Up-Regulation of Carnitine Transporters Helps Maintain Tissue Carnitine Levels in Carnitine Deficiency Induced by Pivalic Acid

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Purpose. Pivalic acid (PVA) forms conjugates with endogenous carnitine and enhances its excretion. The purpose of this study is to determine whether tissue carnitine levels decrease in parallel with plasma levels in carnitine deficiency induced by PVA.
Methods. PVA was orally administered to rats for 5 days. Carnitine levels in plasma, liver, kidney, muscle, and heart were monitored. The tissue uptake clearance (CL_{uptake}) was determined *in vivo* by the integration plot method. Hepatocytes were prepared from control and PVA-treated rats, and the uptake of L-carnitine was determined.
Results. Plasma concentrations of L-carnitine decreased as a result of the enhanced carnitine elimination as pivaloylcarnitine (PCN) when rats were treated with PVA. However, L-carnitine concentrations in liver, muscle, and heart remained relatively constant during the study period. CL_{uptake} increased in liver and muscle and, thus, the rate of carnitine uptake from plasma into these tissues did not change even at low plasma concentrations. This helps maintain carnitine levels in these tissues. Up-regulation of carnitine transporters is suggested to be a mechanism for the increased CL_{uptake}.
Conclusions. In the carnitine deficiency state induced by PVA, increased CL_{uptake} owing to up-regulation of carnitine transporters is suggested to help maintain carnitine levels in some tissues.

KEY WORDS: pivalic acid; carnitine; tissue uptake; carnitine transporter.

INTRODUCTION

Carnitine is an endogenous compound found in higher concentrations in a number of tissues than in plasma. The concentration of carnitine in some tissues is maintained by an active transport system(s), but the mechanism controlling this is not fully understood. Recently, an organic cation transporter, OCTN2, was cloned and shown to be a carnitine transporter. The physiological importance of OCTN2 was demonstrated by analyzing carnitine distribution in Juvenile Visceral Steatosis (JVS) mice with a systemic carnitine deficiency whose carnitine transporter is functionally deficient. Mutation of the OCTN2 gene has been shown to be directly related to carnitine deficiency in mice and humans (1–4).

Carnitine plays an essential role not only in the mitochondrial β-oxidation of long-chain fatty acids but also in enhancing the elimination of endogenous and xenobiotic carboxylic acids by forming conjugates with them. Pivalic acid (PVA), 2,2-dimethylpropionic acid, is one of those compounds that conjugate with carnitine, which is then eliminated in urine as a conjugate, pivaloylcarnitine (PCN). It is often used in the ester-type moiety of prodrugs to improve efficacy after oral administration. After administration of the prodrugs, PVA is released after hydrolysis by esterase(s) in the intestinal tissue during absorption. Melegh *et al.* (5,6) and Holme *et al.* (7,8) have reported a marked reduction in carnitine concentrations in serum and muscle after long-term treatment with PVA-containing prodrugs. They warned of the possibility that enhanced carnitine elimination may cause symptoms similar to carnitine deficiency secondary to organic acidureas. Since then, the safety of drugs of this type has been studied carefully with special emphasis on carnitine metabolism. Soon after the start of the treatment, PCN was detected in urine, and serum carnitine levels fell markedly. However, no side effects related to low carnitine levels have been reported so far, and the serum level returned to the normal range on ending the drug therapy (9–11). As long as the drugs were used as recommended, they were considered to be safe and well-tolerated.

The purpose of this study is to determine whether the tissue carnitine levels decrease in parallel with the plasma levels in rats made carnitine deficient by administering pivalic acid. The tissue uptake of carnitine was also determined in PVA-treated and untreated rats.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (8 to 9 weeks of age, Japan SLC, Shizuoka, Japan) were obtained 1 week before the experiments and fed on a standard laboratory animal chow (MF, Oriental Yeast Co. LTD., Tokyo, Japan). Animals were treated in accordance with the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985).

Reagents

PVA, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and collagenase were purchased from Wako Pure Chemical Industries (Osaka, Japan). L-carnitine was obtained from Tokyo Kasei (Tokyo, Japan), and 1-aminoanthracene (1-AA) and trypsin inhibitor (type I) were from Sigma Chemical Co. (St. Louis, MO), respectively. PCN, isobutyrylcarnitine, and cyclopropylcarnitine were synthesized at the Pharmaceutical Technology Laboratories and Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., according to the method described by Konishi and Hashimoto (12) with slight modification. L-^{3}H-carnitine (carnitine hydrochloride, L-(N-methyl-^{3}H), 85 Ci/mmol), and ^{14}C-inulin (inulin-carboxyl,[carboxyl]-^{14}C-, 93 MBq/g) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) and NEN Research Products (Wilmington, DE), respectively. All other chemicals used were of reagent grade.

Experimental Design

PVA Administration

A dosing solution of PVA (8 mg/ml) neutralized with NaOH was prepared. Rats were given 1 ml/kg of the dosing

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solution (8 mg/kg) orally twice a day at 10 a.m. and 6 p.m. for 5 days. The daily dose of PVA was 16 mg/kg. Rats were kept in metabolic cages and had free access to food and water.

Effect of PVA Administration on Plasma and Tissue Levels of L-Carnitine

On the days after treatment with PVA for 1, 2, 3, 4, and 5 days and on days 1, 3, 5, and 7 after the termination of the treatment, rats were killed by deep anesthesia induced by ethylether. Blood, kidney, liver, heart, and hind limb muscle were collected and frozen immediately in dry ice-acetone and kept at −80 °C until assay. Urine was collected into ice-cold bottles during the experimental period.

Effect of PVA Administration on the Tissue Uptake of L-Carnitine

A second set of experiments were performed to determine the tissue uptake clearance (CL_{uptake}) of L-carnitine to the tissues in the PVA-treated rats and untreated rats. The uptake experiments were performed on the 4th or 5th day of the PVA treatment. PVA was administered to the treated group in the morning and, approximately 3 hours later, the femoral artery and vein of the PVA-treated rats and the untreated rats were cannulated with polyethylene tubing (PE-50). A mixture of L-^{3}H-carnitine and ^{14}C-inulin in 0.5 ml of saline was injected into the femoral vein, and arterial blood was withdrawn at designated times and plasma was separated. After 1, 3, and 5 minutes, rats were sacrificed and kidney, liver, heart, and hind limb muscle were excised immediately. Approximately 100 mg of each tissue sample was weighed and solubilized with Soluene-350® (Packard, Meriden, CT). Atomlight® or Hionicfluor® (Packard, Meriden, CT) was added to the plasma or solubilized tissue samples, respectively, and radioactivity was determined in a liquid scintillation counter. The CL_{uptake} of L-carnitine was determined by integration plot analysis (13). Assuming that the efflux of the radioactive compounds from the tissue is negligible up to 5 minutes, the ratio of the tissue to plasma concentrations of the compounds at time T can be described as follows:

$$C_T(T)/C_p(T) = CL_{uptake}/V_T \times AUC_{(0-T)}/C_p(T) + V_0/V_T$$

where $C_T(T)$, $C_p(T)$, and V_T are the tissue and plasma concentrations at time T and the tissue volume, respectively; $AUC_{(0-T)}$ is area under the plasma concentration curve from time 0 to time T; and V_0 is the volume of distribution in which a rapid equilibrium with the plasma compartment is assumed. The integration plot was obtained by plotting $C_T(T)/C_p(T)$ against $AUC_{(0-T)}/C_p(T)$. The equation indicates that the slope of the integration plot represents the CL_{uptake} of the compound into the tissue of interest per unit volume. An inulin space correction was made for liver, muscle, and heart.

L-Carnitine Uptake into Hepatocytes Isolated from Control and PVA-Treated Rats

The experiments were performed on the 4th and 5th day of PVA treatment. PVA was administered to the treated group in the morning and, approximately 3 hours later, hepatocytes were isolated from PVA-treated rats and control rats by perfusing rat liver with a 0.05% collagenase solution containing 0.005% trypsin inhibitor. The cell suspensions were examined by the trypan blue exclusion test and those with a high percentage of unstained cells (>97%) were used for the uptake experiment. The final incubation medium contained 137 mM NaCl, 5.4 mM KCl, 0.5 mM NaH_2PO_4, 0.42 mM Na_2HPO_4, 4.2 mM $NaHCO_3$, 1.05 mM $MgCl_2$, 0.83 mM $MgSO_4$, 1.26 mM $CaCl_2$, 5 mM glucose, and 10 mM Tris(hydroxymethyl)aminomethane, pH 7.4. The uptake of L-^{3}H-carnitine was initiated by adding the ligand and unlabeled carnitine to the cell suspension preincubated for 2 minutes at 37 °C. At designated times, cells were separated from the medium using a centrifugal filtration technique (14). Briefly, 150 μl of incubation mixture was collected in a sample tube containing 50 μl 3N NaOH covered with 100 μl of silicone oil (density 1.050) and centrifuged in a tabletop centrifuge at 13,750 rpm (Microfuge E; Beckman Instrument, Fullerton, CA). After the pellet of hepatocytes dissolved in the NaOH solution, the tube was cut at the silicone layer. The bottom layer (NaOH layer) was transferred to scintillation vials and neutralized with HCl. Atomlight® was added and the radioactivity was determined. ^{14}C-Inulin was used to correct for the extracellular adhering fluid. The cellular protein was determined using a Bio–Rad protein assay kit (Bio-Rad Laboratories, CA). The uptake clearance ($V_{0,cell}/S$) was fitted to equation (1) by a WinNonlin program (Pharsight Corporation, CA).

$$V_{0,cell}/S = V_{max}/(K_m + S) + P_{diff} \quad (1)$$

where $V_{0,cell}$ is the initial uptake rate of the ligand, S is the concentration of the substrate, K_m is the Michaelis–Menten constant, V_{max} is the maximum uptake velocity, and P_{diff} is the non-saturable uptake clearance. According to Equation (2), $V_{0,cell}/S$ was converted to the permeability-surface area product *in vivo,* PS (mL/min/250 g rat).

$$PS = [V_{0,cell}/S] \cdot (\alpha/\beta) \cdot \gamma \quad (2)$$

where $\alpha = 1.25 \times 10^8$ cells/g liver (15), $\beta = 9.9 \times 10^5$ cells/mg protein (average of 6 preparations), and $\gamma = 11$ g liver/250 g rat (16).

To evaluate the effect of PVA metabolites on the uptake of L-carnitine, 400 μM PVA was added to the incubation mixture and the preincubation time was 10 min.

Renal Clearance of PCN

The renal clearance of PCN was determined to clarify the mechanism responsible for the enhanced carnitine consumption during PVA administration. PCN was infused into the femoral vein at a constant rate. Urine was collected over the time period 100–160 minutes, and the PCN concentration was determined. The plasma concentration was determined at the mid-point, or 130 minutes after the start of infusion. Renal clearance was calculated by dividing the urinary excretion rate by the plasma concentration.

High-Performance Liquid Chromatography (HPLC) Analysis of Carnitine and PCN

The HPLC method to determine carnitine and acylcarnitines in human plasma developed by Longo *et al.* (17) was applied to the assay of carnitine and PCN in rat plasma, urine and tissues. Briefly, sample plasma or 10% tissue homogenate was extracted with acetone/ethanol (3:1), which was then evaporated to dryness and the residue dissolved in phosphate

buffer (pH 3.5). Urine was diluted with phosphate buffer. Samples were derivatized with 1-AA in the presence of EDC as a catalyst. Excess reagent was removed by diethylether and chloroform. The derivatives were analyzed by HPLC with fluorescence detection (Ex. 248 nm and Em. 418 nm). An L-column C18 (4.6 mmϕ × 150 mm, Chemicals Inspection and Testing Institute, Tokyo, Japan) analytical column was used. For the assay of carnitine, the mobile phase composition was acetonitrile:0.15% phosphoric acid (24:76) for the first 11 minutes to elute carnitine. Then, the acetonitrile content was increased to 30% over a 4-minute period, and the composition was maintained from 15 to 35 minutes to elute isobutyrylcarnitine used as an internal standard. For the assay of PCN, mobile phase consisting of acetonitrile and 0.5% phosphoric acid (33:67) was used. Cyclopropylcarnitine was used as an internal standard.

Statistics

All data are given as the mean ± SE. Statistical significance was examined by Student's *t*-test at $P < 0.05$.

RESULTS

During treatment with PVA, 15–19% of the daily dose was excreted in urine as PCN. Although carryover from the last dose was still detected in the urine on the first day after the cessation of treatment, PCN disappeared from urine rapidly (Fig. 1). The plasma and tissue concentrations of carnitine during and after treatment are shown in Table I and Figure 2. The plasma concentration was reduced to 52.5% of the initial value on day 2, without any further reduction during the treatment period. The carnitine concentration in the kidney was reduced in parallel with that in plasma, whereas the concentrations in liver and heart decreased slowly. The lowest concentrations in liver (73.4%) and in heart (77.8%) were observed on day 3 and day 5, respectively. The concentration in muscle remained constant throughout the study period. After the end of treatment, carnitine concentrations returned to the initial levels. As shown in Figure 3, PCN was detected in plasma, kidney, liver, and heart during treatment. The highest concentration was observed in the kidney whereas the concentration in muscle was negligible.

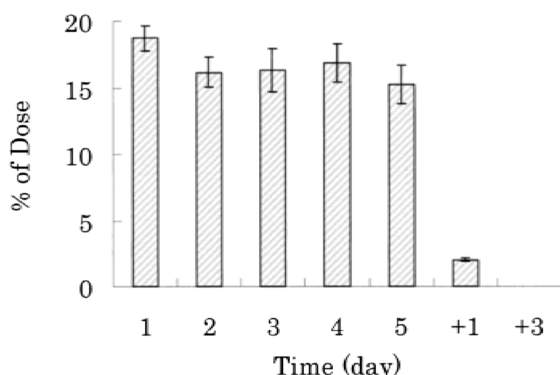

Fig. 1. Daily urinary excretion of PCN in rats treated with PVA for 5 days. Three rats in each group received 8 mg/kg PVA orally twice a day (daily dose was 16 mg/kg) for 5 days. The urinary excretion during PVA administration (from day 1 to day 5) and after the end of treatment (days +1 and +3) was determined. Values are the amount of PCN excreted in urine relative to the daily dose of PVA.

Table I. Plasma and Tissue Free Carnitine Levels Before and After Treatment with Pivalic Acid

	Day 0[a] (μM)	Day 5[b] (μM)	Day 5/Day 0 (%)
Plasma	55.2 ± 2.3	24.0 ± 2.6[c]	43.5
Kidney	637 ± 47	293 ± 22[c]	46.0
Liver	369 ± 17	271 ± 38	73.4
Heart	1180 ± 15	979 ± 114	83.0
Muscle	930 ± 39	878 ± 13	94.4

[a] Untreated rats (n = 3).
[b] Day 5 of treatment with 8 mg/kg pivalic acid twice a day (16 mg/kg/day) (n = 3).
[c] Significantly different from day 0 ($P < 0.05$).

Figure 4 shows the integration plot of L-^{3}H-carnitine uptake into kidney, liver, heart, and muscle in control and PVA-treated rats. The CL_{uptake} calculated from the integration plot is shown in Table II. The CL_{uptake} in liver and muscle increased 2-fold and 6-fold, respectively, whereas the values in kidneys and heart did not change in the PVA-treated rats.

The uptake of L-carnitine into hepatocytes was linear over 10 minutes. The radioactivity in the cells at 7 minutes was used to determine the uptake clearance. Figure 5 demonstrates that the uptake was saturable with a V_{max} and K_m of 25.0 ± 7.8 pmol/min/mg protein and 53.9 ± 14.3 μM (n = 3), respectively. Uptake was enhanced in the hepatocytes prepared from rats treated with PVA. The V_{max} increased significantly, whereas the K_m did not change. The PS values in control and PVA-treated rats were 0.33 ± 0.05 ml/min/rat and 0.80 ± 0.04 ml/min/rat, respectively. The uptake clearance of ^{3}H-L-carnitine at 15 μM in the presence and absence of 400 μM were 0.406 ± 0.027 μl/min/mg protein and 0.379 ± 0.036 μl/min/mg protein (n = 3), respectively.

Table III gives the renal excretion parameters of PCN at a steady-state plasma concentration of PCN. The preliminary experiment, in which the plasma concentration of PCN was determined every 30 minutes for 2 hours, showed that a steady state was achieved after constant infusion of PVA for 1.5 hours. During the study period, 86.9 ± 6.5% of the dose

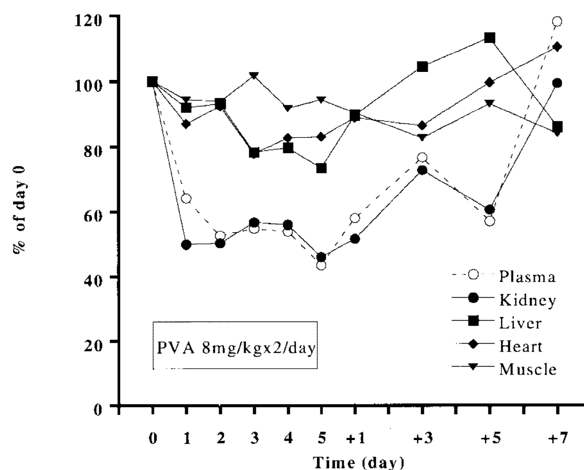

Fig. 2. Effect of PVA treatment on the plasma and tissue concentrations of free carnitine. Three rats in each group received 8 mg/kg PVA orally twice a day (daily dose was 16 mg/kg). Concentrations in each tissue on days 0 and 5 are shown in Table I. Data represent the mean concentrations relative to the initial values.

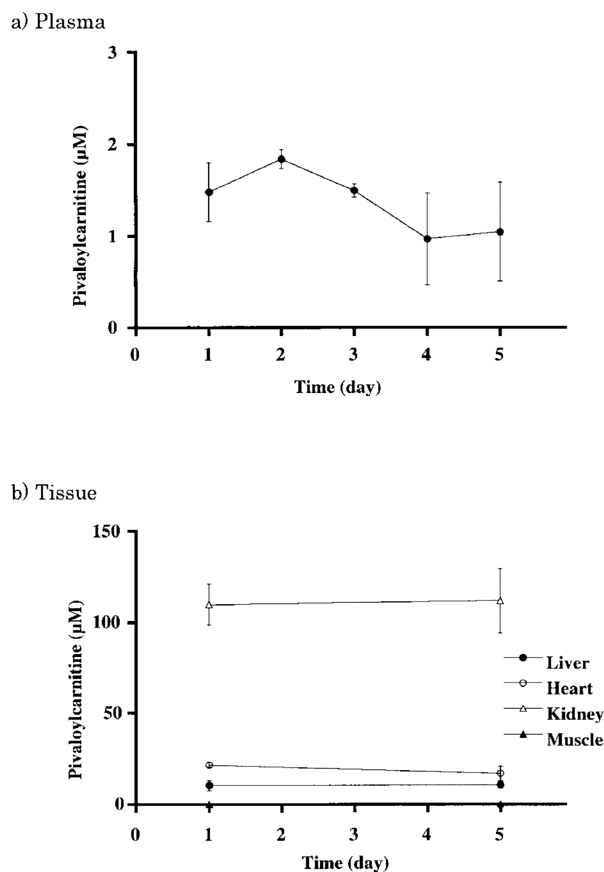

Fig. 3. Plasma (a) and tissue (b) concentrations of PCN in rats treated with PVA for 5 days. Three rats in each group received 8 mg/kg PVA orally twice a day (daily dose was 16 mg/kg).

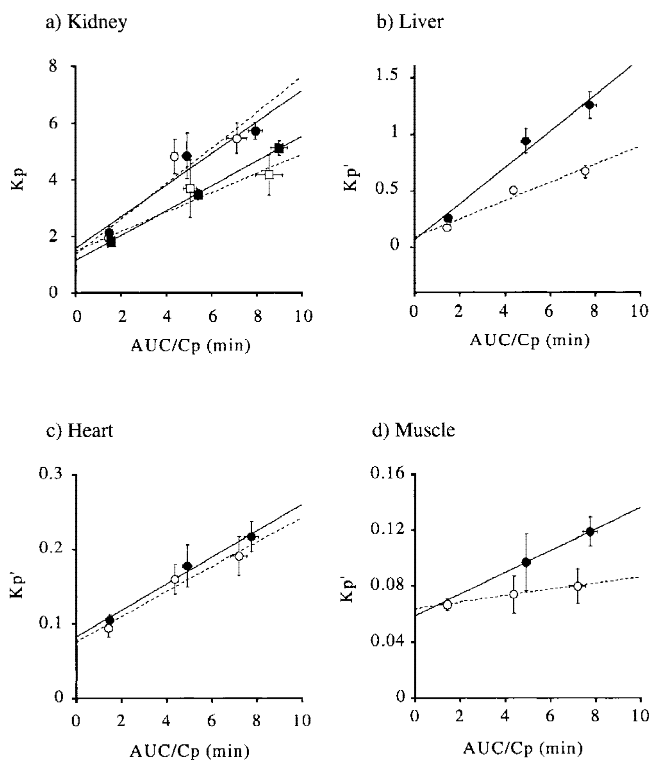

Fig. 4. Integration plot of L-carnitine. L-^{3}H-carnitine and ^{14}C-inulin were administered intravenously to rats. At designated times, plasma and tissue concentrations of radioactivity were determined. Tissue to plasma concentration ratios at 1, 3, and 5 minutes, corrected for distribution in the inulin space (K_p'), were plotted against AUC/C_p at the respective times. The slope of the regression line represents the CL_{uptake} to each tissue. ----○----, K_p or K_p' of L-carnitine in control rats; —●—, K_p or K_p' of L-carnitine in PVA-treated rats; ----□----, K_p of inulin in control rats; —■—, K_p of inulin in PVA-treated rats. Each point represents mean ± SE of 3–5 rats.

was recovered in the urine. The renal clearance of PCN was 696 ± 113 ml/hr/kg (n = 3).

DISCUSSION

Carnitine plays a role not only in fatty acid oxidation but also in eliminating xenobiotic acids from the body by forming conjugates. PVA is one of those compounds that conjugate with carnitine to form PCN and are then eliminated in the urine. It has been reported that long-term administration of PVA-containing prodrugs, such as pivampicillin and pivmecillinam, produces carnitine deficiency (5,7). In some patients with reduced serum carnitine concentrations, hypoketotic hypoglycemia in the fasting state was observed, suggesting the impairment of fatty acid utilization. In this study, we administered 8 mg/kg of pivalic acid orally to rats twice a day for 5 days. The dose level was lower than the no-effect dose determined in the 28-day subacute toxicity study in rats (our unpublished data). During this experimental period, 15–19% of administered PVA was excreted in urine as PCN. The urinary excretion parameters for PCN are shown in Table 3. In contrast to free carnitine, which is conserved by renal tubular reabsorption (18), the renal clearance of PCN was greater than the glomerular filtration rate (GFR) (492 ml/hr/kg, our unpublished data), suggesting that PCN is not reabsorbed as effectively as free carnitine. Thus, conjugation with pivalic acid to form PCN is considered to enhance carnitine consumption. The excess loss of carnitine excreted as PCN was calculated to be 23.5–31.3 μmol/day/kg, which was several times higher than the normal daily carnitine excretion (1–3 μmol/day/rat) (19,20). In our previous study in dogs, the bioavailability of PVA was 88.3%, suggesting that oral absorption was almost complete (21). In rats, after oral administration, PVA was recovered in urine mainly as the glucuronide (our unpublished data). Although we did not determine the other metabolites in urine in this study, PVA is considered to be absorbed almost completely and the fraction which was

Table II. Tissue Uptake Clearance of ^{3}H-L-Carnitine in Control and PVA-Treated Rats

Tissue	Weight (g/250 g rat)[a]	CL_{uptake} (mL/min/rat)[b]	
		Control	PVA treated
Kidney	2.0	1.26 ± 0.44	1.12 ± 0.28
Liver	11.0	0.89 ± 0.19	1.76 ± 0.26
Heart	1.0	0.017 ± 0.003	0.018 ± 0.002
Muscle	125	0.25 ± 0.01	1.50 ± 0.25
Kidney(^{14}C-inulin)	2.0	0.69 ± 0.24	0.88 ± 0.02

[a] Reference 16.
[b] Mean ± calculated SE (calculated from the integration plot shown in Figure 4).

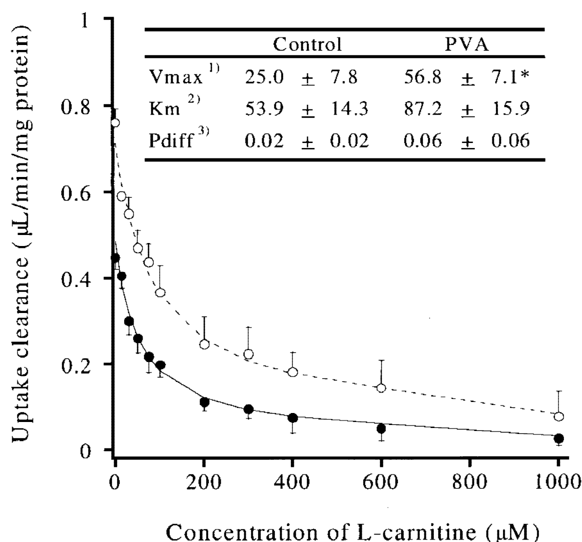

Fig. 5. Uptake of L-carnitine into isolated rat hepatocytes as a function of concentration. Hepatocytes were prepared from control and PVA-treated rats and the initial uptake of L-carnitine was determined at pH 7.4 and 37 °C. Final concentration of L-^{3}H-carnitine 10 nM. L-carnitine concentration ranged from 10 nM to 2.5 mM. —●—, control rats; —○—, PVA-treated rats. 1) Maximum uptake velocity (pmol/min/mg protein); 2) Michaelis–Menten constant (μM); 3) nonsaturable uptake clearance (μL/min/mg protein). * Significantly different from untreated rats ($P < 0.05$).

not recovered as PCN was excreted mainly as the glucuronide.

As a result of enhanced excretion, the carnitine concentration in plasma and some tissues decreased during treatment with PVA. An HPLC method involving pre-column derivatization with 1-AA developed by Longo *et al.* (17) was used successfully to assay carnitine in rat plasma and tissue and PCN in plasma, tissue, and urine. The concentration of carnitine in urine could not be assayed by this method because of unresolved peaks arising from endogenous compounds. The concentrations of carnitine in plasma and tissues before PVA treatment shown in Table 1 were within the range of the reported values (19,20,22,23). The concentrations of PCN in urine determined by this method and those reported by Konishi and Hashimoto (12) agreed well (data not shown).

The plasma carnitine concentration was reduced to approximately 50% the initial value on day 1, without any further reduction during the treatment period. In rats receiving a diet supplemented with varying amounts of carnitine or its precursor, ε-*N*-trimethyllysine, tissue carnitine concentrations were reported to correlate well with the serum level in the

Table III. Urinary Excretion of PCN in Rats[a]

Infusion rate (nmol/hr/kg)	Css[b] (μM)	Excretion[c] (nmol/hr/kg)	Urinary recovery (%)	CLr[d] (ml/hr/kg)
3038 ± 49	3.94 ± 0.49	2634 ± 155	86.9 ± 6.5	696 ± 113

[a] PCN was intravenously infused at a constant rate (n = 3).
[b] Steady-state plasma concentration of PCN.
[c] Urinary excretion rate of PCN.
[d] Renal clearance of PCN.

physiologically normal range (26–69 μM) (23). However, in this study, the carnitine level in tissues other than kidney did not follow that in plasma. Therefore, the plasma carnitine level is not a good predictor of the tissue levels when rats are treated with PVA.

To explain why the carnitine level was maintained relatively constant despite the reduced plasma concentration, we determined the CL_{uptake} of L-^{3}H-carnitine in control and PVA-treated rats. Of the four tissues studied, carnitine is synthesized only in liver. The turnover times before the PVA treatment, calculated as the amount in the tissues divided by the tissue uptake rate ($CL_{uptake} \times C_p$), were 29 minutes, 83 minutes, 39 hours, and 76 hours for kidneys, liver, heart, and muscle, respectively. These values were comparable with those reported by Brooks and Mcintosh (19). The turnover time in liver was possibly overestimated because the rate of biosynthesis was not taken into account.

Kidney

The CL_{uptake} of L-^{3}H-carnitine did not change after treatment with PVA and it was relatively similar to that of ^{14}C-inulin. Because carnitine is highly conserved in the kidney by an active reabsorption mechanism at the renal tubules (18), it is reasonable to assume that glomerular filtration is the rate-limiting step for L-^{3}H-carnitine uptake into the kidney. The CL_{uptake} of ^{14}C-inulin did not change, thus the GFR, as well as the CL_{uptake} of L-^{3}H-carnitine, did not change. As a result, the tissue uptake rate decreased in proportion to the carnitine concentration in plasma and that in kidneys, where the turnover of carnitine is rapid, decreased and quickly reached a new equilibrium.

Liver

The liver carnitine concentration was possibly kept relatively constant by two mechanisms: an increase in CL_{uptake} and stimulation of biosynthesis. Because the CL_{uptake} of liver increased 2-fold in PVA-treated rats, the uptake rate hardly changed even after the plasma concentration decreased to less than 50% the initial value. After the end of PVA treatment, the carnitine concentration in the liver increased gradually to exceed the initial value by 10%, suggesting stimulated biosynthesis.

Heart

The carnitine concentration in heart did not change significantly throughout the experimental period. The CL_{uptake} did not change and so the profile of the carnitine levels during PVA treatment could not be explained by this experiment. The slow turnover time may help maintain the tissue carnitine level during the limited period of PVA treatment.

Muscle

The carnitine concentration in muscle did not change significantly throughout the study period. The tissue uptake rate did not fall owing to the increased CL_{uptake}. In addition to this, the slow turnover time was considered to be attributable to this profile. Diep *et al.* reported carnitine depletion during the administration of pivampicillin for 24 days or 36 days to rats (22). The profiles of the carnitine level were

similar to our data for the first 6 days except that the concentration in kidney remained constant, probably because their value included PCN and ours did not. However, the longer period of treatment resulted in a further decrease in the total carnitine in liver and muscle. Although that in heart appeared to remain constant during treatment, it was reduced compared with that in untreated rats after a 24-day and a 36-day treatment. Bianchi and Davis also showed that in rats treated with PVA for 8 weeks, the tissue total carnitine levels were reduced at all time points tested compared with those in animals treated with sodium bicarbonate as a control, except at 4 days for heart and 4 days and 2 weeks for liver (20). In our experiment, the tissue concentrations appeared to reach steady state by the 5th day of the PVA administration, although longer treatment might have induced a further reduction.

Various tissues accumulate L-carnitine by a saturable uptake process with K_m value comparable to the physiological plasma concentration. Recently, OCTN2 was shown to be a carnitine transporter, which is expressed in tissue including kidney, muscle, heart, and liver (3,24). The increase in CL_{uptake} was partly explained by saturation kinetics. However, CL_{uptake} in the PVA treated rats was higher than that predicted using the reported K_m values for L-carnitine transport in rats (14.8 μM–60 μM) (25–28), especially in muscle. Thus, to study if the kinetic parameters may change in the PVA-treated rats, we determined the L-carnitine transport into hepatocytes. As shown in Figure 5, carnitine uptake into rat hepatocytes was saturable and the K_m value was 53.9 μM in control rats, which was comparable with the physiological plasma concentration of L-carnitine and the reported K_m values for the uptake of L-carnitine into rat heart and muscle (24 μM and 60 μM, respectively) (27,28), but was slightly higher than that measured in rat OCTN2-expressing HRPE (human retinal pigment epithelial) cells (14.8 μM) (26). Repeated doses of PVA enhanced L-carnitine uptake. The V_{max} increased 2-fold without any significant change in K_m, which suggests up-regulation of L-carnitine transporters. The PS values calculated from the kinetic parameters were 0.33 and 0.80 ml/min/rat in the control and PVA-treated rats, respectively, and much lower than the hepatic plasma flow. These values agreed with the CL_{uptake} determined by the integration plot analysis *in vivo,* suggesting that the cellular uptake process mediated by the carnitine transporter is the rate-limiting step for tissue uptake. Thus, the up-regulation of carnitine uptake observed in the *in vitro* study explains the increased CL_{uptake} *in vivo.*

The up-regulation of L-carnitine transport was not explained by the presence of PVA and its metabolites. Preincubation of hepatocytes with 400 μM PVA, at a concentration comparable with the peak concentration of free PVA after oral administration of 20 mg/kg (our unpublished data), did not affect the uptake clearance of L-carnitine. Furthermore, the PCN level, which was highest in the kidneys, a major eliminating organ, followed by heart, an organ reported to be one of the major sites of formation of PCN (29,30), and liver, did not correlate with the increase in CL_{uptake}.

In conclusion, the plasma concentration of carnitine decreased due to enhanced carnitine elimination as PCN when rats were treated with PVA. However, within the study period for 5 days, the plasma concentration is not a good predictor of the tissue concentration of carnitine except for the kidney. The carnitine concentrations in liver, muscle and heart were relatively constant during the study period. The increased CL_{uptake} and unchanged carnitine uptake rate from plasma, even at low plasma concentrations, helped maintain the carnitine level in liver and muscle. Up-regulation of carnitine transporters is suggested to be the mechanism for the increased CL_{uptake}. The concentration in heart did not change during the treatment period, while the CL_{uptake} did not change and the carnitine supply from plasma appeared to decrease.

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