0022-3565/99/2891-0093$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 289, No. 1
Printed in U.S.A.

JPET 289:93–102, 1999

Pharmacokinetic Analysis of the Cardioprotective Effect of 3-(2,2,2-Trimethylhydrazinium) Propionate in Mice: Inhibition of Carnitine Transport in Kidney[1]

MASAMICHI KUWAJIMA, HIDEYOSHI HARASHIMA, MIYUKI HAYASHI, SAORI ISE, MASAKO SEI, KANG-MO LU, HIROSHI KIWADA, YUICHI SUGIYAMA, AND KENJI SHIMA

Department of Laboratory Medicine, School of Medicine (M.K., M.H., M.S., K.L., K.S.) and Faculty of Pharmaceutical Sciences (H.H., S.I., H.K.), The University of Tokushima, Japan; and Faculty of Pharmaceutical Sciences, University of Tokyo, Japan, (Y.S.)

Accepted for publication October 28, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

The site of action of 3-(2,2,2-trimethylhydrazinium) propionate (THP), a new cardioprotective agent, was investigated in mice and rats. I.p. administration of THP decreased the concentrations of free carnitine and long-chain acylcarnitine in heart tissue. In isolated myocytes, THP inhibited free carnitine transport with a K_i of 1340 μM, which is considerably higher than the observed serum concentration of THP. The major cause of the decreased free carnitine concentration in heart was found to be the decreased serum concentration of free carnitine that resulted from the increased renal clearance of carnitine by THP. The estimated K_i of THP for inhibiting the reabsorption of free carnitine in kidneys was 52.2 μM, which is consistent with the serum THP concentration range. No inhibition of THP on the carnitine palmitoyltransferase activity in isolated mitochondrial fractions was observed. These results indicate that the principal site of action of THP as a cardioprotective agent is the carnitine transport carrier in the kidney, but not the carrier in the heart.

It is well known that certain fatty acids and their metabolites cause injury to cardiac muscle cells (Corr et al., 1984). In particular, long-chain acylcarnitine inhibits the Na^+-K^+ ATPase of sarcolemma and the Ca^{++} ATPase of the endoplasmic reticulum, and diminishes the contractility of cardiac muscle (Adams et al., 1979; Dhalla et al., 1992). Hence, the development of drugs for ischemic cardiopathy that suppress the synthesis of long-chain acylcarnitine is in progress (Lopaschuk et al., 1988; Dhalla et al., 1992; Anderson et al., 1995).

3-(2,2,2-trimethylhydrazinium)propionate (THP, MET-88) represents one such drug, which was synthesized by Institute of Organic Synthesis (Riga, Latvia). The cardioprotective effect of THP is well established (Dhar et al., 1996; Kirimoto et al., 1996; Aoyagi et al., 1997; Akahira et al., 1997), and it is in clinical use in Latvia and Russia (Sahartova et al., 1993). In examining the major pharmacological effect of THP, it is important to note that intramyocardial free carnitine and long-chain acylcarnitine levels are decreased when THP is administered to rats and guinea pigs (Simkhovich et al., 1988; Dhar et al., 1996). However, the mechanism by which this reduction is accomplished has not been clarified and, as a result, the site of action of THP remains unknown.

THP is structurally similar to carnitine (Fig. 1). It is, therefore, possible that THP inhibits the transport of free carnitine into the myocyte through the cell membrane. Because free carnitine is a substrate of carnitine palmitoyltransferase (CPT), it is also possible that THP competes with free carnitine at the catalytic site of CPT in cardiac muscle.

To test these hypotheses, the following experiments were performed: 1) THP was administered to mice, and free carnitine and long-chain acylcarnitine concentrations in the blood, heart, and urine were measured and the effect of THP on free carnitine transport to the heart and on free carnitine reabsorption by the kidney were analyzed, 2) myocytes and fibroblasts were prepared and the inhibitory effect of THP on free carnitine transport in these cells was examined, and 3) mitochondria were prepared from heart, and the inhibition of CPT-I and CPT-II activities by THP was examined.

Materials and Methods

Reagents. THP was a gift from Taiho Pharmaceutical Company (Tokushima, Japan). Basal Medium Eagle (BME), Trypsin-EDTA

Received for publication June 30, 1998.
[1] This work was partly supported by grants-in-aid from the Ministry of Education, Science, and Culture of Japan.

ABBREVIATIONS: THP, 3-(2,2,2-trimethylhydrazinium)propionate; CPT, carnitine palmitoyltransferase; BME, Basal Medium Eagle; fp, unbound fraction of free carnitine; K_p, heart to serum concentration ratio; C_{THP}, concentration of THP in serum or medium; C_{car}, concentration of free carnitine in serum or medium; GFR, glomerular filtration rate; CL_r, renal clearance of free carnitine; FBS, fetal bovine serum.

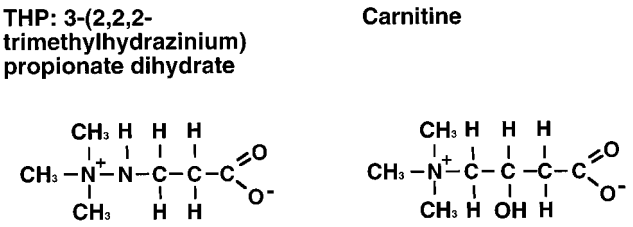

Fig. 1. Structures of THP and carnitine. Left, THP; right, carnitine.

and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY). Dulbecco's minimum essential medium obtained from Handai-Biken (Osaka, Japan). L-[^{3}H]carnitine and [1-^{14}C]acetyl-coenzyme A were obtained from Amersham (Amersham, UK). Unlabeled L-carnitine and RPMI-1640 were obtained from Sigma (Milwaukee, WI). Plastic dishes were obtained from Sumitomo (Tokyo, Japan). Triton X-100 and Clearsol I were obtained from Nakarai (Kyoto, Japan). The protein assay kit was purchased from Pierce (Rockford, IL). The Ultrafree-MC Centrifugal Filter Unit was purchased from Millipore (Bedford, MA). The carnitine assay kit was obtained from Kainos (Tokyo, Japan). A stomach tube was obtained from Natume Seisakusho (Osaka, Japan).

Animals. Age-matched (8–9 weeks old) male C57BL/6J mice (n = 283) weighing 24 to 29 g (Nihon Crea, Japan) were used throughout the study. One- to two-day old baby rats (n = 80) born from 10-week-old Sprague-Dawley rats and weighing 190 to 230 g from SLC (Hamamatsu, Japan) were only used for the preparation of myocytes. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals, and were monitored by the Institutional Animal Care and Use Committee of the University of Tokushima.

Administration of THP. THP was dissolved in water, and 0.5-ml aliquots were orally administered via a stomach tube to each fed mouse under ether anesthesia at 10:00 AM each day. The doses of THP used were set at 0, 100, 200, and 300 mg/kg based on the reported values (Simkhovich et al., 1988; Dhar et al., 1996; Kirimoto et al., 1996).

Collection of Blood and Urine, and Extraction of Heart. Before the administration of THP, and at 1, 2, 4, and 5 days after administration, 0.05 ml of blood was collected by repeated tail cutting at 9:00 to 10:00 AM. Serum was separated and used for the assay for free carnitine and acylcarnitine.

Heart was sampled before the administration of THP, and 1, 2, and 5 days after administration. The heart was removed after an injection of sodium pentobarbital (0.05 mg/g i.p.) and immediately frozen in liquid nitrogen and stored. Free carnitine and acylcarnitine in the heart were measured. Excreted urine was collected in the periods between before administration and 24 h after administration, between 24 and 48 h after administration, and between 96 and 120 h after administration. Each urine sample was collected using a special metabolic cage for mice. Urine was passed through the mesh and collected at the bottom. Both the mesh and the bottom were washed throughout with distilled water and the volume was adjusted to 50 ml. The concentrations of free carnitine and acylcarnitine in each sample were measured.

Measurement of Carnitine. Free carnitine was determined by radioisotopic assay (McGarry and Foster, 1985) or spectrophotometric assay by an enzymatic cycling reaction using a carnitine assay kit (Takahashi et al., 1994). Acylcarnitine in serum and urine was determined as free carnitine after pretreatment with alkali and subsequent neutralization. Short-chain acylcarnitine in a perchloric acid extract and long-chain acylcarnitine in the acid-insoluble fraction from heart were also determined as free carnitine after the same pretreatment.

Effect of THP on CPT Activity. After an injection of sodium pentobarbital (0.05 mg/g i.p.), the heart was removed and mitochondria were prepared from this tissue by the method of McGarry et al. (1983). The mitochondrial fraction obtained was dissolved in a solution containing 5 mM Tris-Cl (pH 7.4) and 150 mM KCl (Sample I). Sample II was prepared by treating Sample I with 0.37% Triton X-100 (Woeltje et al., 1987).

CPT-I activity was measured according to Woeltje et al. (1987). The reaction was started by the addition of Sample I (0.2–0.3 mg protein). The effect of THP on CPT-I activity was examined at final concentrations of 60 μM and 3 mM. CPT-II activity was similarly measured using Sample II (0.4–0.5 mg protein). The effect of THP on CPT-II activity was examined in a similar manner.

Measurement of Free Carnitine Transport Activity in Myocytes and Fibroblasts. Myocytes were prepared by the method of Kaneko and Goshima (1982). In brief, the heart was excised from one- or two-day old Sprague-Dawley rats, minced in BME, and washed twice with PBS (−). The minced tissue was suspended in 0.125% trypsin-EDTA and incubated at 37°C for 15 min. Desegregated cells in the upper layer were collected at the end of three incubation periods.

The cells were resuspended in BME supplemented with 10% FBS and then centrifuged at 600g for 5 min. Pelleted cells were then seeded onto a glass dish containing BME supplemented with 10% FBS, and incubated in humidified 5% CO_2/95% air at 37°C. After 1 h, the undisturbed cells were collected. This procedure was repeated three times. The final untouched cells were plated onto 9.5 cm^2 plastic dishes containing BME supplemented with 10% FBS. The dishes were incubated for 24 h before use.

Fibroblasts were prepared from C57BL/6J mice and free carnitine transport activity was assayed according to Kuwajima et al. (1996). The volume of the incubation medium was 1 ml. The effect of THP was analyzed at final concentrations of 156 μM, 1560 μM, and 3000 μM.

Ultrafiltration. A 0.3 ml aliquot of collected serum was placed on an Ultrafree MC Centrifugal Filter Unit and centrifuged at 3000g for 15 min at 30°C. The carnitine concentration in the solution recovered in the lower chamber (B) and that before filtration (A) were measured by radioisotopic assay, and the unbound fraction (fp) in serum, B/A × 100 (%), was obtained.

Measurement of Blood THP Concentration. On the days of the first, second, and fifth administration, blood was collected from the abdominal aorta at 1, 2, 3, 6, 12, and 24 h after administration of THP after an injection of sodium pentobarbital (0.05 mg/g i.p.). Blood was obtained from one mouse, one time. Serum was separated and 0.15 ml of serum from each of three animals was pooled. THP concentration was measured using methods described by Sahartova et al. (1993).

Kinetic Analysis of Free Carnitine Transport in Heart and Kidney. In the in vivo condition, free carnitine transport in heart was analyzed kinetically by assuming a carrier-mediated transport for the uptake of free carnitine and a passive diffusion for the efflux as shown in Fig. 2A. Competitive inhibition was also assumed for THP based on our preliminary experiments in fibroblasts (data not shown) and the following equation was derived for the kinetic analysis of heart to serum concentration ratio (K_p) of free carnitine by assuming steady-state conditions.

$$K_p = V_{max}/k/[K_m(1+C_{THP}/K_i)+C_{car}] \quad (1)$$

where the V_{max} and K_m represent the maximum transport rate and Michaelis-Menten constant for free carnitine by carrier-mediated transport. K_i represents the inhibition constant by THP and k represents the efflux rate constant. C_{THP} and C_{car} represent the serum concentration of THP and free carnitine.

In myocytes, the inhibition constant of THP for free carnitine transport was also estimated by assuming competitive inhibition by THP based on the following equation:

$$Rate\ of\ uptake = V_{max}\cdot C_{car}/[K_m(1+C_{THP}/K_i)+C_{car}] \quad (2)$$

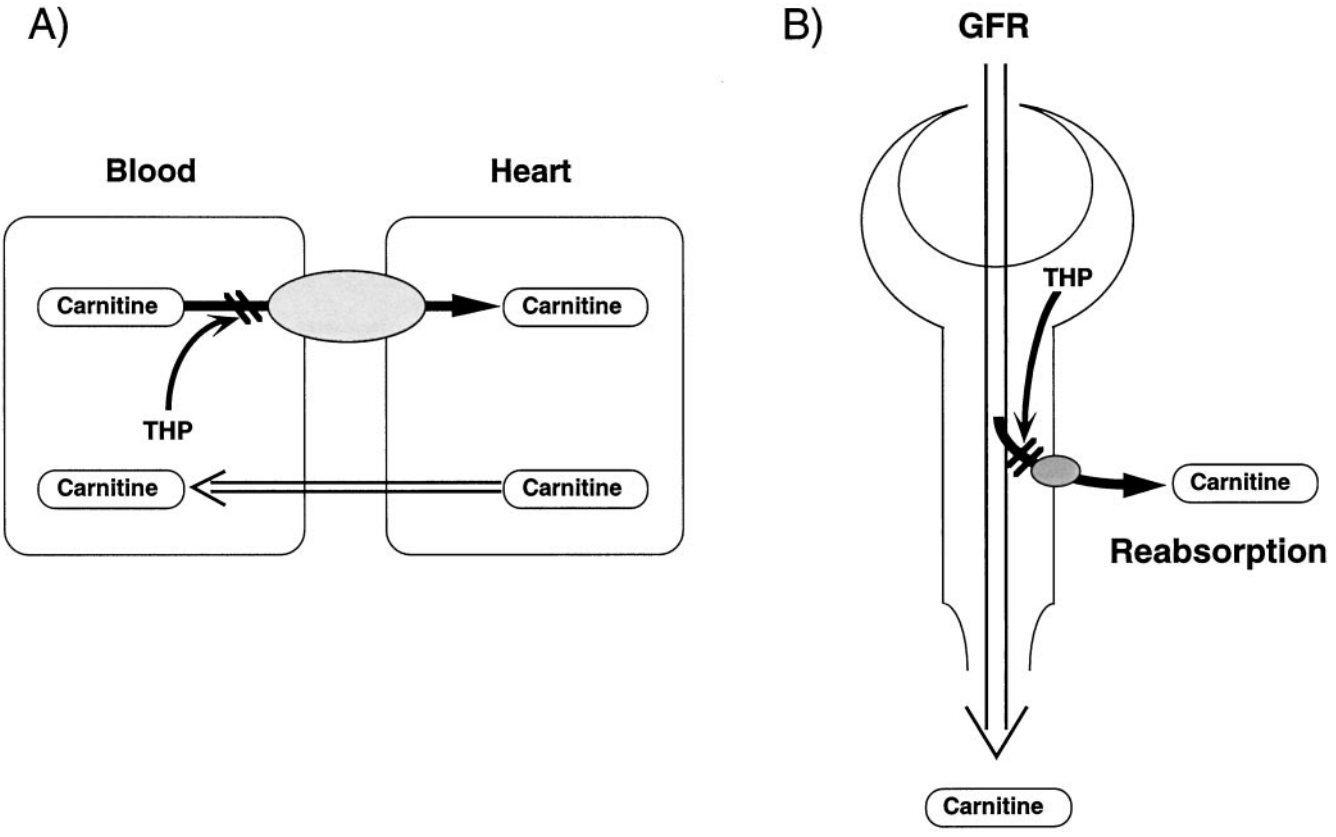

Fig. 2. Kinetic models for the transport of free carnitine and its inhibition by THP. A, distribution to heart. Carrier-mediated transport and passive diffusion were assumed for influx and efflux of free carnitine in heart. Competitive inhibition by THP was assumed. B, reabsorption of free carnitine in kidney. Carrier-mediated transport was assumed for the reabsorption of free carnitine in the apical membrane of proximal tubular cells.

Carnitine transport during renal reabsorption was analyzed kinetically, assuming no renal secretion, as shown in Fig. 2B. Carnitine was filtered with glomerular filtration rate (GFR) and reabsorbed by the carrier-mediated transport system on the apical membrane in proximal tubular cells without renal secretion. The inhibitory effect of THP on the renal clearance of free carnitine (CL_r) can be analyzed according to the following equation:

$$CL_r = \mathrm{GFR} \cdot \mathrm{fp} - V_{max}/[K_m(1 + C_{THP}/K_i) + C_{car}] \quad (3)$$

C_{car} and C_{THP} represent the averaged serum concentration of free carnitine and THP, respectively. Values for V_{max}, K_m, and K_i were simultaneously obtained by the nonlinear least square's method (MULTI) using C_{car} and C_{THP} as independent variables.

Statistical Analysis. Data were analyzed statistically by a two-way ANOVA (dose and time) and a one-way ANOVA followed by posthoc Fisher's Protected least Significant Difference with the level of significance set at $p < .05$. Kinetic analyses were performed by the nonlinear least square's method MULTI (Yamaoka et al., 1981), and kinetic parameters such as K_m and V_{max} values were estimated.

Results

Effects of THP on Carnitine Concentration in Serum and Heart. Serum levels of free carnitine were decreased 1 day after the administration of THP at doses of 100, 200, and 300 mg/kg, respectively (Fig. 3). Five days after administration, serum free carnitine concentrations were further decreased. Based on the two-way ANOVA, no statistical difference with respect to the dose of THP was observed. However, a significant difference was observed with respect to time ($p < .05$). The acylcarnitine/free carnitine ratio in serum was 0.57 ± 0.20 before administration of THP, and no statistical difference with respect to either dose nor time was observed.

In heart, the level of free carnitine was decreased at 2 and 5 days after the administration of THP at a dose of 100, 200, and 300 mg/kg, respectively (Fig. 3). The levels of heart long-chain acylcarnitine were decreased 5 days after the administration of THP at a dose of 100, 200, and 300 mg/kg. The two-way ANOVA showed a significant difference with respect to time ($p < .05$), but not to the dose of THP for heart free carnitine. No significant difference with respect to either dose or time for heart long-chain acylcarnitine was observed.

The concentration of long-chain acylcarnitine in heart was plotted against free carnitine concentration in heart (Fig. 4). Long-chain acylcarnitine concentration in heart increased with an increase in free carnitine levels in heart. This correlation was statistically significant ($p < .0029$) and suggests that THP has no effect on the conversion of free carnitine to long-chain acylcarnitine in heart.

We then examined the effects of THP on the activities of CPT-I and CPT-II, using a mitochondrial fraction from mouse heart. As shown in Table 1, no effects of THP on these CPT activities were observed in the concentration range examined for free carnitine (100–1000 μM) and THP (60–3000 μM).

Transport of Free Carnitine from Serum to Heart. The K_p of free carnitine was then calculated based on the serum and heart concentration as shown in Fig. 5. This parameter reflects the extent of tissue distribution of carnitine. The K_p increased with decreasing levels of serum free

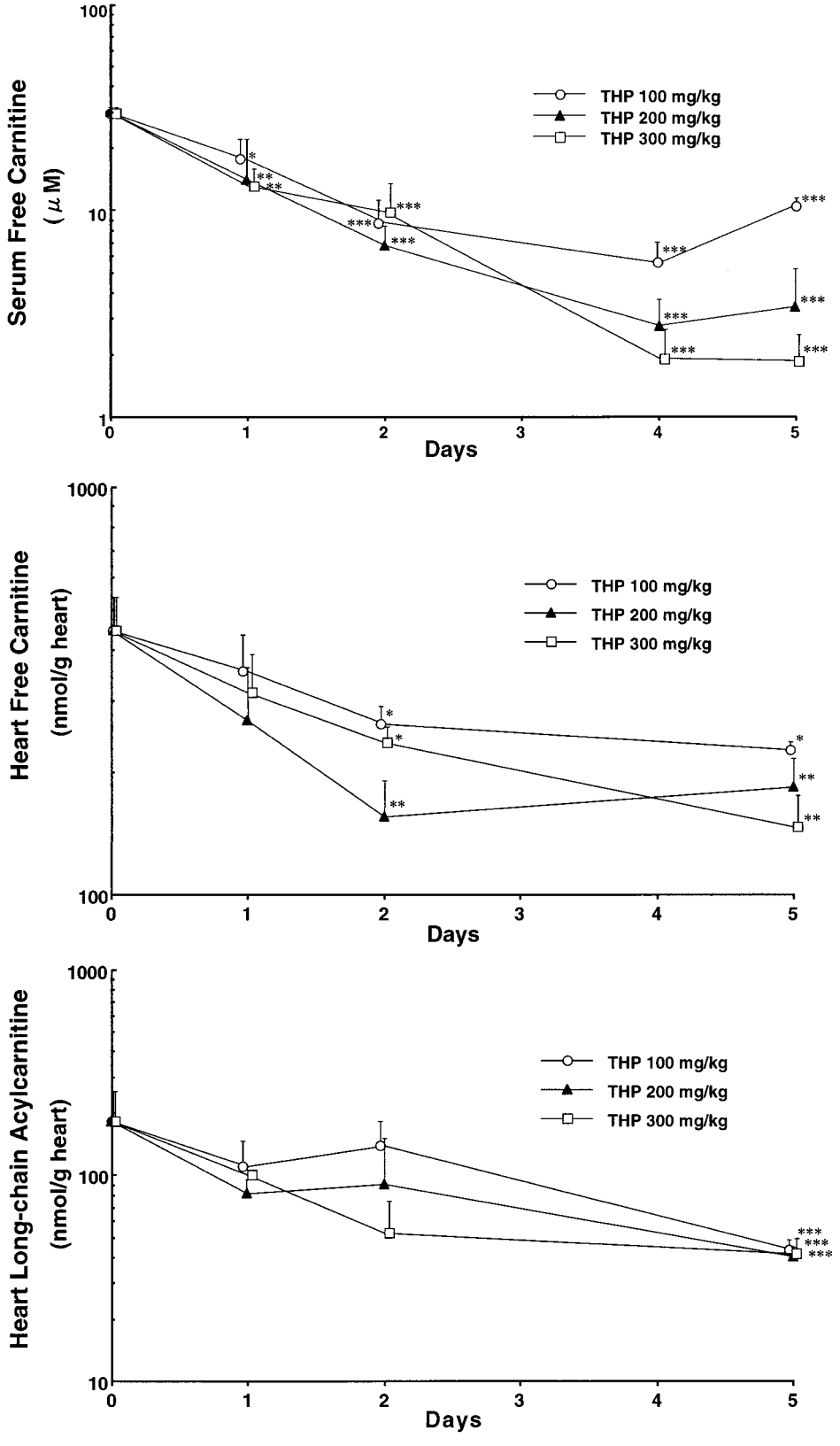

Fig. 3. Time courses for free carnitine or long-chain acylcarnitine in serum and heart after administration of THP. THP doses were 100 mg/kg b.wt. (○-○), 200 mg/kg b.wt. (▲-▲), or 300 mg/kg b.wt. (□-□). Each data point represents the mean ± S.E. (n = 12 at time zero, n = 4 at other times). *p < .05, **p < .01, ***p < .001 compared before and after administration of THP.

carnitine after the administration of THP, although THP would be expected to decrease the free carnitine transport from serum to heart.

The K_p values were analyzed by the model shown in Fig. 2A. The carrier-mediated uptake of free carnitine and the passive diffusion for efflux of free carnitine from heart to blood was assumed for purposes of this analysis. The K_m and V_{max}/k were obtained by the nonlinear least square's analysis and the obtained parameters are summarized in Table 2. Kinetic analysis revealed that the affinity of free carnitine to

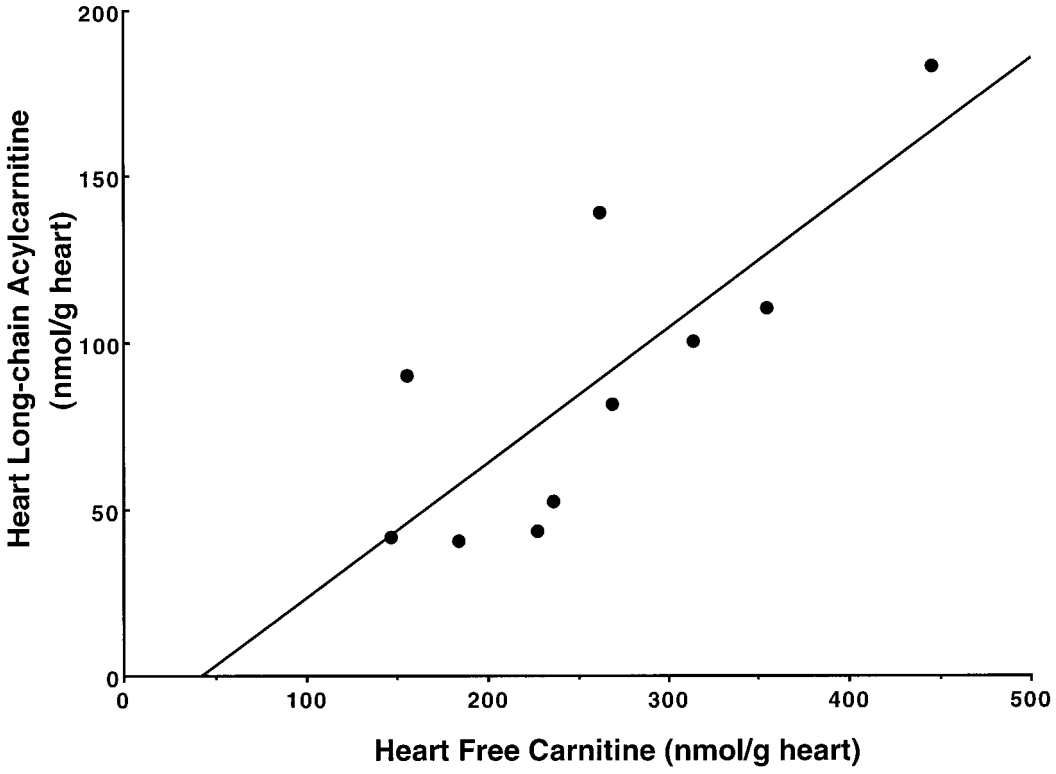

Fig. 4. Relationship between free carnitine and long-chain acylcarnitine in heart. Time courses for free carnitine and long-chain acylcarnitine concentration in heart were plotted. Each data point represents the mean value of Fig. 3. The solid line represents the regression line ($r = 0.809$, $p < .0029$).

TABLE 1
Effect of THP on the activities of CPT-I and CPT-II in mitochondria from mouse heart

	Carnitine	THP	
	μM	*μM*	
CPT-I		60	3000
	1000	99.4 ± 6.9	94.4 ± 3.0
	500	98.0 ± 4.6	102.3 ± 5.2

	Carnitine	THP	
	μM	*μM*	
CPT-II		60	3000
	1000	98.0 ± 2.9	109.1 ± 19.3
	500	97.1 ± 4.1	96.5 ± 2.2
	100	99.3 ± 5.7	97.6 ± 5.7

Activities of CPT-I and CPT-II were measured using the mitochondrial fraction and expressed as percentage of control. The values represent the mean and S.D. of four experiments. Preparation of mitochondrial fraction from mouse heart and assay methods of CPT are described in *Materials and Methods*.

transport carrier on the plasma membrane in heart is much higher than the affinity for THP.

Free carnitine transport characteristics were investigated using myocytes as a carnitine transport system in heart (Fig. 6). Transport activity showed a concentration dependence characterized by a K_m of 93.2 μM and V_{max} of 7.63 pmol/min/mg protein. This transport was inhibited by THP at the 3 mM level.

The estimated K_i for THP for free carnitine transport was 1340 μM (Table 2), which is much larger than the K_m for free carnitine (93.2 μM). These results are consistent with a concentration dependence of the K_p of free carnitine in heart, where K_p increased with decreasing serum free carnitine concentration, even though THP concentration was increasing. Free carnitine transport was also examined using a fibroblast cell line which is known to express the free carnitine transport carrier system (Stieger et al., 1995). The estimated K_m and V_{max} are also summarized in Table 2. The affinity of free carnitine in fibroblast (18.8 μM) is higher than that in myocytes (93.2 μM).

Effect of THP on the Renal Handling of Free Carnitine. These experimental results suggest that the long-chain acylcarnitine in heart, which is generally thought to be a key compound in determining the effect of THP, is not governed by the intracellular effect of THP, but, rather, by extracellular interaction with THP. As shown in Fig. 7, a strong correlation exists between serum free carnitine concentration and long-chain acylcarnitine concentration in heart ($p < .0023$). This relationship suggests that the alteration of serum free carnitine by THP may be a determining factor for the variation of long-chain acylcarnitine in heart.

Renal clearance of free carnitine is an important factor in determining the serum concentration of free carnitine. The relationship between serum free carnitine concentration and its CL_r is shown in Fig. 8. The CL_r was calculated from the urinary excreted free carnitine for 24 h divided by the corresponding area under the concentration-time curve of free carnitine. The acylcarnitine/free carnitine ratio in urine was 1.05 ± 0.51 before administration of THP, and no statistical difference with respect to either dose nor time was observed. The decreased serum free carnitine concentration is easily explained by the increased CL_r with increasing serum THP concentration as shown in Fig. 9. The CL_r increased with increasing THP, suggesting the inhibition of free carnitine reabsorption by THP.

In this analysis, the serum concentration of THP was calculated by measurement of area under the concentration-time curve of THP for 24 h before killing the animals divided by 24. The unbound fraction (free fraction) of free carnitine was measured using the ultrafiltration method; no significant binding of free carnitine to serum protein was detected.

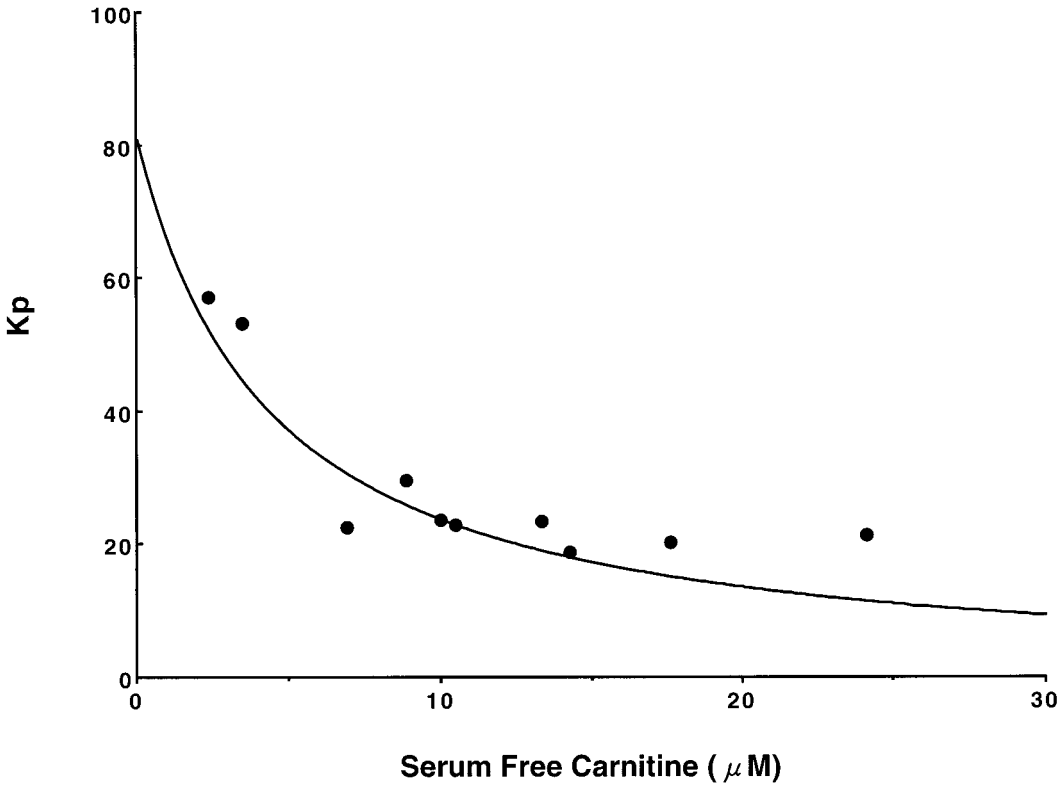

Fig. 5. Relationship between K_p and serum free carnitine. K_p represents the heart to serum concentration ratio of free carnitine based on the data shown in Fig. 3. The solid curve represents the simulation curve using equation (1) fixing the C_{THP} at 90.2 μM.

TABLE 2
Kinetic parameters for carnitine transport in heart and kidney

	K_m for Carnitine	V_{max} [a]	K_i of THP
	μM		μM
Heart	4.00 ± 3.26	350 ± 87.7[b]	1300 ± 6176
Myocyte	93.2 ± 26.2	7.63 ± 1.25[c]	1340 ± 410
Fibroblast	18.8 ± 2.6	6.66 ± 0.39[c]	50.4 ± 7.9
Kidney	1110 ± 580	14200 ± 5800[d]	52.2 ± 26.5

The K_m and V_{max} represent the Michaelis-Menten constant and the maximum rate of transport, respectively as explained in Fig. 2.
Competitive inhibition was also assumed for THP and K_i represents the inhibition constant.
[a] Unit of V_{max} depends on the experimental conditions.
[b] V_{max}/k according to equation (1).
[c] expressed as pmol/min/mg protein.
[d] expressed as nmol/h.

Therefore, the fp was fixed at 1.0 and GFR was fixed at 12 ml/kg (Rozen et al., 1983; Tipping et al., 1997). The kinetic parameters, obtained by the model assumed in Fig. 2B, are summarized in Table 2. As expected, the K_m (1110 μM) was much higher than the K_i (52.2 μM), which governs the alteration of CL_r after the administration of THP.

Discussion

It has been reported that THP decreases intramyocardial free carnitine and long-chain acylcarnitine levels when administered to rats and guinea pigs (Simkhovich et al., 1988; Dhar et al., 1996). We administered THP to mice and found a similar decrease in heart tissue.

Although γ-butyrobetaine, a carnitine-analog, is incorporated into cells (Christiansen and Bremer, 1976), THP, which is also a carnitine-analog, appears to be incorporated into cardiac muscle cells. As a result, it has been generally thought that the mechanism of decreased long-chain acylcarnitine involves the possible competitive inhibition of CPT by THP at its catalytic site (Bremer, 1983). The effect of THP on CPT was examined at 500 and 1000 μM, because heart free carnitine levels are 500 to 700 nmol/g in mouse heart (Kuwajima et al., 1998). The activities of CPT-I and -II were not inhibited by 60 or 3000 μM THP. Therefore, it seems unlikely that THP inhibits CPT activity in the cytosol and acts in a protective manner on cardiac muscle, even if THP is actually transported into the cells. The level of long-chain acylcarnitine in heart is related to that of free carnitine (Fig. 4). This suggests that the decreased level of long-chain acylcarnitine is caused by a decrease in free carnitine level in heart.

Regarding the mechanism by which free carnitine is decreased, it has been reported that THP inhibits the activity of free carnitine synthesizing enzyme, γ-butyrobetaine hydroxylase, in the liver in a noncompetitive manner (Simkhovich et al., 1988). However, the carnitine content in all body tissues, including heart, is determined by not only synthesis in vivo, but also by important factors such as ingestion, absorption, membrane transport, and renal excretion (Borum, 1983). Therefore, even if carnitine synthesis is reduced, the amount of decrease due to this reduced synthesis could be canceled by dietary ingestion and renal resorption, and, as a result, the content in the cardiac muscle may not be substantially decreased.

As shown in Fig. 5, the K_p value becomes higher with decreasing free carnitine concentrations in serum after the administration of THP. The kinetic analysis of carnitine transport in heart in vivo suggests a high affinity for carnitine (K_m = 4.0 μM) and low affinity for THP (K_i = 1300 μM) as summarized in Table 2. In cultured myocytes, we found evidence for the direct inhibition of free carnitine transport by THP. The K_m value for free carnitine in cultured myocytes was 93.2 μM, which is close to the reported value of 60 μM

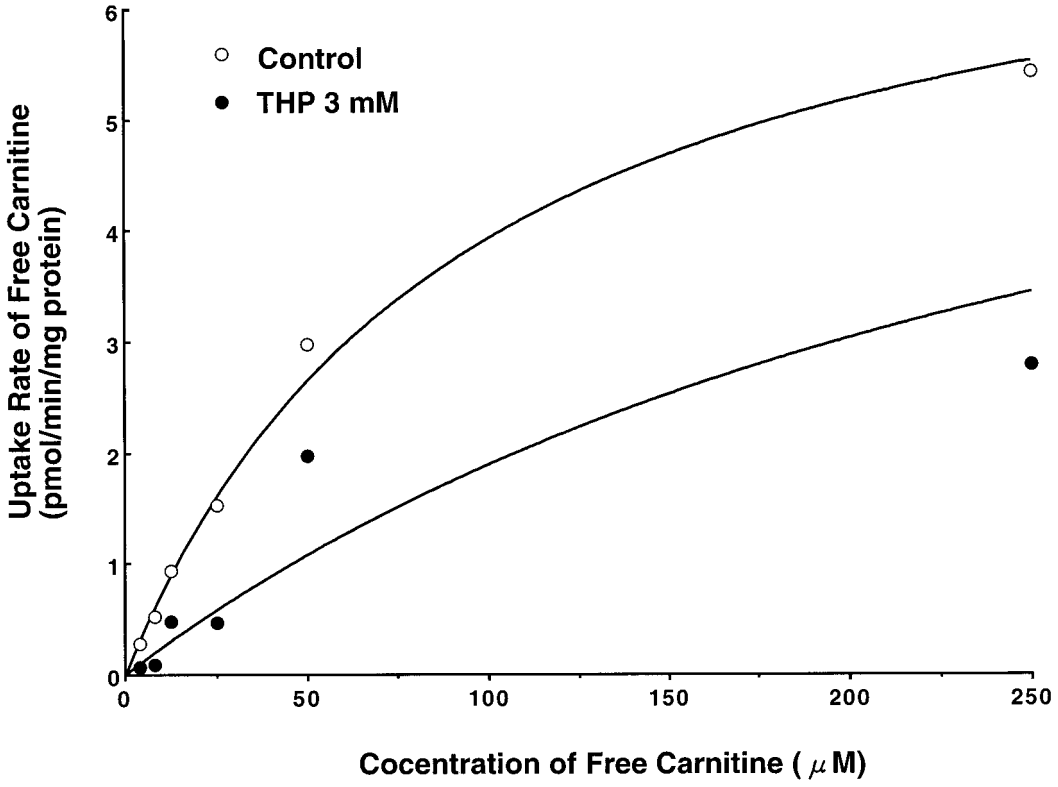

Fig. 6. Concentration-dependent uptake of free carnitine by myocytes. Open and closed circles represent the uptake of free carnitine by myocytes with or without THP (3 mM), respectively. Each data point represents the mean of four experiments. The uptake was measured after 2 h at the indicated concentration. The solid curves represent the fitting curve based on equation (2) using the parameters summarized in Table 2.

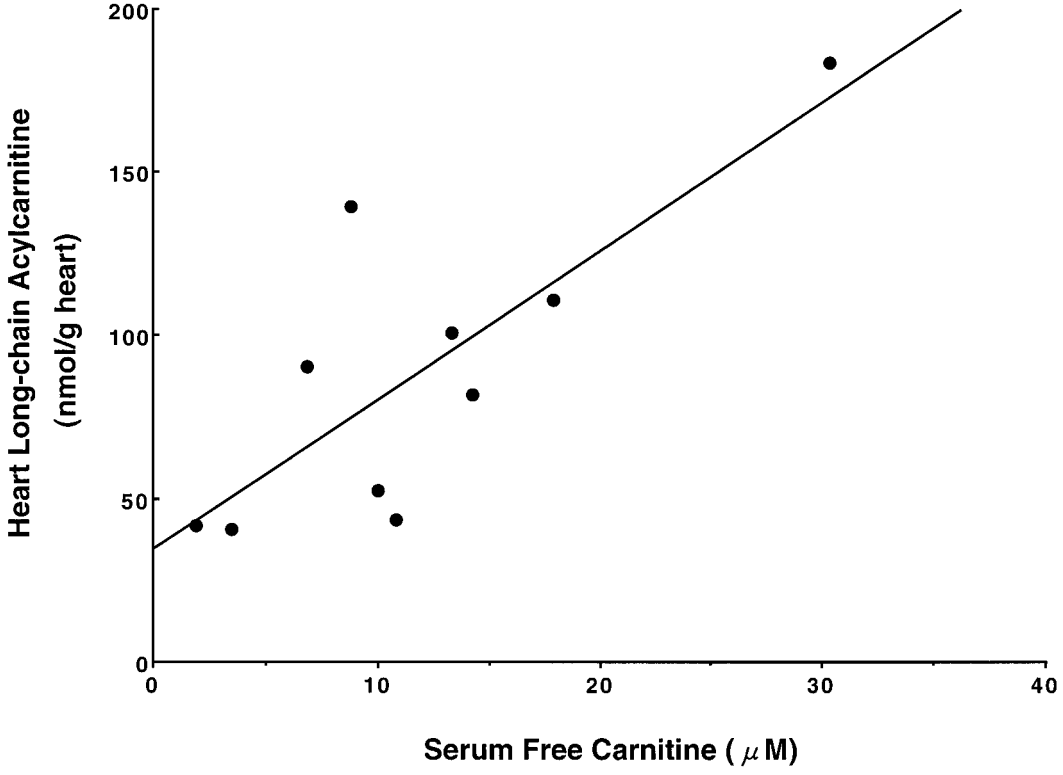

Fig. 7. Relationship between serum free carnitine and heart long-chain acylcarnitine. Each data point represents the mean value of Fig. 3. The solid line represents the regression line ($r = 0.818$, $p < .0023$).

(Bahl et al., 1981). Free carnitine transport was inhibited by THP with a K_i of 1340 μM, which is quite similar to the estimated K_i value in vivo conditions. Thus, the decreased concentration of free carnitine in heart after the administration of THP cannot be explained by the inhibition of free carnitine transport to cardiac muscle cells by THP.

Free carnitine in blood is filtered through the renal glomeruli and reabsorbed into renal proximal tubular cells by means of a carnitine transport carrier, which is localized in the apical side of the tubular cells (Stieger et al., 1995). Free carnitine transport is Na^+-dependent and the K_m for free carnitine has been reported to be 55 μM (Rebouche and

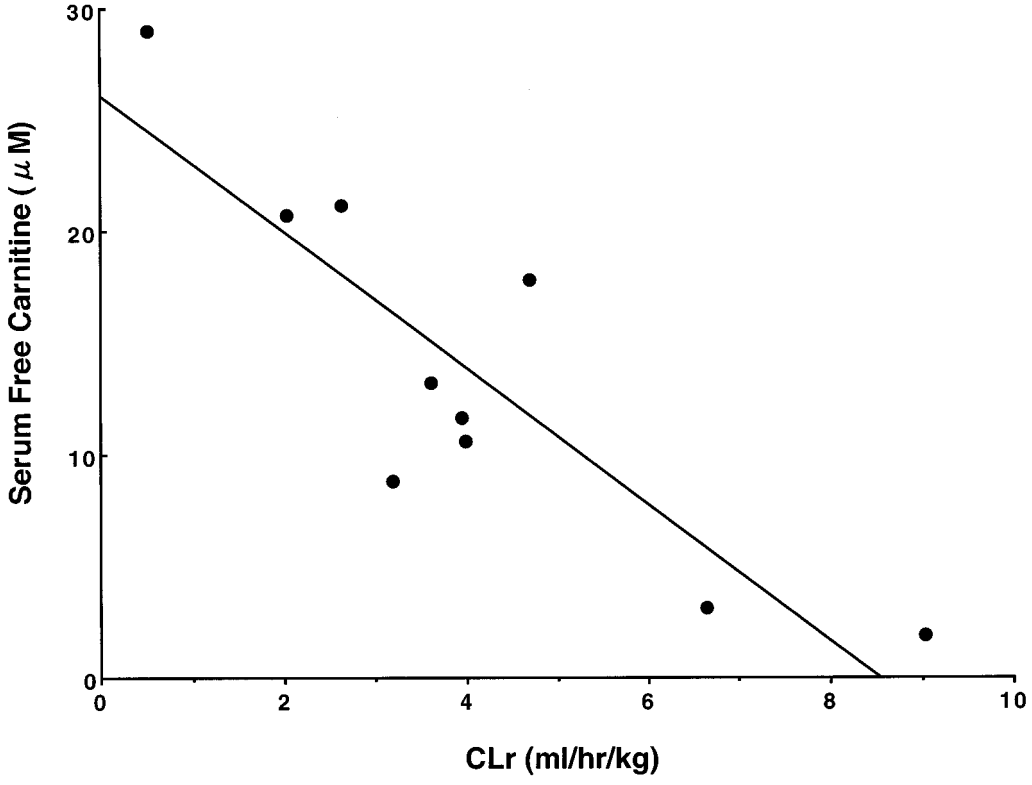

Fig. 8. Relationship between serum free carnitine concentration and renal clearance of free carnitine. The CL_r was calculated from the excreted free carnitine in urine divided by the area under the curve for serum free carnitine concentration for 24 h before killing the animals. The solid line represents the regression line (r = 0.863, $p < .001$).

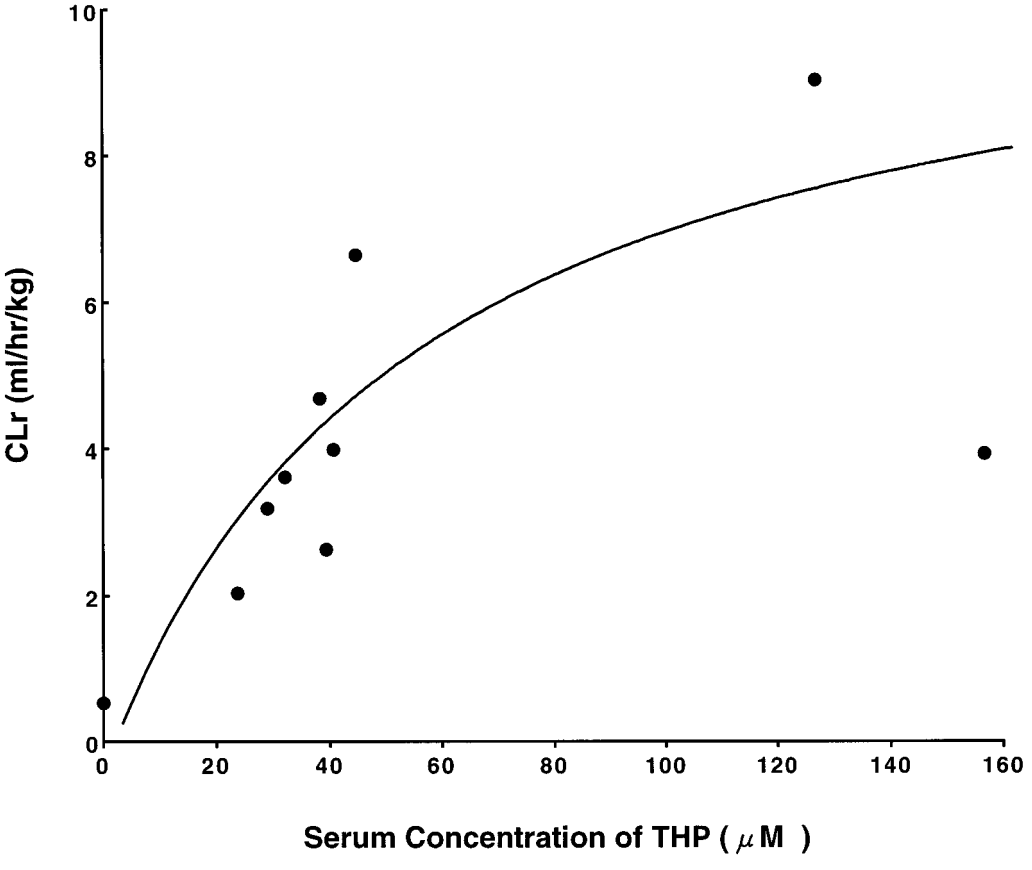

Fig. 9. Relationship between CL_r and serum THP. CL_r increases with increasing serum THP concentration, which was averaged for 24 h before killing animals. The solid curve represents the simulation curve by equation (3) fixing the C_{car} at 50 μM.

Mack, 1984) or 17 μM (Stieger et al., 1995), based on experiments using kidney brush-border membrane vesicles. When this reabsorption system is functioning normally, the blood level appears to be maintained at a constant value (Mancinelli et al., 1995). When THP was given to mice, blood carnitine levels decreased as shown in Fig. 3. Interestingly,

this decrease was rapid, reaching a level below 50% of the level observed at 24 h after THP administration. As the cause of such a rapid decrease, the strong inhibition of renal reabsorption of free carnitine by THP represents the most likely initial event. Hence, the inhibition of renal reabsorption of free carnitine by THP in vivo was examined.

In our present examination, the free carnitine reabsorption rate was approximately 96% under normal conditions of free carnitine concentration at 31 μM. This value is in agreement with the reabsorption rate (about 93% at 30 μM) obtained by experiments involving the renal perfusion system of rats (Mancinelli et al., 1995).

Based on the evidence of competitive inhibition of THP on free carnitine transport in heart and fibroblasts, it is reasonable to assume that the CL_r increased with an increase of THP serum concentration (Fig. 8). We have assumed that there is no secretion of carnitine from serum to urine in this analysis. Recently, it has been reported that free carnitine, derived from acylcarnitine, is secreted into urine to some extent in an isolated perfused rat kidney (Mancinelli et al., 1995; Evans et al., 1997). If this is the case, the estimated K_i for THP (52.2 μM) would be lower. The K_i of THP in kidney is much smaller than the K_m of free carnitine, which is completely opposite to the case in heart. The K_m of free carnitine is much smaller than the K_i of THP in heart. These results demonstrate the heterogeneity of carnitine-transport carriers among organs.

The reason why THP lowers free carnitine transport activity appears to lie with its structural similarity to free carnitine. As examples of inhibition of free carnitine transport activity by carnitine analogs, γ-butyrobetaine, acetylcarnitine, and lysine are known to function as inhibitors (Huth and Shug, 1980; Bahl et al., 1981). However, only THP is used as a drug.

Given the data collected herein, it is possible to discuss speculative adverse reactions of THP. Considering the reactions, the analytical results of Juvenile Visceral Steatosis mice, which are congenitally deficient in free carnitine transport activity may be cited. This type of mice was reported to have a systemic carnitine deficiency (Kuwajima et al., 1991). A defect in renal free carnitine reabsorption in this strain and loss of the saturable uptake of free carnitine transport activity in fibroblasts have also been reported (Horiuchi et al., 1994; Kuwajima et al., 1996).

Juvenile Visceral Steatosis mice show cardiomegaly with age, and an increased number of mitochondria, along with numerous myelin-like structures in their myocytes, as evidenced by histological examination (Miyagawa et al., 1995; Kuwajima et al., 1998). In addition, numerous ragged red fibers and an increased number of mitochondria were found in skeletal muscles by Gomori-trichrome staining (Kaido et al., 1997). An abnormal increase in mitochondria was found even in extrinsic muscle and diaphragm (Narama et al., 1997); in addition, a distinct fatty liver with triacylglycerol accumulation was found (Kuwajima et al., 1991). Therefore, when THP is administered, particularly in a large dose, attention should be paid to injuries to these organs, although the decrease in carnitine levels caused by THP is reversible.

In summary, the principal mechanism for the decreased carnitine concentration in heart did not involve the inhibition of free carnitine transport and/or metabolism in heart, but rather the increased renal clearance of carnitine, possibly by the inhibition of reabsorption. These results also point to the heterogeneity in carnitine transport carriers among organs.

Acknowledgments

We thank Pharmacokinetics Research Laboratory, Taiho Pharmaceutical Co., Ltd., for performing the measurements of THP levels in serum. We also are grateful to Ms. Shiho Nakai and Ms. Misa Bando for skillful secretarial help, and Kouichiro Taniguchi for expert technical assistance.

References

Adams RJ, Cohen DW, Gupte S, Johnson JD, Wallick ET, Wang T and Schwartz A (1979) In vitro effects of palmitylcarnitine on cardiac plasma membrane Na, K-ATPase, and sarcoplasmic reticulum Ca^{2+}-ATPase and Ca^{2+} transport. *J Biol Chem* **254:**12404–12410.

Akahira M, Hara A and Abiko Y (1997) Effect of MET-88, a γ-butyrobetaine hydroxylase inhibitor, on myocardial derangements induced by hydrogen peroxide in the isolated perfused rat heart. *Fundam Clin Pharmacol* **11:**356–364.

Anderson RC, Balestra M, Bell PA, Deems RO, Fillers WS, Foley JE, Fraser JD, Mann WR, Rudin M and Villhauer EB (1995) Antidiabetic agents: A new class of reversible carnitine palmitoyltransferase I inhibitors. *J Med Chem* **38:**3448–3450.

Aoyagi T, Sugiura S, Eto Y, Yonekura K, Matsumoto A, Yokoyama I, Kobayakawa N, Omata M, Kirimoto T, Hayashi Y and Momomura S (1997) Inhibition of carnitine synthesis protects against left ventricular dysfunction in rats with myocardial ischemia. *J Cardiovasc Pharmacol* **30:**468–474.

Bahl J, Navin T, Manian AA and Bressler R (1981) Carnitine transport in isolated adult rat heart myocytes and the effect of 7,8-di OH chlorpromazine. *Circ Res* **48:**378–385.

Borum PR (1983) Carnitine. *Annu Rev Nutr* **3:**233–259.

Bremer J (1983) Carnitine—metabolism and functions. *Physiol Rev* **63:**1420–1480.

Christiansen RZ and Bremer J (1976) Active transport of butyrobetaine and carnitine into isolated liver cells. *Biochim Biophys Acta* **448:**562–577.

Corr PB, Gross RW and Sobel BE (1984) Amphipathic metabolites and membrane dysfunction in ischemic myocardium. *Circ Res* **55:**135–154.

Dhalla NS, Elimban V and Rupp H (1992) Paradoxical role of lipid metabolism in heart function and dysfunction. *Mol Cell Biochem* **116:**3–9.

Dhar PK, Grupp IL, Schwartz A, Grupp G and Matlib MA (1996) Reduction of carnitine content by inhibition of its biosynthesis results in protection of isolated guinea pig hearts against hypoxic damage. *J Cardiovasc Pharmacol Therapeut* **1:**235–242.

Evans AM, Mancinelli A and Longo A (1997) Excretion and metabolism of propionyl-L-carnitine in the isolated perfused rat kidney. *J Pharmacol Exp Ther* **281:**1071–1076.

Horiuchi M, Kobayashi K, Yamaguchi S, Shimizu N, Koizumi T, Nikaido H, Hayakawa J, Kuwajima M and Saheki T (1994) Primary defect of juvenile visceral steatosis (jvs) mouse with systemic carnitine deficiency is probably in renal carnitine transport system. *Biochim Biophys Acta* **1226:**25–30.

Huth PJ and Shug AL (1980) Properties of carnitine transport in rat kidney cortex slices. *Biochim Biophys Acta* **602:**621–634.

Kaido M, Fujimura H, Ono A, Toyooka K, Yoshikawa H, Nishimura T, Ozaki K, Narama I, Kuwajima M (1997) Mitochondrial abnormalities in a murine model of primary carnitine deficiency. *Eur Neurol* **38:**302–309.

Kaneko H and Goshima K (1982) Selective killing of fibroblast-like cells in cultures of mouse heart cells by treatment with a Ca ionophore, A23187. *Exp Cell Res* **142:**407–416.

Kirimoto T, Nobori K, Asaka N, Muranaka Y, Tajima K and Miyake H (1996) Beneficial effect of MET-88, a γ-butyrobetaine hydroxylase inhibitor, on energy metabolism in ischemic dog hearts. *Arch Int Pharmacodyn Thèr* **331:**163–178.

Kuwajima M, Kono N, Horiuchi M, Imamura Y, Ono A, Inui Y, Kawata S, Koizumi T, Hayakawa J, Saheki T and Tarui S (1991) Animal model of systemic carnitine deficiency: Analysis in C3H-H-2° strain of mouse associated with juvenile visceral steatosis. *Biochem Biophys Res Commun* **174:**1090–1094.

Kuwajima M, Lu K-M, Harashima H, Ono A, Sato I, Mizuno A, Murakami T, Nakajima H, Miyagawa J, Namba M, Hanafusa J, Hayakawa J, Matsuzawa Y and Shima K (1996) Carnitine transport defect in fibroblasts of juvenile visceral steatosis (JVS) mouse. *Biochem Biophys Res Commun* **223:**283–287.

Kuwajima M, Lu K-M, Sei M, Ono A, Hayashi M, Ishiguro K, Ozaki K, Hotta K, Okita K, Murakami T, Miyagawa J, Narama I, Nikaido H, Hayakawa J, Nakajima H, Namba M, Hanafusa T, Matsuzawa Y and Shima K (1998) Characteristics of cardiac hypertrophy in the juvenile visceral steatosis mouse with systemic carnitine deficiency. *J Mol Cell Cardiol* **30:**773–781.

Lopaschuk GD, Wall SR, Olley PM and Davies NJ (1988) Etomoxir, a carnitine palmitoyltransferase I inhibitor, protects hearts from fatty acid—induced ischemic injury independent of changes in long chain acylcarnitine. *Circ Res* **63:**1036–1043.

Mancinelli A, Longo A, Shanahan K and Evans AM (1995) Disposition of L-carnitine and acetyl-L-carnitine in the isolated perfused rat kidney. *J Pharmacol Exp Ther* **274:**1122–1128.

McGarry JD and Foster DW (1985) Free and esterified carnitine. Radiometric method, in *Methods of Enzymatic Analysis* (Bergmeyer HU ed) pp 474–481, Academic Press, New York.

McGarry JD, Mills SE, Long CS and Foster DW (1983) Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. *Biochem J* **214:**21–28.

Miyagawa J, Kuwajima M, Hanafusa T, Ozaki K, Fujimura H, Ono A, Uenaka R, Narama I, Oue T, Yamamoto K, Kaidoh M, Nikaido H, Hayakawa J, Horiuchi M, Saheki T and Matsuzawa Y (1995) Mitochondrial abnormalities of muscle tissue in

mice with juvenile visceral steatosis associated with systemic carnitine deficiency. *Virchows Arch* **426:**271–279.
Narama I, Ozaki K, Matsuura T, Ono A, Sei M, Shima K and Kuwajima M (1997) Heterogeneity of histopathologic features in the congenitally carnitine-deficient juvenile visceral steatosis (JVS) mouse. *Biomed Res* **18:**247–255.
Rebouche CJ and Mack DL (1984) Sodium gradient-stimulated transport of L-carnitine into renal brush border membrane vesicles: Kinetics, specificity, and regulation by dietary carnitine. *Arch Biochem Biophys* **235:**393–402.
Rozen R, Scriver CR and Mohyuddin F (1983) Hypertaurinuria in the C57BL/6J mouse: Altered transport at the renal basolateral membrane. *Am J Physiol* **244:** F150–F155.
Sahartova O, Shatz V and Kalvins I (1993) HPLC analysis of mildronate and its analogues in plasma. *J Pharm Biomed Anal* **11:**1045–1047.
Simkhovich BZ, Shutenko ZV, Meirena DV, Khagi KB, Mezapuke RJ, Molodchina TN, Kalvins IJ and Lukevics E (1988) 3-(2,2,2-trimethylhydrazinium) propionate (THP)—a novel γ-butyrobetaine hydroxylase inhibitor with cardioprotective properties. *Biochem Pharmacol* **37:**195–202.
Stieger B, O'Neill B and Krähenbühl S (1995) Characterization of L-carnitine transport by rat kidney brush-border-membrane vesicles. *Biochem J* **309:**643–647.
Takahashi M, Ueda S, Misaki H, Sugiyama N, Matsumoto K, Matsuo N and Murao S (1994) Carnitine determination by an enzymatic cycling method with carnitine dehydrogenase. *Clin Chem* **40:**817–821.
Tipping PG, Kitching AR, Huang XR, Mutch DA and Holdsworth SR (1997) Immune modulation with interleukin-4 and interleukin-10 prevents crescent formation and glomerular injury in experimental glomerulonephritis. *Eur J Immunol* **27:**530–537.
Woeltje KF, Kuwajima M, Foster DW and McGarry JD (1987) Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. II. Use of detergents andantibodies. *J Biol Chem* **262:**9822–9827.
Yamaoka K, Tanigawara Y, Nakagawa T and Uno T (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. *J Pharm Dyn* **4:**879–885.

Send reprint requests to: Dr. Masamichi Kuwajima, M.D., Ph.D., Department of Laboratory Medicine, School of Medicine, The University of Tokushima, 3–18-15 Kuramoto-cho, Tokushima, 770-8503 Japan.