Organic anions, including endogenous compounds and xenobiotics such as drugs, peptides, toxins, and their metabolites, are secreted by organic anion transporters of the liver and kidney (1,2). In the liver, the organic anion-transporting polypeptide (3) and the canalicular multispecific organic anion transporter (cMOAT) (4,5) have been identified. These transporters have multispecificity for substrates and mediate the transport of various anionic substances in the liver (3–6). The organic anion-transporting polypeptide is located on the sinusoidal membrane of the liver and transports several organic anions with different chemical structures, such as bile acids, bromosulfophthalein, conjugated steroid hormones, and ochratoxin A (3). cMOAT, which is a member of the ATP-binding cassette superfamily, is located at the canalicular membrane of hepatocytes and plays a key role in the export of amphiphilic anionic conjugates (4,5). cMOAT has been isolated from rat kidney. The immunogold method showed no labeling for OAT1 in the cytoplasmic vesicles, suggesting that OAT1 may not move together with organic anions into the cells. These results are consistent with previous physiologic and pharmacologic data showing that organic anions, including para-aminohippurate, are taken up by the basolateral Na\(^{+}\)-independent organic anion/dicarboxylate exchanger and excreted at S2 segments. In conclusion, OAT1 was localized to the basolateral membrane of S2 segments of proximal tubules in rat kidneys.

Recently, using a functional expression cloning method, Sekine et al. (18) identified the novel multispecific organic anion transporter 1 (OAT1) from rat kidney. OAT1 cDNA consists of 2294 nucleotides and contains an open reading frame encoding a 551-amino acid residue protein with 12 putative membrane-spanning domains (18). Sweet et al. (19) also identified a renal organic anion transporter, the amino acid sequence of which is 100% identical to that of OAT1. There was no significant sequence homology between OAT1 and the organic anion-transporting polypeptide, but OAT1 showed weak identity (38%) to organic cation transporter 1 (20). OAT1 possesses the same characteristics as the organic anion/dicarboxylate exchanger, which has been predicted by physiologic studies to be at the basolateral membrane of proximal tubules.

mRNA for these hepatic organic anion transporters has also been detected in the kidney, and the transporters may have roles in the transport of the anionic conjugates of lipophilic compounds (9–12). However, the classical renal organic anion transporters, which have been extensively investigated physiologically and pharmacologically, are distinct from these hepatic organic anion transporters. Organic anions, including para-aminohippurate (PAH), are taken up into proximal tubule cells from the peritubular plasma by an organic anion/dicarboxylate exchanger on the basal membrane (1,2,13–15) and are excreted into the urinary fluid through the apical membrane (1,2). The basolateral renal organic anion transporter (PAH transporter) of the proximal tubules also has wide substrate specificity, including drugs and toxins (1,2,16,17).

Abstract. Renal proximal convoluted tubules have an important role, i.e., to excrete organic anions, including numerous drugs and endogenous substances. Recently, multispecific organic anion transporter 1 (OAT1) was isolated from rat kidney. In this study, the cellular and subcellular localization of OAT1 in rat kidney was investigated. Kidneys from normal rats were perfused and fixed with periodate-lysine-paraformaldehyde solution and were then processed for immunohistochemical analysis using the labeled streptavidin-biotin method, preembedding horseradish peroxidase method, and immunogold methods. Light microscopic examination revealed immunostaining for OAT1 in the middle portion of the proximal tubule (S2 segment), but not in the initial portion of the proximal convoluted tubule, next to the glomerulus. Nephron segments other than the S2 segment and the renal vasculature were not stained with antibody to OAT1. Electron-microscopic observation using a preembedding method revealed that OAT1 was exclusively expressed in the basolateral membrane of S2 segments of proximal tubules. The immunogold method showed no labeling for OAT1 in the cytoplasmic vesicles, suggesting that OAT1 may not move together with organic anions into the cells. These results are consistent with previous physiologic and pharmacologic data showing that organic anions, including para-aminohippurate, are taken up by the basolateral Na\(^{+}\)-independent organic anion/dicarboxylate exchanger and excreted at S2 segments. In conclusion, OAT1 was localized to the basolateral membrane of S2 segments of proximal tubules in rat kidneys.

Organic Anion Transporter 1 in Rat Kidney

Immunohistochemical Localization of Multispecific Renal Organic Anion Transporter 1 in Rat Kidney

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Northern blot and in situ hybridization analyses revealed that OAT1 is exclusively expressed in particular segments of proximal tubules, presumably S2 segment (18).

In this study, we raised a polyclonal antibody against the carboxy terminus of OAT1 and investigated the cellular and subcellular localization of OAT1 in rat kidney. We identified OAT1 exclusively in the basolateral membrane domain of S2 segments of proximal tubules.

Materials and Methods

Animal Preparation

For the histochemical studies, three male Sprague Dawley rats (180 to 200 g body wt) were fed standard rat chow and tap water. Animals were anesthetized with pentobarbital (50 mg/kg, intraperitoneally), and the abdominal aorta was cannulated with PE50 tubing (Intramedic, Sparks, MD). The kidneys were briefly perfused with saline, followed by perfusion with periodate-lysine-paraformaldehyde solution (0.01 M NaO₄, 0.075 M lysine, 0.0375 M phosphate buffer, with 2% paraformaldehyde, pH 6.2), through an abdominal cannula. Kidney slices were immersed in periodate-lysine-paraformaldehyde solution overnight at 4°C. The tissue was embedded in wax (polyethylene glycol 400 distearate; Poly-sciences, Warrington, PA) for light microscopic immunohistochemical analysis and in Lowicryl K4M (Electron Microscopy Sciences, Fort Washington, PA) embedding medium for immunogold studies. The remaining tissue was processed for the preembedding immunoperoxidase method.

Antibody

A rabbit polyclonal antibody was raised against a synthesized polypeptide of the carboxy terminus of rat renal OAT1 (18). The specificity of the antibody was analyzed by Western blotting, as described below, and a specific band was detected at 57 kD that corresponded to the OAT1 molecule in the kidney.

Light Microscopic Immunohistochemical Analysis

Wax sections (2 μm) were cut and stained by the labeled streptavidin-biotin method, as described previously (21–23). After dewaxing, the sections were incubated with 3% H₂O₂ for 15 min (to eliminate endogenous peroxidase activity) and then with blocking serum for 15 min. The sections were then incubated with a polyclonal antibody against rat OAT1 (1:200 dilution) for 2 h. The sections were rinsed with Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with the biotinylated secondary antibody against rabbit Ig (Dako, Glostrup, Denmark) for 1 h. After being rinsed with TBST, the sections were incubated for 30 min with horseradish peroxidase (HRP)-conjugated streptavidin solution. HRP labeling was detected using the peroxidase substrate solution with diaminobenzidine (0.8 mM; Dojindo Laboratories). The sections were post-fixed in 2% osmium tetroxide for 1 h at 4°C and embedded in epoxy resin. Sections (1 μm) were cut on an ultramicrotome and stained with toluidine blue for light microscopic analysis. Ultrathin sections (60 to 70 nm) were cut similarly, stained with lead citrate, and photographed on a transmission electron microscope (H-7000; Hitachi, Tokyo, Japan).

Postembedding Immunogold Procedure

Ultrathin sections of the Lowicryl blocks were cut on an ultramicrotome, mounted on colloidal-coated nickel grids, and processed for immunogold labeling as described previously (22,23). Briefly, the sections were incubated with 0.1 M NH₄Cl for 1 h and rinsed with buffer solution (0.02 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.2) for 15 min. The sections were incubated overnight with primary antibody against rat OAT1 (1:200 dilution) at 4°C. After the sections were washed with buffer, 30-nm gold-labeled, goat anti-rabbit IgG secondary antibody (Amersham, Buckinghamshire, England) was applied for 2 h at a dilution of 1:50. The sections were washed again with buffer and then incubated with 2% glutaraldehyde/PBS solution for 30 min. Finally, the sections were rinsed with distilled water, counterstained with uranyl acetate and lead citrate, and examined with an electron microscope.

Western Blotting

As described in detail previously (22,23), the kidneys were removed immediately after perfusion with ice-cold saline and were homogenized on ice with a Teflon-glass tissue homogenizer (Iwaki, Chiba, Japan), in 1 ml of buffer containing 50 mM Tris, pH 7.4, 0.2 mM ethylenediaminetetra-acetic acid, 2 mM leupeptin, 50 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 12,000 x g for 15 min. The supernatants were diluted in sodium dodecyl sulfate (SDS) sample buffer (0.5 M Tris-HCl, pH 6.8, 10% vol/vol glycerol, 10% wt/vol SDS, 5% vol/vol β-mercaptoethanol, and 0.05% wt/vol bromphenol blue). Samples containing 50 μg of protein from the renal cortex and 25 μg of protein from the renal inner medulla were applied to a 8.5% gel, separated by SDS-polyacrylamide gel electrophoresis, and then electroblotted to nitrocellulose membranes. The nitrocellulose membranes were incubated with 5% nonfat dry milk in TBST for 1 h, after incubation with normal rabbit serum for 30 min. The membrane was then incubated with a polyclonal antibody to OAT1 (1:250 dilution) for 2 h. After being rinsed with TBST, membranes were incubated for 1 h with HRP-labeled anti-rabbit IgG antibody (1:2000 dilution). After being rinsed with TBST, blots were processed for detection using diaminobenzidine with 0.3% hydrogen peroxide and 3 mM NiCl₂. To investigate the specificity of the bands, an absorption test was performed. The OAT1 peptide (50 μg/ml) was added to the OAT1-specific antibody solution (1:250 dilution) and incubated for 2 h. Using this preabsorbed antibody, Western blot analysis was performed as described above.
Results

Light Microscopic Analysis of OAT1 in the Kidney

Light microscopic analysis of 2-μm wax sections demonstrated that there was specific immunostaining for OAT1 in some of the proximal tubules (Figure 1a). As shown in Figure 1c, there was no staining for OAT1 in the S1 segment of the proximal convoluted tubules connected to the Bowman’s capsule or in the glomerular cells. In contrast, OAT1 was located in the S2 segment of the proximal tubules but was not observed in distal tubules, cortical collecting ducts, or the vasculature (Figure 1, d and e). OAT1 immunoreactivity was not observed in the S3 segment of the proximal straight tubules, and there was no labeling of tubules in the outer stripe of the outer medulla (Figure 1e). In the longitudinal section of the proximal straight tubules, the upstream portion belonging to the S2 segment showed slight immunoreactivity for OAT1, whereas the S3 segment, with a thicker brush border, was not stained (Figure 1f). No immunostaining for OAT1 was observed in the inner medulla (Figure 1g). The negative control with normal rabbit serum showed no staining (Figure 1b).

Light microscopic examination of 1-μm Epon sections showed immunostaining for OAT1 in the basolateral region of the S2 segment of proximal tubules (Figure 1h). The S1 segments of proximal convoluted tubules, with thicker brush borders, were negative for OAT1 immunostaining (Figure 1h).

Electron Microscopic Observation of OAT1

When examined by electron microscopy using the preembedding method, OAT1 was detected in the basolateral membrane of the S2 segment of proximal tubules, which was recognized by a thinner brush border and prominent basal digitation (Figure 2a). There was no staining for OAT1 in the apical membrane (including the brush border), nucleus, mitochondria, Golgi apparatus, or cytoplasm. High-magnification examination of the S2 segment showed that OAT1 was located exclusively on the basal digitation membrane and the lateral membrane (Figure 2b). The negative control without primary antibody did not show any staining in the kidney, including the S2 segment (Figure 2c). The S1 segment of proximal convoluted tubules, which was identified by thicker brush border and cell bodies, was not stained by the antibody to OAT1 (Figure 2a). The S3 segment of proximal straight tubules, with the thickest brush border and cuboidal cell bodies, was not stained by an antibody against OAT1 (not shown). Both principal cells and intercalated cells of cortical collecting ducts showed no labeling for OAT1. The immunogold method also demonstrated that OAT1 was exclusively located on the basolateral membrane, and not in the cytoplasm or in the cytoplasmic vesicles (Figure 3).

Protein Expression of OAT1

Western blotting was performed to detect OAT1 protein expressed in homogenates of the renal cortex and inner medulla from normal control rats. The antibody to OAT1 detected a specific band that corresponded to the OAT1 protein, with a molecular mass of approximately 57 kD, in the renal cortex but not in the inner medulla (Figure 4). By preincubation of the antibody with OAT1 peptide, the band at 57 kD in the renal cortex was absorbed. The specificity of the antibody for OAT1 was verified by these results.

Discussion

In this study, we have demonstrated that renal multispecific OAT1 is exclusively located at the basolateral membrane of S2 segments of proximal tubules in rat kidney. By light microscopic observation, there was no immunostaining for OAT1 in the outer or inner medulla, indicating that the S3 segments of proximal straight tubules, the loops of Henle, and the outer and inner medullary collecting ducts do not express OAT1. In the cortex, the distal convoluted tubules, macula densa, connecting tubules, cortical collecting ducts, glomeruli, and vasculature showed no staining for OAT1. Only a portion of the proximal convoluted tubules was stained by the antibody against OAT1. The initial portion of the proximal convoluted tubule next to the glomerulus was not stained, indicating that OAT1 is not present in the S1 segments of the proximal tubules, which constitute approximately two-thirds of the pars convoluta. Therefore, OAT1 exists in the S2 segments, which correspond to the remainder of the pars convoluta and the initial portion of the pars recta.

The immunoelectron microscopic analysis also clearly identified the specific localization of OAT1 in the proximal tubules. On the basis of well established ultrastructural characteristics (24), we confirmed that OAT1 is expressed exclusively in the S2 segments of proximal tubules.

Physiologically, the organic anion uptake at the basolateral membrane of the proximal tubules has been shown to be mediated by a tertiary active-transport process (1,2,13–15). PAH, a prototypical organic anion, is transported into cells in an exchange for intracellular dicarboxylates such as α-ketoglutarate (1,2,14). The outwardly directed gradient of dicarboxylates is maintained by both intracellular metabolism and a Na+/dicarboxylate cotransporter, the transport energy of which is derived from basolateral Na+/K+-ATPase activity (13,15). Using an expression cloning method, the basolateral organic anion/dicarboxylate exchanger (OAT1) molecule has been identified from Xenopus oocytes, which were co-injected with Na+/dicarboxylate cotransporter cRNA (rNaDC-1) and rat kidney poly(A)+ RNA (18). The immunohistochemical localization of OAT1 is consistent with the previous physiologic and functional findings that PAH is excreted primarily via S2 segments of proximal tubules, rather than S1 or S3 segments (1,2,25,26). PAH excretion has also been detected in S3 segments, although to a minor extent (25). Shpun et al. (13) have demonstrated that PAH transporters are fewer in S3 segments than in S2 segments, and they may not be identical in the two segments, as suggested by functional studies. Therefore, there may be other forms of organic anion transporters that excrete PAH in S3 segments.

The OAT1 in the basolateral membrane of proximal tubules has broad substrate selectivity, including β-lactam antibiotics (cephaloridine), diuretics (furosemide and ethacrynic acid),
Figure 1. Light micrographs illustrating immunostaining for organic anion transporter 1 (OAT1) in rat kidney. Immunostaining for OAT1 is observed in the proximal convoluted tubules in the cortex (a). The negative control with normal rabbit serum shows no staining (b). High magnifications of the glomerulus and the initial portion of the proximal convoluted tubule (c) and the proximal convoluted tubule and cortical collecting duct (d) are also shown. There is no staining for OAT1 in the renal artery or proximal straight tubules in the outer stripe of the outer medulla (e) and the inner medulla (g). A longitudinal section of the proximal straight tubules, through the cortex to the outer stripe (f), and a 1-µm section illustrating OAT1 in proximal convoluted tubule, using preembedding procedure (h), are also shown. Magnification: ×100 in a, b, and g; ×200 in c, d, and f; ×50 in e; and ×480 in h.
nonsteroidal anti-inflammatory drugs (indomethacin), probenecid, methotrexate, captopril, and endogenous substances such as cAMP, cGMP, prostaglandin E2, α-ketoglutarate, and urate (18). Recently, Saito et al. (27) identified another renal organic anion transporter, OAT-K1, which is a 669-amino acid protein with a 74-kD molecular mass that mediates the transport of methotrexate. However, other organic anions, such as PAH, taurocholate, prostaglandin E2, and leukotriene C4, were not transported by OAT-K1 (27), indicating that OAT-K1 is distinct from the multispecific organic anion transporter (PAH transporter). Human MRP, which transports many anionic conjugates of lipophilic substances with glutathione, glucuronate, or sulfate (8,28–31), has been shown to be localized in the basolateral membrane of pig kidney cells transfected with MRP cDNA (32). These results suggest that there are several organic anion transporters expressed in the proximal tubule, and substrates may differ among these organic anion transporters.

After basolateral uptake, organic anions move across the cytoplasm via two possible mechanisms, i.e., a vesicle- and cytoskeleton-mediated mechanism and simple diffusion (1).

In this study, we demonstrated that the subcellular localization of OAT1 was limited to the basolateral membrane and not the apical membrane or the cytoplasm of S2 segments. The cytoplasmic vesicles were not stained by the antibody to OAT1. This suggests that OAT1 may not absorb the organic anions via the endocytotic pathway or have a mechanism of membrane recycling, as shown for the vacuolar H^+-ATPase and the water channel in collecting ducts (24,33).

The mechanism of organic anion excretion toward the lumen is not clearly understood. The brush-border vesicle

Figure 2. Electron micrograph illustrating OAT1 immunostaining, using the preembedding immunoperoxidase procedure. (a) OAT1 immunostaining is present in the S2 segment of proximal tubule. There is no labeling of the S1 segment. (b) High magnification of basal area of the S2 segment stained for OAT1. (c) The negative control for S2 segments shows no staining for OAT1. Magnification: ×4,800 in a; ×15,000 in b; and ×5,000 in c.
studies showed two luminal organic anion transport mechanisms, i.e., potential difference-driven facilitated diffusion and anion exchange (1). The other possible mechanism mediating organic anion secretion across the luminal membrane involves export by primary active transporter(s). Fluorescence imaging studies of organic anion transport showed that potential difference-driven facilitated diffusion is not a major factor in the transport of organic anions across the luminal membrane of killifish proximal tubules (34). MRP1 (7) and the distinct hepatocyte canalicular isoform MRP2 (4,8), which belong to the ATP-binding cassette superfamily of transporters, also transport certain amphiphilic organic anions. MRP1 and MRP2 share only 49% amino acid identity, but their substrate specificities are very similar (8,31). Rat MRP2 has an apical localization in proximal tubules (10), although MRP1 has been localized to the basolateral membrane (32). P-glycoprotein, which mediates the ATP-dependent export of a broad spectrum of lipophilic, primarily cationic substrates (35), has been found in the brush border of proximal tubules of the kidney (36). MRP2 and P-glycoprotein may have roles in the apical secretion of conjugated substances; however, the substrates for MRP2 and P-glycoprotein do not include the characteristic organic anions that are transported by proximal tubules. In this study, we could not detect OAT1 in the apical membrane of proximal tubules. It is possible that isoforms of OAT1 may exist on the apical membrane of proximal tubules. Additional studies are required to identify the organic anion transporter responsible for the luminal exit of organic anions that are taken up via OAT1 in the S2 segments of proximal tubules.
In conclusion, multispecific OAT1 is expressed in the S2 segments of proximal tubules in rat kidney, a finding that is in agreement with previous physiologic observations. The subcellular localization of OAT1 was exclusively on the basolateral membrane of S2 segments and there was no expression in the cytoplasmic vesicles, suggesting that organic anions may not be absorbed via an endocytotic pathway.

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References


