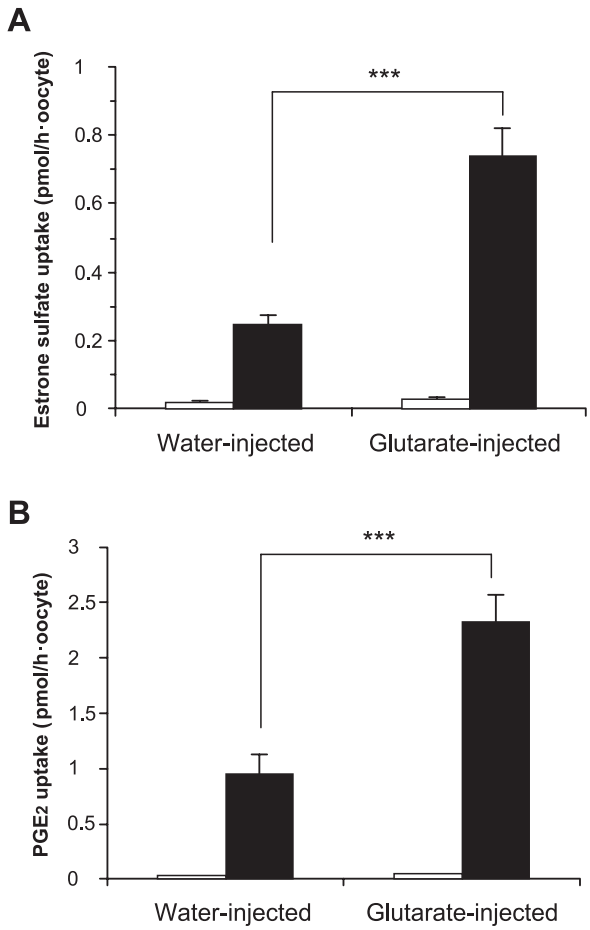

Fig. 3. Effects of glutarate on mOat3-mediated transport. *Trans*-stimulatory effect of glutarate on the uptake of [^{3}H]estrone sulfate (ES) (A) or [^{3}H]PGE_2 (B) via mOat3. Control (open column) and mOat3-expressing oocytes (closed column) were injected with 50 mM unlabeled glutarate (right columns) or water (left columns) and incubated for 5 min on ice. Then the oocytes were incubated with [^{3}H]ES (100 nM) or [^{3}H]PGE_2 (5 nM) and the amount of [^{3}H]ES or [^{3}H]PGE_2 accumulated for 1 h was determined (mean ± S.E.M., n = 8 – 10). ***P<0.001.

control oocytes (Fig. 4, left two columns). In addition, as would be expected for an exchanger, [^{3}H]PGE_2 efflux via mOat3 was significantly *trans*-stimulated by unlabeled glutarate in the medium (10 mM) (Fig. 4, right two columns).

Among the OAT isoforms, OAT3 as well as OAT1 are known to be polyspecific organic anion transporters that are responsible for the basolateral uptake of various organic anions (9 – 14). Although functional analysis of an Oat3 knockout mouse model strongly suggested that murine Oat3 was present in the basolateral membrane of renal proximal tubular cells (18), its exact localization in the kidney has not been demonstrated yet. Therefore, to clarify the intrarenal localization, we raised an antibody against the mOat3 C-terminal region and performed immunohistochemical analysis.

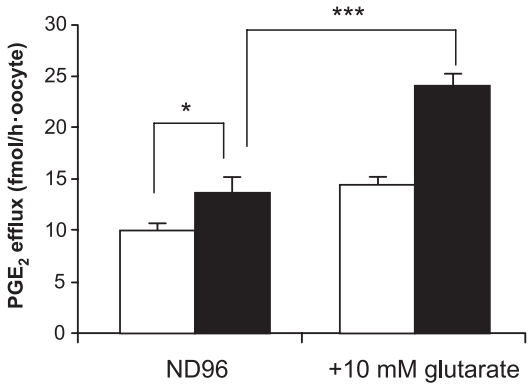

Fig. 4. mOat3-mediated butyrate efflux. *Trans*-stimulatory effect of glutarate on the efflux of [^{3}H]PGE_2 via mOat3. Control (open column) and mOat3-expressing (closed column) oocytes were injected with [^{3}H]PGE_2. After washing, the oocytes were incubated with 10 or 0 mM unlabeled glutarate. The amount of [^{3}H]PGE_2 effluxed for 1 h was determined. *P<0.05, ***P<0.001.

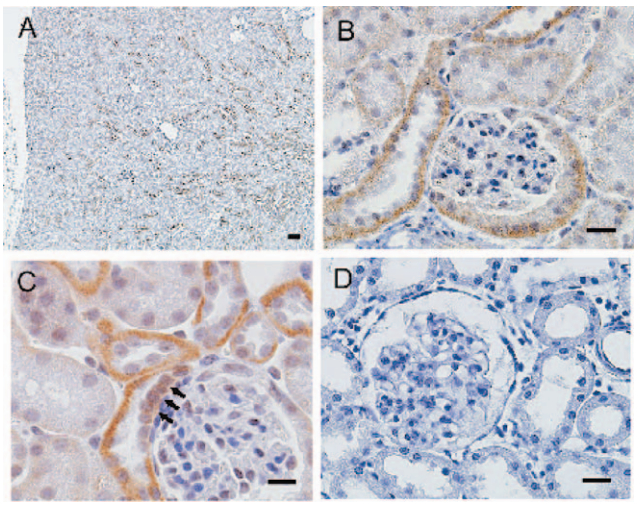

Fig. 5. Immunohistochemical analysis of Oat3 in mouse kidney. Two-micrometer sections were incubated with a polyclonal antibody against mOat3. Basolateral membrane of proximal tubules and that of distal tubules and collecting ducts were stained (proximal < distal), and no staining was observed in the glomeruli. Immunoreactivity in macula densa was also observed (arrows) at the basolateral side as well as the cytoplasm. (400×). Scale bars = 150 μm for A, 20 μm for B to D.

As shown in Fig. 5A, broad immunoreactivities of mOat3 were observed throughout the cortex under low magnification. There was no staining in the glomerulus. Under high magnification, mOat3 was found to be localized not only at the basolateral membrane of the proximal tubules but also at the same side of the distal tubules and of the collecting ducts (Fig. 5B). Interestingly, the intensities of mOat3 immunoreactivity seem stronger in the distal tubules and collecting ducts than in the proximal tubules. In addition, mOat3 immunoreactivity was detected in MD cells (Fig. 5C). By preincubation of the antibody with mOat3 peptide, the immunoreactivity disappeared (Fig. 5D). The specificity of the antibody for mOat3 was verified by these results.

Discussion

In this study, we analyzed mOat3-mediated PGE_2 transport properties and the intrarenal localization of mOat3 to determine whether mOat3 contributes to the basolateral transport of PGE_2 in distal nephron including MD cells.

PGE_2 is a major prostanoid derived from COX metabolism and modulates salt and water homeostasis in the kidney. In the renal cortex, COX-1 expression predominates in the collecting duct, vascular tissue, and glomerular mesangial cells, while COX-2 is expressed and presumably mediates PG production in the MD and surrounding cortical thick ascending limb (cTAL) cells (28 – 31). MD cells are in direct contact with the vascular pole of the same glomerulus from which the filtrate originates. They sense changes in tubular NaCl concentration and send signals to control preglomerular vascular resistance and glomerular filtration rate in a process named tubuloglomerular feedback (TGF). MD cells also control the renin release from juxtaglomerular granular cells (28 – 30). COX-2-derived PGs may participate in MD-mediated control of juxtaglomerular function, particularly in high renin states such as low salt intake, loop diuretic treatment, and renovascular hypertension (29, 31). PGE_2 produced by MD cells has been suggested to be the mediator of renin release induced by low luminal NaCl concentration (30 – 36). In addition, PGE_2, as a potent vasodilator, may also modulate preglomerular vascular resistance (3, 37) and TGF (38). Recently, it has been reported that PGE_2 release from MD cells is important in the control of renin release and

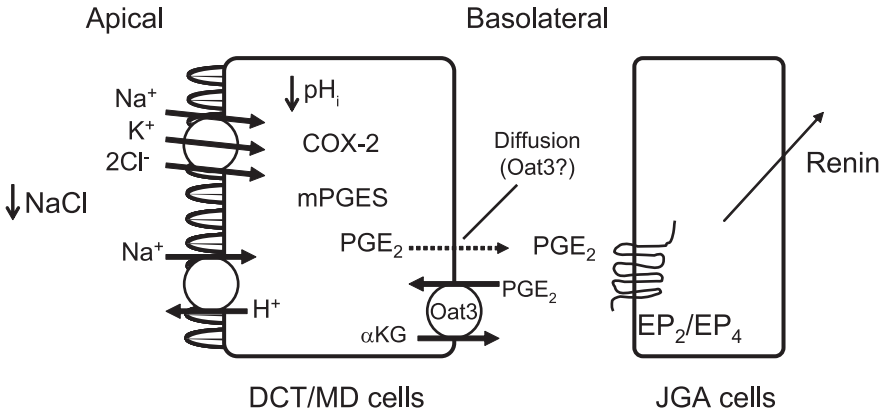

Fig. 6. Model of macula densa (MD) signaling. A marked decrease in tubular NaCl concentration, particularly under low salt diet, causes activation of the PG-synthesizing machinery, including COX-2 and microsomal PGE_2 synthase (mPGES) and PGE_2 release across the basolateral membrane via diffusion (or by Oat3). Oat3 is likely to be a transporter responsible for the rapid re-uptake of released PGE_2 using the outwardly directed gradient of dicarboxylates such as α-ketoglutarate (αKG).

renal vascular resistance during salt deprivation (4). However, little is known about the molecular mechanism of the transport of PGE_2 in distal nephron including MD cells.

The OAT family plays important roles in the elimination of a variety of endogenous substances, xenobiotics and their metabolites (9 – 14). We previously reported that human OATs (OAT1 to OAT4) mediate the transport of PGs (8). At present, there is little information on the functional properties of mouse Oat isoforms, particularly mOat3 (19 – 21), although Oat3 knockout mice have been generated several years ago (18). Among the OATs, OAT3 is likely to be a transporter responsible for the basolateral transport of PGE_2 in distal nephron including MD cells based on its broad localization in the rat kidney (15). In this regard, the current results support this possibility. mOat3 mediates both the uptake and the efflux of PGE_2 as shown in Figs. 2 – 4. Furthermore, mOat3 functions as an exchanger for both directions (Figs. 3 and 4). As shown in Fig. 5, mOat3 is localized at the basolateral membrane of proximal tubules, distal tubules including MD cells, and collecting ducts. These characteristics are compatible with the PGE_2 transport pathway in MD cells (Fig. 6).

As shown in Figs. 3 and 4, glutarate, a non-metabolized dicarboxylate, *trans*-stimulated mOat3-mediated PGE_2 transport in both directions. Taking the existence of the outwardly directed dicarboxylate gradient in tubular cells into account, endogenous dicarboxylates such as α-ketoglutarate (αKG) seem to contribute to the uptake of PGE_2 into cells. This supports the idea that Oat3 at the basolateral membrane of distal nephron functions as a re-uptake pathway of released PGE_2. As Pritchard mentioned (39), αKG is the most abundant within the proximal tubular cell. However, to date, there is no information concerning its concentration in distal nephron. Furthermore, energy-utilizing processes are different from segment to segment, based on the observation of ATP production in microdissected nephron segments shown by Uchida and Endou (40). Therefore, we could not exclude the possibility that Oat3 functions as an efflux pathway for PGE_2. As αKG is unlikely to be the endogenous counterion for PGE_2 efflux in MD cells, it seems necessary to identify such an endogenous counterion(s) for PGE_2 efflux in MD cells to further consider the role of PGE_2 as a signal, although the efflux of PGE_2 occurs without the counterion (Fig. 4).

Recently, Soodvilai et al. reported that the exposure of PGE_2 enhanced the OAT3-mediated estrone sulfate transport in isolated rabbit renal proximal tubules (41). Although no PGE_2-receptor isoform was detected in proximal tubules and distal tubules in renal cortex (3), this phenomenon seems compatible for the role of re-uptake of PGE_2 to maintain its extracellular concentration.

The K_m value for PGE_2 (1.48 μM) is different in humans and mice (more than 4-fold difference, ref. 8). The reason for this may be due to the interspecies difference in the interactions of OAT3/Oat3 with this substance or the difference in the expression system, that is, mammalian expression system for hOAT3 (8) versus *Xenopus* oocytes expression system for mOat3 (this study).

The generation of gene knockout animals could provide new information on the contribution of individual transporters in intact organs. Knockout mice for Oat3 have been generated several years ago (18). This model revealed the loss of organic anion transport and indicated the importance of drug uptake of Oat3 in the kidney and choroids plexus (42), although no morphological changes were found. Considering the novel role of mOat3 as a basolateral transport pathway of PGE_2 in MD cells, it would be interesting to observe changes in phenotype in mice under high renin states such as salt deficiency, administration of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, diuretic administration, or experimental renovascular

hypertension.

Since the molecular cloning of OAT1, first isoform of the OAT family, OATs were recognized mainly as an influx pathway of numerous endogenous and exogenous organic anions (12 – 14). However, recent studies by Aisf et al. unveiled the novel role of OAT3 as an efflux pathway of cortisol in endocrine tissues such as adrenocortical cells (43, 44). Therefore, the maintenance of homeostasis through the efflux of some endogenous substances such as PGs and steroid hormones would be another important role of the OAT family (9).

In conclusion, the current results indicate that mOat3 may play an important role in the basolateral efflux pathway of PGE_2 in the renal tubules including MD cells that may constitute one of the indispensable steps for renin release and the regulation of the TGF mechanism.

Acknowledgments

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the Japan Society for the Promotion of Science; Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation; Mutual Aid Corporation for Private Schools of Japan; the Salt Science Research Foundation (No. 0524); the Japan Foundation of Applied Enzymology; Astellas Foundation for Research on Metabolic Disorders; Gout Research Foundation of Japan; The Ichiro Kanehara Foundation; The Shimabara Science Promotion Foundation; Kyorin University School of Medicine (Kyorin Medical Research Award 2006); and Health and Labor Sciences Research Grants for Research on Advanced Medical Technology: Toxicogenomics Project.

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