Functional Involvement of Rat Organic Anion Transporter 3 (rOat3; Slc22a8) in the Renal Uptake of Organic Anions

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ABSTRACT

Our previous kinetic analyses have shown that the transporter responsible for the renal uptake of pravastatin, an HMG-CoA reductase inhibitor, differs from that involved in its hepatic uptake. Although organic anion transporting polypeptides are now known to be responsible for the hepatic uptake of pravastatin, the renal uptake mechanism has not been clarified yet. In the present study, the involvement of rat organic anion transporter 3 (rOat3; Slc22a8) in the renal uptake of pravastatin was investigated. Immunohistochemical staining indicates the basolateral localization of rOat3 in the kidney. rOat1- and rOat3-expressed LLC-PK1 cells exhibited specific uptake of p-aminohippurate (PAH) and pravastatin, respectively, with the Michaelis-Menten constants (Km values) of 60 μM for rOat1-mediated PAH uptake and 13 μM for rOat3-mediated pravastatin uptake. Saturable uptake of PAH and pravastatin was observed in kidney slices with Km values of 69 and 11 μM, respectively. The difference in the potency of PAH and pravastatin in inhibiting uptake by kidney slices suggests that different transporters are responsible for their renal uptake. This was also supported by the difference in the degree of inhibition by benzylpenicillin, a relatively selective inhibitor of rOat3, for the uptake of PAH and pravastatin by kidney slices. These results suggest that rOat1 and rOat3 are mainly responsible for the renal uptake of PAH and pravastatin, respectively.

The kidney plays an important role in the urinary excretion of drugs and their metabolites via glomerular filtration and tubular secretion (Burckhardt and Wolff, 2000; Inui et al., 2000; Sekine et al., 2000; Van Aubel et al., 2000; Dresser et al., 2001). The first step in the tubular secretion is the uptake from blood through the basolateral membrane of the epithelial cells in the proximal tubules. p-Aminohippurate (PAH) has been shown to be efficiently taken up by the kidney from the blood via the renal organic anion transporter on the basolateral membrane (Ulrich and Rumrich, 1993). Ulrich and Rumrich (1993) have thoroughly investigated the substrate specificity of the renal uptake mechanism for PAH by examining the inhibitory effect of various compounds using an in situ kidney perfusion technique. They demonstrated that the renal organic anion transporter for PAH on the basolateral membrane has broad substrate specificity. Recently, rat organic anion transporter 1 (rOat1) has been isolated from rat kidney by expression cloning using Xenopus laevis oocytes (Sekine et al., 1997). Functional characterization has shown that the transport of PAH via rOat1 involves an exchange of dicarboxylate (Sekine et al., 1997), which is consistent with the transport property of an organic anion transporter on the basolateral membrane of proximal tubules. rOAT1 has broad substrate specificity and accepts various drugs such as nonsteroidal anti-inflammatory drugs, β-lactam antibiotics, methotrexate, and antiviral drugs and various endogenous organic anions such as cyclic nucleotides, prostaglandins, dicarboxylates, and folate (Burckhardt and Wolff, 2000; Inui et al., 2000; Sekine et al., 2000; Van Aubel et al., 2000; Dresser et al., 2001).

To date, rOat2 and rOat3 have been isolated as isoforms of rOat1 in rats (Sekine et al., 1998; Kusuhara et al., 1999). Northern blot analyses indicated that rOat2 is expressed predominantly in the liver and only weakly in the kidney (Sekine et al., 1998), and that rOat3 is expressed in the liver, kidney, brain, but only weakly in the eye (Kusuhara et al., 1999). Functional characterization shows that substrates of rOat3 include organic anions, such as estrone sulfate, 17β-estradiol-17β-glucuronide, ochratoxin A, PAH, and an organic cation, cimetidine (Kusuhara et al., 1999; Sugiyama et al., 2001). Human OAT3 (hOAT3) is predominantly expressed in the kidney and localized to the basolateral mem-

ABBREVIATIONS: PAH, p-aminohippurate; PBS, phosphate-buffered saline; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; PCG, benzylpenicillin; DBSP, dibromosulphthalein.
brane of the proximal tubules (Cha et al., 2001). The basolateral localization of hOAT3 suggests its involvement in the renal uptake of organic anions. However, it is not yet known whether rOat3/hOAT3 is involved in the renal uptake of organic anions nor is there any information about the contribution of rOat3 to the total renal uptake of organic anions including PAH.

Pravastatin, a hydrophilic HMG-CoA reductase inhibitor, exhibits relatively selective inhibition of hepatic cholesterol synthesis compared with other more highly lipophilic drugs. We have already demonstrated that efficient transport systems are involved both in its renal and hepatic uptake in rats (Yamazaki et al., 1996). The involvement of the organic anion transporting polypeptide family (rOatp1, rOatp2, and hLST1/hOATP-C/hOATP2) in the hepatic uptake of pravastatin has already been demonstrated (Hsiang et al., 1999; Tokui et al., 1999), however, the transporters responsible for the renal uptake of pravastatin have not been identified yet. It has been demonstrated that PAH inhibits the renal, but not the hepatic, uptake of pravastatin, suggesting involvement of the OAT family in its renal uptake (Yamazaki et al., 1996). In the present study, we have found that pravastatin is transported by rOat3, but not by rOat1, using cDNA transfected cells, and examined the involvement of rOat3 in the renal uptake of organic anions using pravastatin as a model ligand.

Materials and Methods

Materials. [3H]pravastatin (45.5 Ci/mmol), [14C]pravastatin (14.4 mCi/mmol), and unlabeled pravastatin were kindly donated by Sankyo (Tokyo, Japan). [3H]PAH (4.08 Ci/mmol), [3H], and [14C]mannitol (19.9 Ci/mmol and 51 mCi/mmol, respectively) were purchased from New England Nuclear (Boston, MA). Unlabeled PAH was purchased from Sigma (St. Louis, MO), and unlabeled benzylpenicillin and dibromosulfophthalein were from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were analytical grade and commercially available.

Antiserum and Western Blot Analysis. Anti-rOat3 serum was raised in rabbits against a synthetic peptide consisting of the 16 carboxyl-terminal amino acids of rOat3 coupled to keyhole limpet hemocyanine at its carboxyl-terminal via an additional tyrosine. Membrane fractions were prepared from rat kidney and rOat3-expressed LLC-PK1 cells as described previously (Nakajima et al., 2000). The membrane fractions were diluted with 3% Red loading buffer (BioLabs, Hertfordshire, UK). They were boiled for 3 min then loaded onto a 10% SDS-polyacrylamide electrophoresis gel with a 4.4% stacking gel. Proteins were electroblotted onto a polyvinylidene difluoride membrane (Pall, NY) using a blotter (Trans-blot; Bio-Rad, Richmond, CA) at 15 V for 1 h. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 h at room temperature. After washing with TBS-T, the membrane was incubated with anti-rOat3 serum (dilution 1:1000). The membrane was allowed to bind a horseradish peroxidase-labeled anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) diluted 1:2000 in TBS-T for 1 h at room temperature followed by washing with TBS-T. No band was detected in the crude membrane fraction of rOat1-expressed LLC-PK1 cells indicating that rOat3 antisera does not react with rOat1.

Immunofluorescence Study. Frozen sections from male Sprague-Dawley rats for immunofluorescence study were prepared after fixed in acetone (−20°C). Sections were incubated with anti-rOat3 antibodies for 1 h at room temperature, washed three times with PBS (140 mM NaCl and 10 mM phosphate, pH 7.4), and subsequently incubated with the secondary antibodies for 1 h at room temperature. Sections were washed twice with PBS and incubated with SYTO61 (Molecular Probes, Eugene, OR) for 20 min and were mounted in VECTASHIELD (Vector Laboratories, Burlingame, CA). Antibodies were diluted with PBS at the following dilutions: anti-rOat3 serum at 1:10, fluorescein isothiocyanate-labeled anti-rabbit IgG (Vector Laboratories, Burlingame, CA) at 1:100. The nucleus was stained by SYTO61 diluted with PBS (1:1000).

Cell Culture. rOat1- and rOat3-expressed LLC-PK1 cells were established as described previously by us (Sugiyama et al., 2001). LLC-PK1 cells were grown in M199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 (Invitrogen) at 37°C with 5% CO2 and 95% humidity on the bottom of a dish. Cells were seeded in 12-well plates at a density of 1.2 × 105 cells/well. Cell culture medium was replaced with culture medium supplemented with 5 mM sodium-butyrate 24 h before transport studies to induce the expression of rOat1 and rOat3. In this study, LLC-PK1 cells between the 5th and 22nd passages were used.

Transport Studies. Transport studies were carried out as described previously (Sugiyama et al., 2001). Uptake was initiated by adding medium containing 1 μM [3H]PAH or 0.5 μM [3H]pravastatin after cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min. The Krebs-Henseleit buffer consists of 142 mM NaCl, 23.8 mM NaHCO3, 4.83 mM KCl, 0.96 mM KH2PO4, 1.20 mM MgSO4, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl2 adjusted to pH 7.4. The uptake was terminated at a designed time by adding ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. Then, cells were washed twice with 1 ml of ice-cold Krebs-Henseleit buffer, dissolved in 500 μl of 0.2 N NaOH, and kept overnight. Aliquots (450 μl) were transferred to scintillation vials after adding 100 μl of 1 N HCl. The radioactivity associated with the cells and medium was determined by liquid scintillation counting after adding 2 ml of scintillation fluid (NACALAI TESQUE, Kyoto, Japan) to the scintillation vials. The remaining 50 μl of the aliquots of cell lysate were used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard.

Ligand uptake is given as the cell-to-medium concentration ratio determined as the amount of ligand associated with the cells divided by the medium concentration. Specific uptake was obtained by subtracting the uptake into vector-transfected cells from the uptake into cDNA-transfected cells. Kinetic parameters were obtained using the following equation

\[

\nu = \frac{V_{max} \times S}{(K_m + S)}

\]

where \( \nu \) is the uptake velocity of the substrate (pmol/min/mg of protein), \( S \) is the substrate concentration in the medium (μM), \( K_m \) is the Michaelis-Menten constant, and \( V_{max} \) is the maximum uptake velocity.
the Michaelis-Menten constant (µM), and \( V_{\text{max}} \) is the maximum uptake rate (pmol/min/mg of protein). Inhibition constants (\( K_i \) values) of a series of compounds were obtained by examining their inhibitory effects on the rOat1- and rOat3-mediated uptake assuming competitive inhibition using the following equation

\[
\frac{CL}{CL + I/K_i}
\]

where CL represents the uptake clearance and the subscript (+I) represents the value in the presence of inhibitor. I represents the concentration of inhibitor (µM). The substrate concentration was low compared with its \( K_m \) value in the inhibition study. Fitting was performed by the nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981) and the Damping Gauss Newton Method algorithm was used for fitting.

Uptake by Kidney Slices. Uptake studies were carried out as described in a previous report (Urakami et al., 1999). Slices (0.3 mm thick) of whole kidneys from male Sprague-Dawley rats were put in ice-cold oxygenated incubation buffer. The incubation buffer consists of 120 mM NaCl, 16.2 mM KCl, 1 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), and 10 mM NaH\(_2\)PO\(_4\)/Na\(_2\)HPO\(_4\) adjusted to pH 7.5. Two slices, each weighing 10 to 20 mg, were randomly selected and then incubated in the 12-well plate with 1 ml of oxygenated incubation buffer in each well after slices had been preincubated with incubation buffer for 5 min. The uptake study of 1 µM [\( ^3\)H]PAH and 0.5 µM [\( ^{14}\)C]pravastatin was carried out at 37°C. [\( ^3\)H] and [\( ^{14}\)C]mannitol (1 µM) were used to estimate the adherent water of the kidney slice in each experiment. After incubating for an appropriate time, each slice was rapidly removed from the incubation buffer, washed in ice-cold saline, blot-

Fig. 2. Immunofluorescence localization of rOat3 in the kidney. Cryosections of kidney from male Sprague-Dawley rats were incubated with the rOat3 antiserum and stained by fluorescein isothiocyanate-labeled anti-rabbit IgG. Nucleuses were stained by SYTO61. The basolateral membrane of the proximal tubule was stained (A), but no staining was observed in the medulla (B). In addition, no staining was observed in either cortex or medulla incubated with normal rabbit serum (C and D). Original magnifications in A through D, 10×.
tet on filter paper, weighed, and dissolved in 1 ml of soluene-350 (Packard Instruments, Downers Grove, IL) at 50°C for 3 h. The radioactivity was determined in a liquid scintillation counter after adding 10 ml of scintillation fluid (Hionic Flour; Packard Instruments).

Ligand uptake was given as the amount of ligand associated with the slice divided by the medium concentration. The $K_m$ and $K_i$ values were obtained as described previously.

## Results

### Western Blot Analysis

The expression of rOat3 in the transfected cells and kidney plasma membrane were confirmed by Western blot analyses. An antiserum against rOat3 recognized approximately 54- and 65-kDa proteins in the membrane fractions from rOat3-expressed LLC-PK1 cells and kidney, respectively (Fig. 1A). The molecular mass of rOat3 in the kidney was slightly greater than that in rOat3-expressed LLC-PK1 cells. The band was abolished when preabsorbed antiserum for rOat3 was used (Fig. 1B), suggesting that the positive bands were specific for the antigen peptide. No expression of rOat3 was observed in vector-transfected LLC-PK1 cells (Fig. 1A, lane 2).

### Immunofluorescence Localization of rOat3

The localization of rOat3 in the kidney was investigated by immunofluorescence analysis. Specific immunostaining for rOat3 was observed in the basolateral membrane of the proximal tubular cells (Fig. 2A), but no staining was observed in the medulla (Fig. 2B). Both cortex and medulla treated with normal rabbit serum were not stained (Fig. 2, C and D).

### Uptake of PAH and Pravastatin by Transfectants

The time profiles of the uptake of PAH by rOat1- and rOat3-expressed and vector-transfected LLC-PK1 cells are shown in Fig. 3A. Transfection of rOat1 results in an increase in the uptake of PAH, but does not affect the uptake of pravastatin. On the contrary, transfection of rOat3 results in an increase in the uptake of pravastatin, but not PAH (Fig. 3C). The $K_m$ and $V_{max}$ values were determined by kinetic analyses; the $K_m$ and $V_{max}$ values of PAH for rOat1-mediated transport were found to be $59.5 \pm 5.0$ μM and $1.34 \pm 0.02$ nmol/min/mg of protein, respectively (Table 1; Fig. 3B). Nonsaturable component was seen in the Eadie-Hofstee plot even for the specific uptake of pravastatin by rOat3 (Fig. 3D). This is due to the increase in the uptake extrapolated at time 0 in rOat3-expressed LLC-PK1 cells by unknown reason (Fig. 3C). The $K_m$ and $V_{max}$ values of pravastatin for the saturable component and uptake clearance for the nonsaturable component were $13.4 \pm 2.4$ μM, $50.5 \pm 7.6$ pmol/min/mg of protein, and $0.53 \pm 0.06$ ml/min/mg of protein, respectively (Table 1; Fig. 3D).

### Uptake of PAH and Pravastatin by Kidney Slices

Figure 4, A and C, show the time profiles of the uptake of PAH and pravastatin by kidney slices, respectively. The uptake of PAH and pravastatin increased linearly over 30 and 20 min, respectively. Eadie-Hofstee plots of these ligands are shown in Fig. 4, B and D. The $K_m$, $V_{max}$, and $P_{diff}$ values for the uptake of PAH were found to be $69.0 \pm 8.6$ μM, $12.8 \pm 1.2$ nmol/min/g of kidney, and $0.011 \pm 0.001$ ml/min/g of kidney, respectively. The corresponding parameters for the uptake of pravastatin were found to be $11.4 \pm 3.1$ μM, $2.04 \pm 0.45$ nmol/min/g of kidney, and $0.028 \pm 0.002$ ml/min/g of kidney, respectively.
TABLE 1

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<tr>
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<th>PAH Uptake</th>
<th>Pravastatin Uptake</th>
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<td></td>
<td>Oat1-LLC</td>
<td>Kidney Slice</td>
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<tr>
<td>PAH</td>
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<tr>
<td>$K_m$ (μM)</td>
<td>59.9 ± 5.0</td>
<td>69.0 ± 8.6</td>
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<tr>
<td>pravastatin</td>
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<tr>
<td>$K_i$ (μM)</td>
<td>1150 ± 480</td>
<td>937 ± 492</td>
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<tr>
<td>PCG</td>
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<tr>
<td>$K_i$ (μM)</td>
<td>418 ± 42</td>
<td>1930 ± 330</td>
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<tr>
<td>DBSP</td>
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<td>$K_i$ (μM)</td>
<td>2.74 ± 0.26</td>
<td>22.2 ± 5.8</td>
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**Discussion**

In the present study, we have demonstrated the basolateral localization of rOat3 in the kidney and examined the contribution of rOat1 and rOat3 to the renal uptake of PAH and pravastatin. The expression of rOat3 was studied in the cDNA-transfected LLC-PK1 cells and the kidney (Fig. 1). Two bands were detected in the crude membrane fraction of rOat3-expressed LLC-PK1 cells. Because these bands were abolished by incubating the antiserum with the antigen and were not detected in the vector-transfected cells, they are related to rOat3. Two bands were also detected in the plasma membrane fraction from the kidney at a similar molecular weight. The difference in the molecular size of two bands may be due to the difference in the degree of glycosylation, however, the physiological meaning remains to be clarified. Immunofluorescence studies revealed that rOat3 was localized to the basolateral membrane of the renal proximal tubular cells (Fig. 2), which is consistent with the localization of hOAT3 in the kidney (Cha et al., 2001).
rOat1- and rOat3-expressed LLC-PK1 cells exhibited specific uptake of PAH and pravastatin, respectively (Fig. 3, A and C). The $K_i$ value of PAH for the uptake of pravastatin by rOat3-expressed cells was 1.4 mM, which was 23-fold greater than the $K_m$ value for the rOat1-mediated uptake of PAH ($K_m = 60 \mu M$) (Table 1; Fig. 3B). In contrast to PAH, pravastatin exhibited a much higher affinity for rOat3 than for rOat1. The $K_i$ value of pravastatin for the uptake of PAH by rOat1-expressed cells was 1.2 mM which is 86-fold greater than the $K_m$ value for the rOat3-mediated uptake of pravastatin ($K_m = 13 \mu M$) (Table 1; Fig. 3D). Taking these kinetic parameters into consideration, PAH and pravastatin appear to be a relatively specific substrate of rOat1 and rOat3, respectively.

These in vitro transport studies suggest that the renal uptake of PAH and pravastatin is accounted for by rOat1 and rOat3, respectively. This was confirmed by a mutual inhibition study for the uptake of PAH and pravastatin by kidney slices and by examining the inhibitory effect of PCG on their uptake. PCG has been suggested to be a selective inhibitor of rOat3 from transport studies using X. laevis oocytes, in which the inhibition constant of PCG for rOat1-mediated PAH transport was 1.68 mM (Jariyawat et al., 1999), whereas rOat3-mediated transport was completely inhibited by PCG at 1 mM (Kusuhara et al., 1999). More quantitative inhibition experiments in our present study using cDNA transfected cells revealed a great difference in the $K_i$ values of PCG for rOat1 and rOat3 (418 and 52.8 $\mu M$, respectively) (Table 1; Fig. 5). DBSP is a potent, but nonspecific, inhibitor of rOat1 and rOat3 (Table 1; Fig. 5). A mutual inhibition study was carried out on the uptake of PAH and pravastatin by kidney slices. As shown in Table 1, there was 30-fold difference in the $K_m$ and $K_i$ values of PAH for the uptake of PAH and pravastatin by kidney slices (Table 1). An 85-fold difference was observed in the $K_i$ and $K_m$ values of pravastatin for the uptake of PAH and pravastatin by kidney slices (Table 1). These results indicate that different uptake systems are responsible for the renal uptake of PAH and pravastatin. This was supported by the difference in the degree of inhibition by PCG. PCG is a more potent inhibitor for the uptake of pravastatin by kidney slices, and the $K_i$ value was 21-fold smaller than that for the uptake of PAH. In addition, kinetic parameters ($K_m$ and $K_i$ values) of PAH, pravastatin, and PCG for PAH uptake by kidney slices were almost comparable with those for the uptake by rOat1-expressed LLC-PK1 cells, whereas the kinetic parameters for the uptake of pravastatin by kidney slices were almost comparable with those of for the uptake by rOat3-expressed cells (Table 1; Fig. 5). However, the $K_i$ values of DBSP determined in kidney slices were about 7-fold greater than those observed in the transfectants by unknown reason (Table 1). The difference in the $K_i$ value of DBSP may be accounted for by the reduced concentration of DBSP in the uptake buffer due to adsorption and/or uptake by kidney slices. Taking all re-
sults presented in this manuscript into consideration, we can conclude that rOat1 is mainly responsible for the renal uptake of PAH, whereas the renal uptake of pravastatin is mainly accounted for by rOat3 (Fig. 6). The importance of rOat3 in the renal uptake of other organic anions and an organic cation, cimetidine, remains to be clarified. In future studies, it will be necessary to examine its contribution to the total renal uptake and to investigate its substrate specificity using gene expression systems.

It is generally accepted that amphipathic organic anions are eliminated by the liver and small and hydrophilic organic anions are eliminated by the kidney. This is partly achieved by the uptake mechanism governing an initial process of overall elimination. The OATP family has been considered to play a major role in the hepatic uptake of amphipathic organic anions (Meier et al., 1997; Muller and Jansen, 1997; Suzuki and Sugiyma, 2000). As described previously, substrates of rOat1 include hydrophilic and small molecules, and do not overlap with those of the OATP family. In contrast, many of the substrates of rOat3 are also substrates of the OATPs (Meier et al., 1997; Muller and Jansen, 1997; Suzuki and Sugiyma, 2000). The overlap in substrates between OATPs and rOat3 but not rOat1 has prompted us to propose that the substrates of rOat1 mainly distribute to the kidney, whereas those of rOat3 distribute not only to the kidney but also to the liver, because these are recognized also by rOatps. Further quantitative studies are required to confirm this hypothesis by examining the uptake of common substrates of rOat3 and OATPs by the liver and kidney.

The renal clearance of pravastatin exceeds the glomerular filtration rate, suggesting that it undergoes tubular secretion (Singhvi et al., 1990). Although the molecular mechanism for the excretion process of organic anions has not been identified, several candidate transporters for pravastatin are available (Fig. 6). Multidrug resistance associated protein 2, a primary active transporter, which is expressed on the bile canalicular membrane and the brush border membrane of the intestine and extrudes amphipathic organic anions into the luminal side (Keppler and Konig, 1997; Suzuki and Sugiyama, 1998; Mottino et al., 2000). It has been demonstrated to be also expressed on the brush border membrane of proximal tubules (Schaub et al., 1997). In addition, rOatp1, rOat-K1, and rOat-K2 are also candidates for the renal excretion of amphipathic organic anions in rats (Bergwerk et al., 1996; Inui et al., 2000), because they mediate bidirectional transport (Li et al., 1998; Inui et al., 2000).

In conclusion, we have demonstrated that rOat3 is involved in the renal uptake of pravastatin on the basolateral membrane of the proximal tubules.

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**References**


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