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Role of Na⁺/L-carnitine Transporter (OCTN2) in Renal Handling of Pivaloylcarnitine and Valproylcarnitine Formed during Pivalic Acid-containing Prodrugs and Valproic Acid Treatment

Shuichi OHNISHI*, Noboru OKAMURA **, Shingo SAKAMOTO, Hiroshi HASEGAWA, Ryo NORIKURA, Eri KANAOKA, Kouji TAKAHASHI, Kazutoshi HORIE, Kiyoshi SAKAMOTO and Takahiko BABA

Developmental Research Laboratories, Shionogi & Co., Ltd., Japan

Summary: Pivalic acid and valproic acid decreases L-carnitine concentration in the body via urinary excretion of their acylcarnitines, pivaloylcarnitine (PC) and valproylcarnitine (VC). To obtain an information about the mechanism of the physiological response, we investigated the renal handling of these acylcarnitines by Na⁺/L-carnitine cotransporter, OCTN2 using the isolated perfused rat kidney, rat OCTN2 (rOCTN2) and human OCTN2 (hOCTN2) expressing cells. In the perfused rat kidney, PC and VC were strongly reabsorbed with an efficiency comparable to L-carnitine, and these reabsorption were inhibited by 1 mM L-carnitine, suggesting that the interaction of L-carnitine with PC and VC reabsorption would be responsible for renal handling of these acylcarnitines in rats. The rOCTN2-mediated uptake of PC was lower than that of L-carnitine, whereas rOCTN2-mediated uptake of VC was as high as that of L-carnitine, indicating that contribution of rOCTN2 in renal handling of PC and VC would be different. Furthermore, hOCTN2-mediated uptake of these acylcarnitines was markedly lower than that of L-carnitine. On the other hand, both PC and VC inhibited L-carnitine reabsorption in the perfused rat kidney and their concentration-dependent inhibition was also observed for rOCTN2 and hOCTN2. These results suggest that low renal reabsorption and interaction of hOCTN2 for these acylcarnitines might possibly affect the decrease of L-carnitine concentration in humans.

Keywords: pivalic acid; valproic acid; carnitine transporter; acylcarnitine; renal excretion

Introduction

L-Carnitine (Fig. 1) is an essential compound for the transport of long chain fatty acids into mitochondria and subsequent production of cellular energy by β-oxidation.1) It exists in the body as the free form or acylcarnitine, which is a conjugate produced from L-carnitine and acyl-CoA by carnitine acyltransferase.2) The concentration of L-carnitine in the body is controlled by various factors such as its biosynthesis mainly in the liver, its absorption from dietary sources, conversion to acylcarnitine from L-carnitine, renal excretion of L-carnitine or acylcarnitine, renal reabsorption of L-carnitine or acylcarnitine and release of L-carnitine from acylcarnitine.3–5) Most of the L-carnitine and acylcarnitine is filtered at the glomerulus in the kidney, because these protein bindings are negligible.3) In the kidney, a high-affinity Na⁺/L-carnitine cotransporter, OCTN2 is expressed in the brush border membranes of the proximal tubule cells and is responsible for efficient reabsorption of filtered L-carnitine and acetylcarnitine (AC), which is the predominant form of acylcarnitine.3–6,10) Primary carnitine deficiency is characterized by a low L-carnitine level in plasma and tissues and induces clinical symptoms such as progressive cardiomyopathy, hypoketotic hypoglycemic encephalopathy and myopathy.11) Functional defects due to mutations of the OCTN2 gene have been observed in the primary carnitine deficiency patients.12,13) Therefore, the transport activity of L-carnitine and acylcarnitine by OCTN2 plays an important role in maintaining L-carnitine concentration in plasma and tissues. The L-carnitine concentration in the plasma and tissues has been reported to decrease during administration of prodrugs such as pivampicillin, pivmecillinam, cefditocene, etc.
Chemical structure of L-carnitine, PC and VC are shown at physiological pH.

Fig. 1. Chemical structure of L-carnitine, PC and VC. The chemical structure of L-carnitine, PC and VC are shown at physiological pH.

We have recently reported the mechanism for the decrease of L-carnitine concentration caused by pivalic acid and valproic acid based on the transport characteristics of PC and VC by the porcine kidney epithelial cell line, LLC-PK₁, which functionally expresses Na⁺/L-carnitine cotransporter in the apical membranes.²⁰ PC and VC were not substrates for Na⁺/L-carnitine cotransporter in LLC-PK₁ cells, and VC inhibited L-carnitine uptake by LLC-PK₁ cells in a noncompetitive manner. These findings suggest that the recognition and interaction of Na⁺/L-carnitine cotransporter are important factor for the decrease of L-carnitine concentration. In the present study, to clarify the in vivo contribution of OCTN2, we examined the transport characteristics of PC and VC using the isolated perfused rat kidney and rat OCTN2 (rOCTN2) expressing cells. The perfused rat kidney is a useful system for investigating the drug transport and drug-drug interaction in the intact kidney, and has been adapted for analysis of organic anion transporter, organic cation transporter and P-glycoprotein (MDR1).²⁷⁻³² Furthermore, to examine the molecular mechanism for the decrease of L-carnitine concentration in humans, transport assays of PC and VC were performed using human OCTN2 (hOCTN2) expressing cells.

Methods

Materials: L-[Methyl-³H]carnitine hydrochloride (2.96–3.07 TBq/mmol) was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). [Carboxyl-¹⁴C]inulin-carboxyl (74 MBq/g) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Unlabeled L-carnitine was purchased from NACALAI TESQUE, INC. (Kyoto, Japan). Pivaloyl-L-[methyl-³H]carnitine hydrochloride (188 GBq/mmol) and valproyl-L-[methyl-³H]carnitine hydrochloride (188 GBq/mmol) were synthesized by acylation of L-[methyl-³H]carnitine hydrochloride with the corresponding acid chloride at Shionogi & Co., Ltd. Unlabeled pivaloyl-L-carnitine and unlabeled valproyl-L-carnitine were also synthesized by acylation of L-carnitine hydrochloride with the corresponding acid chloride at Shionogi & Co., Ltd. All other agents were of reagent grade.

Preparation of the isolated perfused rat kidney: All animal experiments were approved by the institution Animal Care and Use Committee of Shionogi & Co., Ltd. A single perfusion of rat kidney was performed as reported previously.²⁷⁻³² The right kidney of Jcl:SD rat (male, body weight: 308 g–416 g, CLEA Japan, Inc, Tokyo, Japan) was isolated by the method of Nishitsutsuji-Uwo et al.¹⁹ and the ureter, vena cava inferior and renal artery were cannulated. Thereafter, the kidney was
perfused with Krebs-Henseleit bicarbonate buffer (KHBB) containing bovine erythrocytes (hematocrit: 12.0%–14.5%), 5% bovine serum albumin (BSA), amino acids (0.5 mM methionine, 2.0 mM alanine, 2.0 mM glycine, 2.0 mM serine, 1.0 mM arginine, 2.0 mM proline, 1.0 mM isoleucine and 3.0 mM aspartic acid), 0.1% glucose, 3% mannitol and 0.05% creatinine. An appropriate volume of gas with 5% CO₂–95% air was infused into the KHBB. During the initial 20-min equilibration time of the kidney, the perfusate plasma flow rate was adjusted to approximately 5.0 ml/min. Appropriate volumes of the perfusate and urine were collected before administration of the drugs. The perfusate plasma was obtained by centrifugation of the perfusate. Creatinine concentrations in plasma and urine were measured by the alkaline picrate method using the Creatinine Test-WAKO (Wako Pure Chemical Industries, Osaka, Japan). Sodium concentrations were determined using a selective ion electrode (pH meter F-8 AT, Horiba, Ltd., Kyoto, Japan) and glucose concentrations were determined by the o-toluidine method using the Glucose Test-WAKO (Wako Pure Chemical Industries). The glomerular filtration rate (GFR) was determined from the creatinine clearance. The urine flow rate, GFR and fractional reabsorption of sodium and glucose were used as indices to monitor the viability of the perfused kidney.

**Multiple indicator dilution experiment in the isolated perfused rat kidney:** The renal handling of L-carnitine, PC and VC was investigated from single-pass outflow curves of each indicator simultaneously injected into the renal artery. Evans-blue labeled albumin (1.7 mg/mL Evans blue and 5% BSA) was used as a marker for vascular space and [14C]inulin (2.84 mg/mL, 0.21 MBq/mL) was used as a marker for extracellular space and glomerular filtration. Each test compound containing [3H]carnitine (0.5 μM, 1.48 MBq/mL), [3H]PC (4.9 μM, 0.74 MBq/mL) or [3H]VC (4.9 μM, 0.74 MBq/mL) was mixed with Evans-blue labeled albumin and [14C]inulin in KHBB. After a bolus injection of the mixture (0.05 mL) via the renal artery, venous effluent and urine samples were collected up to 15 min as described previously. The perfusate plasma was obtained by centrifugation of the blood. Immediately after collection of venous effluent and urine samples, the kidney was excised, weighed for wet weight, and then homogenized with water. An aliquot of perfusate plasma, urine and homogenate was sampled for determination of radioactivity. After addition of 10 mL of Pico-Fluor 40 (PerkinElmer Life and Analytical Sciences), the radioactivity of [3H]labeled test compounds and [14C]inulin was simultaneously determined with a liquid scintillation counter (Tri-carb 2200CA, PerkinElmer Life and Analytical Sciences). Evans blue-labeled albumin in plasma was determined by spectrophotometry (595 nm). Since drug molecules which bind to erythrocytes are generally consi-
bated with 2 mL of incubation medium containing L-[^3]H)carnitine (10 nM, 30.7 kBq/mL), L-[^3]H)PC (114 nM, 17.2 or 21.4 kBq/mL) or L-[^3]H)VC (100 nM, 15.1 or 11.9 kBq/mL) at 37°C for the desired time. After incubation, the medium was aspirated and the cells were washed twice with ice-cold HBS (pH 7.4). The cells were solubilized in 1 mL of 1 N NaOH, and an aliquot (100 or 500 µL) was sampled for determination of radioactivity. After addition of 10 mL of Pico-Fluor 40, the radioactivity was determined with a liquid scintillation counter (Tri-carb 3100TR, PerkinElmer Life and Analytical Sciences). The protein content of the cells solubilized in 1 N NaOH was determined by a minor modification of the Lowry’s method using a Protein Assay Rapid Kit wako (Wako Pure Chemical Industries) with BSA as a standard.

**Data analysis:** The kinetic parameters for L-carnitine, PC and VC uptake by rOCTN2 and hOCTN2 were calculated by fitting the data to the following equation:

\[
V = \frac{V_{\text{max}} \cdot S}{K_m + S} + K_d \cdot S
\]

where \( V \) is the uptake rate of the substrate (nmol/mg protein/min), \( V_{\text{max}} \) is the maximum uptake rate (nmol/mg protein/min), \( S \) is the substrate concentration in the incubation medium (µM), \( K_m \) is the Michaelis constant (µM) and \( K_d \) is the coefficient constant of the nonspecific uptake (µL/mg protein/min).

IC\(_{50}\) values (µM) for inhibition of PC and VC on L-carnitine uptake were calculated by fitting the data to the following equation:

\[
V = V_{\text{max}} - (V_{\text{max}} - V_0) \cdot \frac{I}{I + IC_{50}}
\]

where \( V \) is the uptake rate of the substrate (fmol/mg protein/min), \( V_{\text{max}} \) is the maximum uptake rate (fmol/mg protein/min), \( V_0 \) is the nonspecific uptake rate (fmol/mg protein/min) and \( I \) is the inhibitor concentration (µM).

The \( K_i \) value (µM) for competitive inhibition of L-carnitine uptake was calculated by fitting the data to the following equation:

\[
V = \frac{V_{\text{max}} \cdot S}{K_m \cdot (1 + I/K_i) + S}
\]

where \( V \) is the uptake rate of the substrate (nmol/mg protein/min), \( V_{\text{max}} \) is the maximum uptake rate (nmol/mg protein/min), \( S \) is the substrate concentration in the incubation medium (µM), \( K_m \) is the Michaelis constant (µM), \( I \) is the inhibitor concentration (µM) and \( K_i \) is the coefficient constant of the nonspecific uptake (µL/mg protein/min).

Curve-fitting was carried out using nonlinear-least squares regression analysis with KaleidaGraph for Windows (Synergy Software, Reading, PA).

**Results**

**Experimental conditions of the kidney perfusion:** The perfusate flow rate, urine flow rate, GFR and fractional reabsorption of sodium and glucose in the present perfused rat kidney are summarized in Table 1. Perfusion flow rates were controlled to similar values in all experiments, although the value was significantly increased in effect of 1 mM VC on L-carnitine reabsorption. Significant increase for fractional reabsorption of glucose was observed in effect of 1 mM L-carnitine on PC reabsorption. However, the values (93.7%–97.5%) in all experiments were consistent with those (93.3%–98.5%) reported previously. In the other indices for kidney function, the values of all experiments were not different from those of the control.

**Renal handling of L-carnitine in the isolated perfused rat kidney:** Figs. 2A and 2B show typical venous and urinary outflow curves of Evans-blue labeled albumin, inulin and L-carnitine after a simultaneous bolus injection into a renal artery. The renal venous outflow curve of L-carnitine showed a lower peak and broader shape than those of the other reference com-

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**Table 1. Experimental conditions of the isolated perfused rat kidney**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Condition</th>
<th>PFR (mL/min)</th>
<th>UFR (mL/min)</th>
<th>GFR (mL/min)</th>
<th>FR(_{\text{Na}}) (%)</th>
<th>FR(_{\text{Glc}}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Carnitine</td>
<td>Control (6)</td>
<td>5.29 ± 0.27</td>
<td>0.098 ± 0.015</td>
<td>0.410 ± 0.074</td>
<td>97.0 ± 0.3</td>
<td>93.7 ± 1.5</td>
</tr>
<tr>
<td>L-Carnitine, 1 mM (3)</td>
<td>5.48 ± 0.35</td>
<td>0.119 ± 0.035</td>
<td>0.455 ± 0.064</td>
<td>97.1 ± 1.5</td>
<td>96.1 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>PC, 1 mM (3)</td>
<td>4.96 ± 0.04</td>
<td>0.131 ± 0.057</td>
<td>0.419 ± 0.190</td>
<td>95.3 ± 1.5</td>
<td>93.8 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>VC, 1 mM (3)</td>
<td>6.38 ± 0.05*</td>
<td>0.137 ± 0.019</td>
<td>0.336 ± 0.028</td>
<td>93.0 ± 1.8</td>
<td>94.7 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>Control (3)</td>
<td>5.94 ± 0.06</td>
<td>0.118 ± 0.022</td>
<td>0.374 ± 0.045</td>
<td>97.4 ± 1.3</td>
<td>95.6 ± 0.3</td>
</tr>
<tr>
<td>L-Carnitine, 1 mM (3)</td>
<td>5.83 ± 0.19</td>
<td>0.089 ± 0.032</td>
<td>0.353 ± 0.103</td>
<td>99.2 ± 0.4</td>
<td>97.5 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td>VC</td>
<td>Control (3)</td>
<td>5.69 ± 0.29</td>
<td>0.136 ± 0.015</td>
<td>0.428 ± 0.021</td>
<td>94.6 ± 1.3</td>
<td>94.3 ± 0.2</td>
</tr>
<tr>
<td>L-Carnitine, 1 mM (3)</td>
<td>5.92 ± 0.45</td>
<td>0.140 ± 0.044</td>
<td>0.439 ± 0.093</td>
<td>95.5 ± 2.3</td>
<td>95.6 ± 1.4</td>
<td></td>
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</tbody>
</table>

Values are the mean ± SE of three or six independent experiments. Numbers in parentheses represent the number of experiments. Each parameter was determined according to the procedure indicated in Methods section. Abbreviations are: PFR, plasma flow rate; UFR, urine flow rate; GFR, glomerular filtration rate; FR\(_{\text{Na}}\), fractional reabsorption of Na; FR\(_{\text{Glc}}\), fractional reabsorption of glucose. *p < 0.05: significantly different from control value.
Renal Handling of Acylcarnitine by Carnitine Transporter

Fig. 2. Typical renal venous outflow curves (A and C) and urinary excretion rate vs. times curves (B and D) for L-carnitine, inulin and albumin in the isolated perfused rat kidney.

In the absence (A and B) or presence (C and D) of 1 mM L-carnitine, A and C show renal venous outflow curves, and B and D show urinary excretion rate vs. times curves after simultaneous bolus injection of 0.5 μM [3H]carnitine (○), [14C]inulin (●) and Evans blue-albumin (▲). The plasma concentration represents as fraction of concentration at each time point to total recovery in plasma, urine and kidney. Urinary excretion rate represents as fraction of radioactivity per minute at each time point to total recovery in plasma, urine and kidney.

pounds. The mean artery-to-vein transit time of L-carnitine (7.0 ± 0.5 sec, mean ± SE) tends to be greater than those of albumin (2.0 ± 0.1 sec) and inulin (5.6 ± 0.5 sec). On the other hand, the urinary excretion of L-carnitine (FE = 1.0 ± 0.1%) was lower than that of inulin (FE = 3.3 ± 0.4%) and the FE/FF value of L-carnitine (0.30 ± 0.03) was less than unity (Fig. 3). The effect of excess unlabeled L-carnitine on L-carnitine reabsorption in the isolated perfused rat kidney was also examined. In the presence of 1 mM L-carnitine, the venous outflow curves were not changed (Fig. 2C), whereas urinary excretion of L-carnitine was markedly increased (Fig. 2D). The FE/FF values of L-carnitine in the presence of 1 mM L-carnitine were close to unity (Fig. 3).

Effect of PC and VC on L-carnitine reabsorption in the isolated perfused rat kidney: Excess unlabeled PC and VC inhibited L-carnitine reabsorption in the isolated perfused rat kidney. Urinary excretion of L-carnitine was markedly increased in the presence of 1 mM PC and 1 mM VC and these FE/FF values were close to unity (Fig. 3).

Renal handling of PC and VC in the isolated perfused rat kidney: Renal handling of PC and VC in the isolated perfused rat kidney was investigated. The renal venous outflow curves of PC and VC were similar to that of L-carnitine (data not shown). The FE/FF values of PC and VC (0.30 ± 0.06 and 0.16 ± 0.07, respectively) were less than unity (Fig. 3). In addition, urinary excretion of PC and VC in the presence of 1 mM L-carnitine was markedly increased and these FE/FF values were almost unity. These findings demonstrate that PC and VC would be strongly reabsorbed in the isolated perfused rat kidney like L-carnitine.

Characteristics of PC and VC uptake by rOCTN2 and hOCTN2: The rOCTN2 and hOCTN2-mediated uptake of PC and VC was investigated in the presence or absence of Na⁺. In the case of PC uptake by rOCTN2-transfected CHO cells (CHO-rOCTN2 cells), the PC uptake by mock-transfected cells in the presence of Na⁺ tends to increase comparing with that by CHO-rOCTN2.
cells in the absence of Na\(^+\) (Fig. 4A). However, the increase was not statistically significant for the data other than the PC uptake after 5 min-incubation. In other cases, the PC and VC uptake by mock-transfected cells in the presence of Na\(^+\) slightly decreased comparing with that by OCTN2-transfected cells in the absence of Na\(^+\) (Figs. 4B, 4C and 4D). Therefore, the possible involvement of endogenous transport activity by CHO and HEK293 cells seems to be very low. The uptake of PC by CHO-rOCTN2 cells in the presence of Na\(^+\) was markedly stimulated compared with that by mock-transfected cells (Fig. 4A). Similarly, the uptake of VC by CHO-rOCTN2 cells in the presence of Na\(^+\) was markedly greater than that by mock-transfected cells (Fig. 4B). In both cases, uptake of these acylcarnitines by CHO-rOCTN2 cells was markedly reduced in the absence of Na\(^+\) (Figs. 4A and 4B). Similar results were obtained with hOCTN2-transfected HEK293 cells (HEK293-hOCTN2 cells, Figs. 4C and 4D). These results demonstrate that PC and VC could be substrates for both rOCTN2 and hOCTN2.

Fig. 5 reveals the concentration dependence of L-carnitine, PC and VC uptake by rOCTN2 and hOCTN2. In all cases, the specific uptake was calculated by subtracting the nonspecific uptake from the total uptake. Eadie-Hofstee plots gave a single straight line (Fig. 5, insets), suggesting the involvement of a single saturable uptake system. With the use of nonlinear-least squares regression analysis, kinetic parameters were calculated according to the Michaelis-Menten equation and these parameters were summarized in table 2. For rOCTN2, the uptake clearance (\(V_{\text{cat}}/K_m\)) of PC was about 4-fold lower than that of L-carnitine due to about 6-fold increase of \(K_m\) value for rOCTN2. On the other hand, the uptake clearance of VC by rOCTN2 was almost same as that of L-carnitine. For hOCTN2, the uptake clearance of PC and VC was about 7-fold and 20-fold lower than that of L-carnitine, respectively. In this case, there was remarkable change of \(K_m\) value for hOCTN2 (about 20-fold increase for both PC and VC).

**Effects of PC and VC on L-carnitine uptake by rOCTN2 and hOCTN2:** Concentration-dependent inhibitions of PC and VC were observed for L-carnitine uptake by rOCTN2 and hOCTN2 (Figs. 6A and 6B, respectively). For rOCTN2, the calculated \(I_{50}\) values of PC and VC were 51.9 and 10.4 \(\mu\)M, respectively. For hOCTN2, the \(I_{50}\) values of PC and VC were 308 and 458 \(\mu\)M, respectively. The kinetic mode for inhibitions of PC and VC were examined for hOCTN2 (Figs. 7A and 7B). The apparent \(K_m\) and \(V_{\text{cat}}\) values for L-carnitine uptake in the absence of these inhibitors were 8.01 \(\mu\)M and 0.450 nmol/mg protein/min, respectively. The presence of PC and VC caused increase of \(K_m\) values (23.4 and 27.7 \(\mu\)M for PC and VC, respectively), whereas no change of the \(V_{\text{cat}}\) values (0.440 and 0.461 nmol/mg protein/min for PC and VC, respectively) was observed. These results show that PC and VC could inhibit L-carnitine uptake in a competitive manner. Therefore, the calculated \(K_m\) values of PC and VC were 282 and 458 \(\mu\)M, respectively, which were similar to the \(I_{50}\) values.

**Discussion**

Administration of pivalic acid-containing prodrugs and valproic acid has been reported to induce a decrease of L-carnitine concentration in the body.\(^{14-17,21-23}\) Although urinary loss of PC and VC is well known to be the predominant cause of the symptom,\(^{16,20,21}\) renal handling of PC and VC is not largely clarified. In renal proximal tubules, a high affinity Na\(^+\)/L-carnitine cotransporter, OCTN2 mediates reabsorption of L-carnitine and acylcarnitine and maintains the L-carnitine level in plasma and tissues.\(^{3,4,6-10}\) Therefore, we investigated the transport characteristics of PC and VC in the isolated perfused rat kidney, rOCTN2 and hOCTN2 transiently expressing cells to get an information about the molecular mechanism for the decrease of L-carnitine concentration.

We demonstrated that L-carnitine is reabsorbed in the isolated perfused rat kidney. The FE/FF value of L-carnitine (0.30 \(\pm\) 0.03) was less than unity (Fig. 3), indicating that reabsorption rather than secretion could be the
Fig. 4. Na⁺-dependent PC and VC uptake by rOCTN2 (A and B) and hOCTN2 (C and D). OCTN2 transfected (●) or mock transfected (▲) cells were incubated at 37°C for a desired time with 114 nM [³H]PC (A and C) and 100 nM [³H]VC (B and D) in the presence of Na⁺. [³H]PC and [³H]VC uptake by OCTN2 transfected cells was also determined in the absence (○) of Na⁺. In the Na⁺-free medium, the NaCl of the incubation medium was replaced with N-methyl-D-glucamine. The radioactivity of the solubilized cells was determined. Values are the mean ± SE of three monolayers. If the error bar is not shown, it is smaller than the symbol. **p < 0.01, *p < 0.05: significantly different from the control value.

Table 2. Kinetic parameters for L-carnitine, PC and VC uptake by rOCTN2 and hOCTN2

<table>
<thead>
<tr>
<th>Species</th>
<th>Kinetic parameter</th>
<th>L-Carnitine</th>
<th>PC</th>
<th>VC</th>
</tr>
</thead>
<tbody>
<tr>
<td>rOCTN2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kₘ (μM)</td>
<td></td>
<td>26.0</td>
<td>159</td>
<td>13.0</td>
</tr>
<tr>
<td>Vₘₐₓ (nmol/mg protein/min)</td>
<td>0.155</td>
<td>0.242</td>
<td>0.0554</td>
<td></td>
</tr>
<tr>
<td>Vₘₐₓ/Kₘ (μL/mg protein/min)</td>
<td>5.96</td>
<td>1.52</td>
<td>4.25</td>
<td></td>
</tr>
<tr>
<td>Kₑ (μL/mg protein/min)</td>
<td>2.44</td>
<td>0.804</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>hOCTN2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kₘ (μM)</td>
<td></td>
<td>8.01</td>
<td>166</td>
<td>168</td>
</tr>
<tr>
<td>Vₘₐₓ (nmol/mg protein/min)</td>
<td>0.450</td>
<td>1.29</td>
<td>0.440</td>
<td></td>
</tr>
<tr>
<td>Vₘₐₓ/Kₘ (μL/mg protein/min)</td>
<td>56.2</td>
<td>7.76</td>
<td>2.62</td>
<td></td>
</tr>
<tr>
<td>Kₑ (μL/mg protein/min)</td>
<td>1.34</td>
<td>0.354</td>
<td>0.349</td>
<td></td>
</tr>
</tbody>
</table>

Kₘ and Vₘₐₓ values were calculated according to the Michaelis-Menten equation from concentration dependence of L-carnitine, PC and VC uptake by rOCTN2 and hOCTN2 in Fig. 5.

Furthermore, in vivo contribution of OCTN2 was examined from comparison of PC and VC transport activity by the isolated perfused rat kidney and CHO-rOCTN2 cells. Transport activity of PC and VC in the isolated perfused rat kidney was same as L-carnitine as described above (Fig. 3). The uptake clearance of PC by rOCTN2 was lower than that of L-carnitine (Table 2), suggesting that predominant process in the renal tubular transport of L-carnitine. In addition, reabsorption of L-carnitine was markedly inhibited by 1 mM L-carnitine (Fig. 3). Mancinelli et al. demonstrated that filtered L-carnitine and AC is efficiently reabsorbed by a saturable transport process in the isolated perfused rat kidney. These findings indicate that the present perfused rat kidney could be a suitable experimental system for renal reabsorption of L-carnitine and acylcarnitine.

PC and VC were reabsorbed with the same transport activity as L-carnitine in the isolated perfused rat kidney, and urinary excretion of PC and VC was markedly elevated in the presence of 1 mM L-carnitine (Fig. 3). These findings suggest that interaction of L-carnitine with PC and VC reabsorption would be responsible for renal handling of these acylcarnitines in rats. However, whether the interaction is the main mechanism for renal handling of PC and VC is not known, because the present L-carnitine concentration (1 mM) was much higher than physiological plasma L-carnitine concentration (30–50 μM). In the isolated perfused rat kidney, the FE/FF value of VC (1.68 ± 0.18) in the presence of 1 mM L-carnitine was quite higher than unity, suggesting that renal tubular secretion would be responsible for renal handling of VC.
Fig. 5. Concentration dependence of L-carnitine, PC and VC uptake by rOCTN2 (A, C and E) and hOCTN2 (B, D and F). L-[3H]Carnitine (A and B), [3H]PC (C and D) and [3H]VC (E and F) uptake by OCTN2 transfected cells was measured at various concentrations at 37°C for 5 or 15 min. The radioactivity of the solubilized cells was determined. The solid and dotted lines represent the estimated overall and nonsaturable uptake based on the fitting data, respectively. Values are the mean ± SE of three monolayers. If the error bar is not shown, it is smaller than the symbol. Inset: Eadie-Hofstee plots of the uptake after correction for the estimated nonsaturable component. V, uptake rate (nmol/mg protein/min); S, substrate concentration (μM).
transporter(s) other than rOCTN2 could be responsible for the renal handling of PC in rats. On the other hand, the uptake clearance of VC was close to that of L-carnitine (Table 2), indicating that the transport activity of VC by rOCTN2 could contribute to its renal reabsorption in vivo. Thus, contribution of OCTN2 in renal handling of acylcarnitines is likely to be dependent on their structure. Previously, we showed that PC and VC are not transported by Na+/L-carnitine cotransporter in LLC-PK1 cells derived from the porcine kidney. In the present study, PC and VC were substrates of rOCTN2 (Fig. 4). These findings demonstrate that there could be species difference in substrate recognition of acylcarnitine by Na+/L-carnitine cotransporter. Furthermore, strong inhibition of 1 mM PC and 1 mM VC on L-carnitine reabsorption was observed in the present perfused kidney (Fig. 3). L-Carnitine uptake by rOCTN2 was also inhibited by PC and VC in a concentration-dependent manner (Fig. 6A), suggesting that competition between these acylcarnitines and L-carnitine for rOCTN2-mediated reabsorption could occur in vivo.

The hOCTN2-mediated transport characteristics of PC and VC was examined to obtain an insight into clinical importance of OCTN2 in their renal excretion processes. The uptake clearance of PC and VC was markedly lower than that of L-carnitine due to their low affinity for hOCTN2 (Table 2). It has been reported that urinary PC excretion is the predominant elimination process of pivalic acid in human. Melegh et al. demonstrated that urinary PC clearance approximated creatinine clearance in children receiving a 7-day course of pivampicillin (2 g/day) and implied lack of PC reabsorption in kidney. Furthermore, Brass et al. reported that the mean plasma PC concentration after administration of 400 mg cefditoren pivoxil twice daily for 14 days is around 7 µM and the concentration is much lower than the Km value (166...
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μM) of PC for hOCTN2 (Table 2). Therefore, these findings indicate that urinary loss of filtered PC due to low reabsorption activity by hOCTN2 might possibly be related to the decrease of L-carnitine concentration induced by pivalic acid. However, as indicated in our rat study, it is possible that other transporter(s) could mainly contribute to the renal handling of PC in humans. In renal brush border membranes, various transporters including sodium-glucose cotransporter (SGLT1 and 2) and peptide transporter (PEPT1 and 2) have been reported to mediate renal reabsorption of endogenous compounds and drugs. Therefore, further investigation seems to be necessary to clarify the mechanism of PC excretion in kidney. VC was also excreted in urine following chronic treatment with valproic acid. Thus, the present result suggests that urinary excretion of filtered VC due to low reabsorption by hOCTN2 would induce the decrease of L-carnitine concentration by valproic acid.

Both PC and VC inhibited competitively L-carnitine uptake by hOCTN2 with $K_i$ values of 282 and 458 μM, respectively (Figs. 6B and 7). The $K_i$ value of PC was markedly higher than the plasma PC concentration (about 7 μM) in pivalic acid-treated patients. It has also been reported that the urinary L-carnitine excretion following administration of pivalic acid-containing prodrugs is negligible. Accordingly, these results indicate that PC could not affect renal reabsorption of L-carnitine. On the other hand, the case of valproic acid may be different because urinary L-carnitine and AC have been detected in children on chronic valproic acid treatment. Thus, the case of valproic acid may be different because urinary L-carnitine and AC have been detected in children on chronic valproic acid treatment. Therefore, VC might interfere in L-carnitine reabsorption by hOCTN2.

In conclusion, these data suggest that contribution of rOCTN2 to renal handling of PC and VC could be different. In addition, low reabsorption and interaction of hOCTN2 for these acylcarnitines may be related to the decrease of L-carnitine concentration caused by pivalic acid and valproic acid.

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References

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