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Research Paper

Involvement of Recognition and Interaction of Carnitine Transporter in the Decrease of L-Carnitine Concentration Induced by Pivalic Acid and Valproic Acid

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Purpose. Prodrugs with pivalic acid and valproic acid decrease L-carnitine concentration in plasma and tissues by urinary excretion of acylcarnitine as pivaloylcarnitine (PC) and valproylcarnitine (VC), respectively. We investigated the role of the Na^+/L-carnitine cotransporter in the porcine kidney epithelial cell line, LLC-PK_1 for the decrease of L-carnitine concentration.
Methods. The uptake of L-[^{3}H]carnitine, acetyl-L-[^{3}H]carnitine (AC), L-[^{3}H]PC and L-[^{3}H]VC were investigated in LLC-PK_1 cells seeded in a 6-well culture plate.
Results. L-Carnitine and AC uptake in LLC-PK_1 cells exhibited Na^+ dependency. The K_m values for L-carnitine and AC uptake were 11.0 and 8.18 μM, respectively. These results indicated expression of Na^+/L-carnitine cotransporter in LLC-PK_1 cells. PC and VC inhibited Na^+/L-carnitine cotransporter in the competitive (K_i = 90.4 μM) and noncompetitive (K_i = 41.6 μM) manners, respectively. PC and VC uptake by Na^+/L-carnitine cotransporter were not observed in LLC-PK_1 cells.
Conclusions. These data suggested that PC and VC formed in the body could not be reabsorbed in the kidney, resulting in the decrease of L-carnitine concentration. In addition, inhibition of L-carnitine reabsorption by VC with lower K_i value could induce the decrease of L-carnitine concentration. Collectively, the recognition and interaction of Na^+/L-carnitine cotransporter are important factors for carnitine homeostasis.

KEY WORDS: carnitine homeostasis; carnitine transporter; LLC-PK_1 cells; pivalic acid; valproic acid.

INTRODUCTION

L-Carnitine plays an important role in the transport of long chain fatty acids into mitochondria for β-oxidation (1). It is usually accumulated in the body by biosynthesis and dietary intake. Renal reabsorption of L-carnitine by a high-affinity L-carnitine transporter in the brush-border membranes of proximal tubule cells is an important physiological process in L-carnitine family homeostasis (2,3), because L-carnitine is likely to be filtered at the glomerulus in the kidney due to negligible protein binding (4). The recently identified hOCTN2 has been suggested to be a high affinity Na^+/L-carnitine cotransporter in various tissues including the kidney, heart, skeletal muscle and placenta (5,6). Especially in the kidney, hOCTN2 is the key for maintaining the serum L-carnitine concentration. It has been reported that mutations of the hOCTN2 gene cause primary systemic carnitine deficiency, which is characterized by low concentrations of L-carnitine in plasma and tissues (7,8). The clinical symptoms of this disease include cardiac myopathy and skeletal myopathy.

L-Carnitine is conjugated with acyl-CoA by carnitine acyltransferase to form acylcarnitine (9). Thus, L-carnitine is present in the body as the free form or as acylcarnitine, which predominantly consists of acetyl-L-carnitine (AC) in the physiological condition (9). AC has been reported to be a reserved form to maintain the plasma L-carnitine concentration by conversion of AC to L-carnitine (10) These compounds are likely to be filtered at the glomerulus in the kidney due to negligible protein binding such as L-carnitine (4). Furthermore, it has been reported that renal reabsorption of AC could be mediated by hOCTN2 (10–12). Therefore, transport of acylcarnitine by hOCTN2 plays an important role in preserving and/or restoring homeostasis of L-carnitine and its derivatives.

Pivalic acid, trimethylacetic acid, has been used in prodrugs to increase oral bioavailability by adding lipophilicity. Treatment of prodrugs with pivalic acid such as pivampicillin, pivmecillinam, cefditoren pivoxil and cefcapene pivoxil has been reported to decrease L-carnitine

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ABBREVIATIONS: AC, acetyl-L-carnitine; PC, pivaloylcarnitine; VC, valproylcarnitine.

concentration in plasma and tissues (13–16). Pivalic acid liberated from prodrugs is metabolized by conjugation with L-carnitine to form pivaloylcarnitine (PC), which is one of the acylcarnitines (17,18). In humans, pivalic acid is predominantly excreted as PC into urine, resulting in reduction of the L-carnitine concentration in plasma (15,19). Valproic acid, 2-propylpentanoic acid, is widely used as an anticonvulsant mainly in pediatric patients. Treatment of valproic acid has been reportedly associated with a decrease in plasma L-carnitine concentration due to urinary excretion of valproylcarnitine (VC), which is one of the acylcarnitines (20–22). Thus, the renal excretion process of acylcarnitines including PC and VC in the kidney could be an important factor in the decrease of L-carnitine concentration induced by pivalic acid and valproic acid. However, the precise mechanisms in renal handling of PC and VC are still unknown.

The porcine kidney epithelial cell line, LLC-PK_1 has been used extensively as a model for transport and accumulation of organic cations in renal proximal tubules (23,24). We have showed that Na^+/L-carnitine cotransporter is functionally expressed in the apical membranes of LLC-PK_1 cells (25), and that L-carnitine uptake activity in the apical membranes of LLC-PK_1 cells is functionally similar to OCTN2. These findings have suggested that LLC-PK_1 cells are appropriate as a model to assess L-carnitine transport. The present study was undertaken to investigate the detailed mechanism in the decrease of L-carnitine concentration caused by pivalic acid and valproic acid.

MATERIALS AND METHODS

Materials

L-[Methyl-^{3}H]carnitine hydrochloride (3.00 TBq/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). 3-O-Acetyl-L-[methyl-^{3}H]carnitine hydrochloride (2.40 TBq/mmol) was obtained from Moravek Biochemicals (Brea, CA). Pivaloyl-L-[methyl-^{3}H]carnitine hydrochloride (188 GBq/mmol, radiochemical purity determined by radio-HPLC: 99.9%), valproyl-L-[methyl-^{3}H]carnitine hydrochloride (188 GBq/mmol, radiochemical purity determined by radio-HPLC: 99.8%), unlabeled pivaloyl-L-carnitine and valproyl-L-carnitine were synthesized by acylation of L-carnitine hydrochloride with the corresponding acid chloride by the Labeled Compounds Synthesis Group of Shionogi & Co., Ltd. Unlabeled L-carnitine and acetyl-L-carnitine were purchased from Sigma-Aldrich (St. Louis, MO). All other agents were of reagent grade.

Cell Culture

LLC-PK_1 cells (ATCC CL-101) were obtained from the American Type Culture Collection (Manassas, VA). They were propagated by Dulbecco's Modified Eagle's medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin G and 100 μg/mL streptomycin (Invitrogen) on 100 mm plastic culture dishes (Becton Dickinson, Franklin Lakes, NJ) in an atmosphere of 5% CO_2–95% air at 37°C. Subculturing was done every week using 0.25% trypsin and 1 mM EDTA (Invtirogen). In general, 100 mm plastic culture dishes were inoculated with 1×10^6 cells in 10 mL of complete culture medium. In this study, LLC-PK_1 cells were used between passages 201 and 222.

Uptake Experiment

The LLC-PK_1 cells were seeded on a 6-well culture plate (well size: 9.6 cm^2, Becton Dickinson) at a density of 2.5×10^5 cells/well. Uptake experiments were carried out at 6 or 7 days after seeding. The culture medium was replaced with Dulbecco's phosphate buffered saline (D-PBS) containing (in mM) 138 NaCl (Na^+ containing buffer) or 138 *N*-methyl-D-glucamine chloride (Na^+ free buffer), 3 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$, 0.5 $MgCl_2$ and 3 glucose. The pH of the medium was adjusted by the addition of a solution of HCl or NaOH (Na^+ containing buffer) and KOH (Na^+ free buffer). In general, the cells were preincubated for 30 min at 37°C with 2 mL of incubation medium (pH 7.4). After removal of the medium, the cells were incubated with 2 mL of incubation medium containing L-[^{3}H]carnitine (5 nM, 15.0 kBq/mL), L-[^{3}H]AC (5 nM, 15.7 kBq/mL), L-[^{3}H]PC (114 nM, 21.4 kBq/mL) and L-[^{3}H]VC (100 nM, 18.8 kBq/mL) for the desired time at 37°C. After incubation, the medium was aspirated and the cells were washed twice with ice-cold D-PBS (pH 7.4). The cells were dissolved in 1 mL of 1 N NaOH, and an aliquot (500 μL) was sampled for determination of radioactivity. After addition of 10 mL of Pico-Fluor 40 (PerkinElmer Life and Analytical Sciences, Wellesley, MA), the radioactivity was determined with a liquid scintillation counter (Tri-carb 2200CA or Tri-carb 3100TR, PerkinElmer Life and Analytical Sciences).

Protein Assay

The protein content of the cells solubilized in 1 N NaOH was determined by a minor modification of the Lowry's method (26) with the use of Protein assay rapid kit (Wako Pure Chemical Industries, Osaka, Japan) with bovine serum albumin as a standard.

Data Analysis

The kinetic parameters for L-carnitine and AC uptake by LLC-PK_1 cells were calculated by fitting the data to the following equation:

$$V = \frac{V_{max} \cdot S}{K_m + S} + K_d \cdot S$$

where V is the uptake rate of the substrate (pmol/mg protein/15 min), S is the substrate concentration in the incubation medium (μM), K_m is the Michaelis-Menten constant (μM), V_{max} is the maximum uptake rate (pmol/mg protein/15 min) and K_d is the coefficient constant of the nonspecific uptake (pmol/mg protein/15 min/μM).

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

$$V = \frac{V_{max} \cdot S}{K_m \cdot \left(1 + \frac{I}{K_i}\right) + S} + K_d \cdot S$$

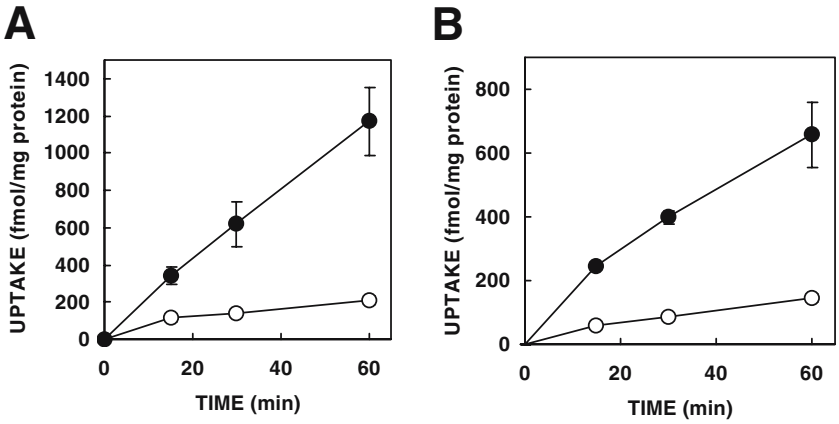

Fig. 1. Na^+-dependent L-carnitine and AC uptake by LLC-PK_1 cells. LLC-PK_1 cells were incubated at 37°C for the desired time with L-[^{3}H]carnitine (A: 5 nM, 15.0 kBq/mL, pH 7.4) and L-[^{3}H]AC (B: 5 nM, 15.7 kBq/mL, pH 7.4) in the presence (●) or absence (○) of NaCl. In the Na^+-free medium, the NaCl of the incubation medium was replaced with *N*-methyl-D-glucamine. Then, the radioactivity of the solubilized cells was determined. Each value represents the mean ± SD of three monolayers. If the error bar is not shown, it is smaller than the symbol.

where V is the uptake rate of the substrate (pmol/mg protein/30 min), V_{max} is the maximum uptake rate (pmol/mg protein/30 min), K_d is the coefficient constant of the nonspecific uptake (pmol/mg protein/30 min/μM) and I is the inhibitor concentration (μM). K_i value (μM) for noncompetitive inhibition of L-carnitine uptake was calculated by fitting the data to the following equation:

$$V = \frac{V_{max} \cdot S}{(K_m + S) \cdot \left(1 + \frac{I}{K_i}\right)} + K_d \cdot S$$

A curve-fitting has been carried out with the use of nonlinear-least squares regression analysis by WinNonlin software (Pharsight Corp., Mountain View, CA).

RESULTS

Characteristics of L-Carnitine and AC Uptake in LLC-PK_1 Cells

First, we examined the time course of L-carnitine uptake in the presence or absence of Na^+ (Fig. 1A). The L-carnitine uptake in the presence of Na^+ was much higher than that in the absence of Na^+. The Na^+-dependent uptake was linear up until 60 min and it was dependent on its concentration (Fig. 2A). The specific uptake was calculated by subtracting the nonspecific uptake from the total uptake. Eadie-Hofstee plots gave a single straight line (Fig. 2, inset), 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. The apparent K_m and V_{max}

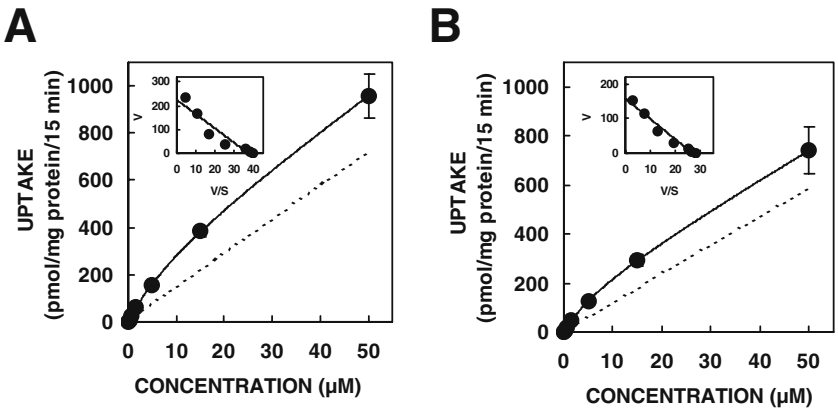

Fig. 2. Concentration dependence of L-carnitine and AC uptake by LLC-PK_1 cells. L-[^{3}H]Carnitine (A) and L-[^{3}H]AC (B) uptake by LLC-PK_1 cells was measured at various concentrations for 15 min at 37°C. Then, the radioactivity of the solubilized cells was determined. The *solid* and *dotted lines* represent the estimated overall and nonsaturable uptake based on the fitting data, respectively. Each value represents the mean ± SD of six 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 nonsaturable component. V, the uptake rate (pmol/mg protein/15 min); S, the substrate concentration (μM).

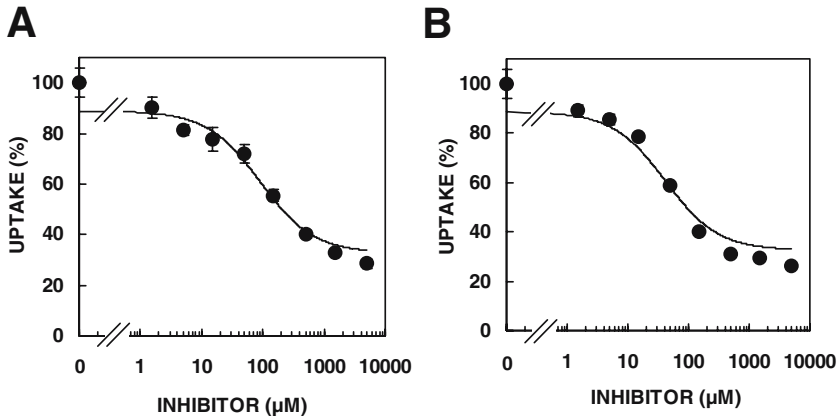

Fig. 3. Concentration-dependent inhibition of L-carnitine uptake by PC and VC in LLC-PK_1 cells. LLC-PK_1 cells were incubated at 37°C for 30 min with L-[^{3}H]carnitine in the presence of increasing concentrations of PC (A) and VC (B), respectively. Then, the radioactivity of the solubilized cells was determined. The *solid lines* represent the estimated overall uptake based on the fitting data. Each value represents the mean ± SD of three monolayers. If the error bar is not shown, it is smaller than the symbol.

values for L-carnitine uptake were 11.0 μM and 282 pmol/mg protein/15 min, respectively.

Figure 1B shows the uptake of AC, which is a typical acylcarnitine, in LLC-PK_1 cells. The AC uptake was Na^+-dependent and it was linear up until 60 min as was observed for L-carnitine uptake. Figure 2B illustrates the concentration dependency of AC uptake in LLC-PK_1 cells. The K_m and V_{max} values calculated for AC uptake were 8.18 μM and 177 pmol/mg protein/15 min, respectively.

Effects of PC and VC on L-Carnitine Uptake in LLC-PK_1 Cells

Figure 3 shows the inhibitory effects of PC and VC on L-carnitine uptake in LLC-PK_1 cells. Concentration-dependent inhibitions of PC and VC were observed for L-carnitine uptake. The kinetic mode of inhibitions by PC and VC were examined (Fig. 4). The apparent K_m and V_{max} values for L-carnitine uptake in the absence of these inhibitors were 5.73 μM and 399 pmol/mg protein/30 min, respectively. The presence of PC caused the increase of the K_m value (21.5 μM), whereas no change of the V_{max} value (382 pmol/mg protein/30 min) was observed. In the presence of VC, the V_{max} value (152 pmol/mg protein/30 min) decreased without affecting the K_m value (5.09 μM). These results show that PC and VC could inhibit L-carnitine uptake in the competitive and noncompetitive manners, respectively. Therefore, the calculated K_i values of PC and VC were 90.4 and 41.6 μM, respectively. Furthermore, no inhibitory effects of pivalic acid and valproic acid on L-carnitine uptake were found in LLC-PK_1 cells (data not shown).

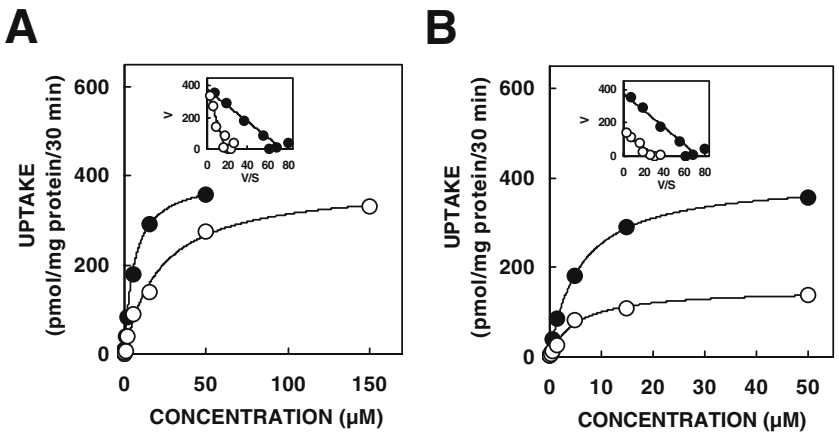

Fig. 4. Kinetic analysis of inhibition of L-carnitine uptake by PC and VC in LLC-PK_1 cells. L-[^{3}H]Carnitine uptake by LLC-PK_1 cells was measured at various concentrations for 30 min at 37°C in the presence (○) or absence (●) of 100 μM PC (A) and 50 μM VC (B). Then, the radioactivity of the solubilized cells was determined. The *lines* represent the estimated uptake based on the fitting data after correction for the nonsaturable component. Each value represents the mean of three monolayers. *Inset*: Eadie-Hofstee plots of the uptake after correction for the nonsaturable component. *V*, the uptake rate (pmol/mg protein/30 min); *S*, the substrate concentration (μM).

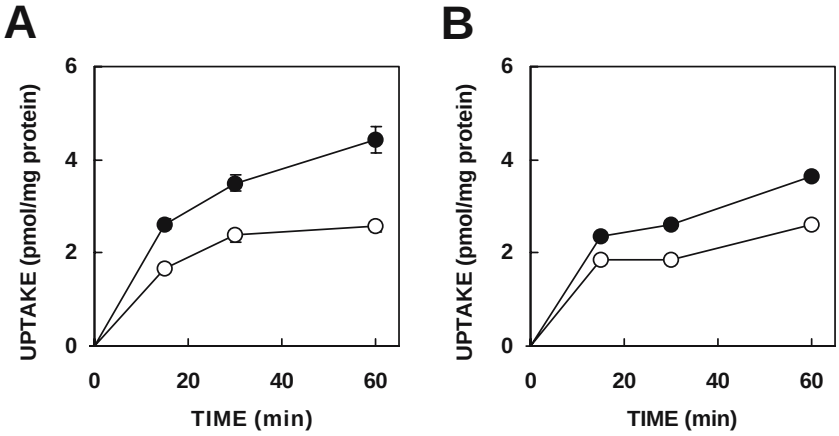

Fig. 5. Effect of Na^+ on PC and VC uptake by LLC-PK_1 cells. LLC-PK_1 cells were incubated at 37°C for the desired time with L-[^{3}H]PC (A: 114 nM, 21.4 kBq/mL, pH 7.4) and L-[^{3}H]VC (B: 100 nM, 18.8 kBq/mL, pH 7.4) in the presence (●) or absence (○) of NaCl. Then, the radioactivity of the solubilized cells was determined. Each value represents the mean ± SD of three monolayers. If the error bar is not shown, it is smaller than the symbol.

Uptake of PC and VC in LLC-PK_1 Cells

The uptake of PC and VC in LLC-PK_1 cells was investigated (Figs. 5 and 6). The Na^+ dependence of PC and VC uptake was slightly observed, but it was much lower than that of L-carnitine uptake (Fig. 5). The uptake of PC and VC was not inhibited by excess unlabeled L-carnitine (Fig. 6). These data indicated that PC and VC are not transported by Na^+/L-carnitine cotransporter in LLC-PK_1 cells.

DISCUSSION

Prodrugs with pivalic acid and valproic acid have been reported to decrease L-carnitine concentration due to urinary excretion of PC and VC, which are acylcarnitines formed by conjugation of L-carnitine with pivalic acid and valproic acid, respectively (13–16,19–22). The recently identified hOCTN2 is a high affinity Na^+/L-carnitine cotransporter and contributes to maintaining L-carnitine plasma level in circulation by reabsorption of filtrated L-carnitine and acylcarnitine in renal proximal tubules (5,6,12). Therefore, to clarify the involvement of OCTN2 in the decrease of L-carnitine concentration, we examined the uptake activity of PC and VC in LLC-PK_1 cells.

The uptake of L-carnitine in LLC-PK_1 cells was markedly stimulated in the presence of Na^+ (Fig. 1A). Our kinetic analysis indicated that L-carnitine uptake was of high affinity (K_m = 11.0 μM) (Fig. 2A). The K_m value was consisted with that reported by Tamai *et al.* using hOCTN2-overexpressing cell lines (K_m = 4.3 μM) (5) and that suggested previously in LLC-PK_1 cells (K_m = 7.8 μM) (25).

As AC is the predominant acylcarnitine in the body (9), we investigated its transport characteristics as a typical acylcarnitine in LLC-PK_1 cells. The present result demonstrated that the Na^+/L-carnitine cotransporter in LLC-PK_1 cells mediated AC uptake in a Na^+-dependent manner

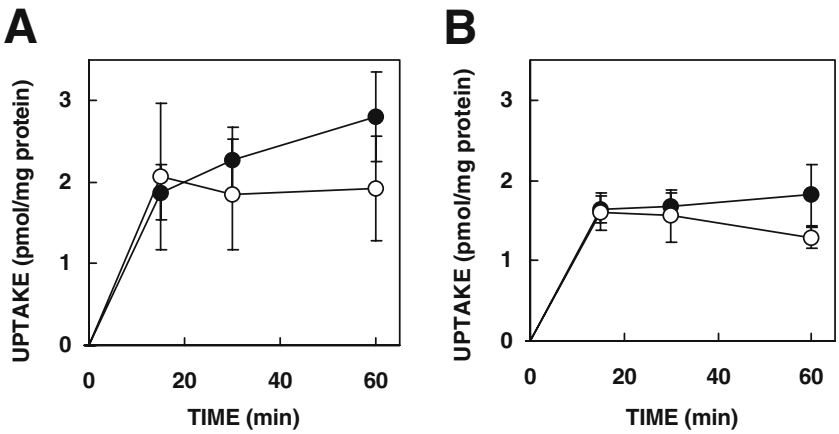

Fig. 6. Effect of L-carnitine on PC and VC uptake by LLC-PK_1 cells. LLC-PK_1 cells were incubated at 37°C for the desired time with L-[^{3}H]PC (A) and L-[^{3}H]VC (B) in the presence (○) or absence (●) of 1 mM L-carnitine. Then, the radioactivity of the solubilized cells was determined. Each value represents the mean ± SD of three monolayers. If the error bar is not shown, it is smaller than the symbol.

(Fig. 1B). AC uptake by this transporter was of high-affinity with a K_m value of 8.18 μM (Fig. 2B). AC is likely to be reabsorbed by the saturable renal tubular process in humans (10) and rats (11). Ohashi *et al*. has demonstrated that Na^+ dependent AC uptake by hOCTN2 was of high affinity (K_m = 8.50 μM) (12). Our data appear to be comparable to these findings *in vivo* and *in vitro*. Because the plasma concentration of AC is around 5 μM (9,13), the high affinity Na^+/L-carnitine cotransporter plays an important role in the reabsorption of AC filtered in the glomerulus. Collectively, the existence of Na^+/L-carnitine transporter in the apical membrane of LLC-PK_1 cells was confirmed from transport characteristics of L-carnitine and AC. Therefore, it was suggested that this experimental model is appropriate for investigating the transporter.

PC and VC inhibited L-carnitine uptake by Na^+/L-carnitine cotransporter in LLC-PK_1 cells with K_i of 90.4 and 41.6 μM, respectively (Figs. 3 and 4). The urinary L-carnitine excretion following administration of prodrugs with pivalic acid is reported to be negligible (13–16). PC is a competitive inhibitor for Na^+/L-carnitine cotransporter with the higher K_i value than the K_m value of L-carnitine uptake. (Fig. 4A). 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 (13). The concentration is much lower than the K_i value of PC for the Na^+/L-carnitine cotransporter. Therefore, these findings suggest that there would be no substantial interference with L-carnitine reabsorption by the renal tubular PC following administration of prodrugs with pivalic acid at therapeutic doses. On the other hand, the case with valproic acid may be different as free L-carnitine is reported to be excreted into urine in epileptic children on chronic valproate treatment (27,28). The present result showed that VC inhibited noncompetitively L-carnitine uptake (Fig. 4B). Tein *et al*. also reported that L-carnitine uptake in cultured human skin fibroblasts was inhibited by exposure to valproic acid for 14 days (29). They suggested that the inhibition would be related to the formation of VC by conjugation of L-carnitine with valproic acid in the fibroblasts. Therefore, VC would cause significant inhibition of L-carnitine reabsorption in the kidney. The inhibitory effect of straight chain acylcarnitine was examined by Molstad *et al*. and Ohashi *et al*. using cultured human heart cells and hOCTN2 transfected HEK293 cells, respectively (12,30). They found that hexanoyl-L-carnitine (5 or 20 μM) and octanoyl-L-carnitine (5 μM), which are straight chain acylcarnitines with a similar number of carbons as PC and VC, inhibited about 80% of L-carnitine uptake by Na^+/L-carnitine cotransporter. Therefore, these findings suggest that the affinity of branched chain acylcarnitine such as PC and VC for Na^+/L-carnitine cotransporter would be lower than that of straight chain acylcarnitine.

In the present study, we first demonstrated that PC and VC are not substrates for Na^+/L-carnitine cotransporter in LLC-PK_1 cells (Figs. 5 and 6). It has been reported that urinary PC excretion is the predominant route of pivalic acid elimination in humans (15,19). Melegh *et al*. found 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 the kidney (15). In addition, PC does not cause inhibition of L-carnitine reabsorption in the kidney as described above. These findings indicate that formation of PC and subsequent lack of reabsorption of PC in the kidney could be a key mechanism for the decrease of L-carnitine concentration induced by prodrugs with pivalic acid. VC was also detected in the urine of patients undergoing chronic valproic acid therapy (20). VC would cause significant inhibition of L-carnitine reabsorption in the kidney as described above. Therefore, the present result suggests that both lack of reabsorption of VC and inhibition of L-carnitine reabsorption by VC in the kidney following formation of VC are likely to contribute to the decrease of L-carnitine concentration in patients following valproic acid administration. However, further investigations are necessary to clarify the detailed mechanisms of the decrease of L-carnitine concentration by valproic acid, because valproic acid can form various metabolites in mammalian tissues in contrast to pivalic acid (31,32).

In conclusion, these data suggested that PC and VC formed in the body could not be reabsorbed in the kidney, resulting in the decrease of L-carnitine concentration. In addition, inhibition of L-carnitine reabsorption by VC with lower K_i value could induce the decrease of L-carnitine concentration. Collectively, the recognition and interaction of Na^+/L-carnitine cotransporter are important factors for carnitine homeostasis.

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