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Effect of γ-butyrobetaine on fatty liver in juvenile visceral steatosis mice

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Abstract

We pharmacokinetically examined the effect of γ-butyrobetaine, a precursor of L-carnitine, on the change of fatty acid metabolism in juvenile visceral steatosis (JVS) mice, which have systemic L-carnitine deficiency due to lack of L-carnitine transporter activity. The concentrations of total free fatty acid (FFA), palmitic acid and stearic acid in the liver of JVS mice were significantly higher than those in wild-type mice. After intravenous administration of γ-butyrobetaine (50 mg kg^{-1}), the concentration of L-carnitine in the plasma of JVS mice reached about twice that of the control level and levels in the brain, liver and kidney were also significantly increased, whereas those in wild-type mice hardly changed. Although the plasma concentrations of FFA in both types of mice were unchanged after administration of γ-butyrobetaine, the concentrations of palmitic acid and stearic acid were significantly decreased. In particular, the liver concentration of FFA in JVS mice was decreased to the wild-type control level, accompanied by significant decreases in long-chain fatty acids, palmitic acid and stearic acid, whereas those in wild-type mice were not changed. These results suggest that γ-butyrobetaine can be taken up into organs, including the liver, of JVS mice, and transformed to L-carnitine. Consequently, administration of γ-butyrobetaine may be more useful than that of L-carnitine itself for treatment of primary deficiency of carnitine due to a functional defect of the carnitine transporter.

Introduction

It is well known that L-carnitine plays an important role in the transport of long-chain fatty acids across the mitochondrial inner membrane for β-oxidation and energy metabolism (Bremer 1962; Fritz & Yue 1964). All mammals take up L-carnitine from foods or synthesize L-carnitine from a precursor, γ-butyrobetaine, in the liver, kidney and brain (Vaz et al 1998), and reabsorb more than 90 % of unchanged L-carnitine in the kidney (Bernardini et al 1985). Clinically, primary chronic L-carnitine deficiency causes hepatic encephalopathy or cardiomyopathy in combination with skeletal myopathy (Breningstall 1990; Scholte et al 1990). Waber et al (1982) found that deficiency of L-carnitine blocks mitochondrial oxidation of fatty acids to carbon dioxide in all tissues or to ketones in the liver, resulting in lipid accumulation in hepatic cytosol.

Previously, we found that homozygous mutant mice (juvenile visceral steatosis (JVS) mice) show systemic L-carnitine deficiency (Koizumi et al 1988). The mice develop a fatty liver within 5 days of birth, with subsequent hyperammonaemia and

hypoglycaemia by 3 weeks after birth. Kuwajima et al (1991) found that endogenous L-carnitine concentrations in plasma, liver and muscle of JVS mice were markedly lower than in wild-type mice. Recently, we have suggested that the L-carnitine transporter is lacking or functionally deficient in JVS mice, because the renal reabsorption, intestinal absorption and tissue distribution of L-carnitine are significantly lower in JVS mice than in wild-type mice (Yokogawa et al 1999a). Further, we cloned *OCTN2* as a new member of the organic cation transporter family (Tamai et al 1998) and demonstrated that the transporter gene in JVS mice had a point mutation that abolished the Na^+-dependent carnitine transport activity. (Nezu et al 1999; Yokogawa et al 1999b). Therefore, it is thought that fatty liver and cardiomyopathy in JVS mice are attributable to the change of fatty acid metabolism arising from the deficiency of L-carnitine due to the lack of carnitine transporter activity. There are many reports that L-carnitine deficiency syndromes are improved by L-carnitine administration. (Breningstall 1990; Scholte et al 1990; Iliceto et al 1995; Yoshimine et al 1997). However, the effectiveness of L-carnitine treatment for diseases based on L-carnitine transporter deficiency is intrinsically restricted by the low transport activity in tissues.

In this study, we pharmacokinetically examined the effect of γ-butyrobetaine, a precursor of L-carnitine, on the change of fatty acid metabolism in the liver of JVS mice.

Materials and Methods

Materials

L-Carnitine hydrochloride and γ-butyrobetaine were purchased from Sigma Chemical Co., St Louis, MO. All other chemicals, which were purchased from Wako Pure Chemical Industries, Osaka, Japan and Sigma Chemical Co., were of reagent grade and were used without further purification. Total free fatty acid (FFA) and unchanged L-carnitine were determined by using a Free Fatty Acid Kit (Wako Pure Chemical Industries) and a Free Carnitine Kit (Kainos Co., Tokyo, Japan), respectively.

Mouse experiments

JVS mice were originally found among mice of the C3H.OH strain in our laboratory (Koizumi et al 1988). The autosomal recessive mutant gene, *jvs*, was then backcrossed into C57BL/6 mice (CLEA, Tokyo, Japan), and C57BL/6-*jvs* mice (Yokogawa et al 1999a) were used as JVS mice in this study. Mice were weaned at around 1 month and maintained on a regular laboratory chow, CE-2 (CLEA, Tokyo, Japan). Before weaning, JVS mice were given a subcutaneous injection of carnitine (50 mg kg^{-1}) daily for 1 month, and thereafter every week. Eight-week-old JVS male mice (*jvs/jvs*), about 20 g, were used at 4 days after the last carnitine injection. As control mice, age-matched wild-type male mice (+/+), about 20 g, were used.

The dose of L-carnitine or γ-butyrobetaine was fixed at 50 mg kg^{-1}, and was injected via the jugular vein in a volume of 50 μL. Serial blood samples were collected from the intra-orbital venous plexus of individual mice under light ether anaesthesia, using heparinized capillary tubes, at designated time intervals during the experiment. The plasma was separated by centrifugation and stored at −30°C until assay. For determination of the liver concentration of FFA and saturated fatty acids, the mice were killed by decapitation at 4 h after a single intravenous injection of γ-butyrobetaine. The liver was quickly excised, rinsed well with ice-cold saline, blotted dry and weighed.

Assay for unchanged L-carnitine

The excised tissues were homogenized with four volumes of ice-cold 0.25 M sucrose (3 mM tris-HCl (pH 7.4), 0.1 mM EDTA). All subsequent steps were performed at 4°C. Then the homogenate was centrifuged at 25 000 *g* for 30 min. Thereafter the supernatant (200 μL) was mixed with 15% trichloroacetic acid (96 μL) and centrifuged at 25 000 *g* for 10 min. The supernatant was adjusted to pH 7.5 by adding 1 M KOH. The unchanged L-carnitine concentrations in plasma and tissues were measured by using a Free Carnitine Kit according to the method of Takahashi et al (1994). The method involved an enzymatic cycling technique with NADH, thio-NAD^+, and carnitine dehydrogenase, and measured the increase of absorbance at 415 nm of thio-NADH produced at 37°C during the reaction.

Assay for saturated fatty acids

Concentrations of saturated fatty acids (myristic acid, palmitic acid, stearic acid and arachidic acid) in plasma and liver were determined by gas chromatography–mass spectrometry (GC-MS, Model GC-17 system Class 5000, Shimadzu, Kyoto, Japan) (Magni et al 1994). A sample of plasma (20–50 μL) was mixed with 100 μL of methanol containing 50 μg mL^{-1} of heptadecanonic acid as an internal standard, and then extracted into 250 μL of chloroform. After evaporation of the solvent, the residue was dissolved in 2 mL of n-hexane containing 75 μL of trimethylsilyl-diazomethane and the solution

was mixed at room temperature for 30 min. After evaporation of the solvent, the residue was dissolved in 180 μL of n-hexane, and then 5 μL of the solution was subjected to GC-MS. A sample of liver (0.05–0.2 g) was homogenized with 400 μL of methanol and 250 μL of chloroform. The sample was analysed in the same manner as described for plasma.

Analyses were carried out in the selected-ion monitoring (SIM) mode, monitoring ions at m/Z 242, m/Z 270, m/Z 298 and m/Z 326 for myristic acid, palmitic acid, stearic acid and arachidic acid, respectively. The monitoring ion for heptadecanonic acid as the internal standard was at m/Z 284. Chromatographic separation of the fatty acid was achieved with a capillary column (ULBON HR-1701; 25 cm × 0.32 mm i.d.; Sinwakako Co., Kyoto, Japan) in a gas chromatograph equipped with a splitless injector. The oven temperature was set at 50°C for 3 min and then programmed to 270°C at 6°C min^{-1}.

Statistical analysis

The data were analysed using Student's t-test to compare the unpaired means of two sets of data. The number of determinations (N) is noted in each table and figure. A P value of 0.05 or less was taken to indicate a significant difference between sets of data.

Results

Endogenous fatty acid concentration in plasma and liver

The concentrations of total FFA and various saturated fatty acids (myristic acid, palmitic acid, stearic acid and arachidic acid) in plasma and liver of wild-type and JVS mice are shown in Table 1. The concentration of FFA in the liver of JVS mice was about 4-fold higher than that in wild-type mice, whereas the plasma concentrations were the same in the two types of mice. The plasma and liver concentrations of palmitic acid and stearic acid in JVS mice were significantly higher than those in wild-type mice.

Disposition of L-carnitine in wild-type and JVS mice

The plasma concentrations of L-carnitine in JVS mice, at 4 days after the last L-carnitine injection, and in wild-type mice were 1.33 ± 0.66 and 4.49 ± 0.32 (mean ± s.d., μg mL^{-1}), respectively. Figure 1 shows the time-course of plasma concentrations of L-carnitine after intravenous administration of L-carnitine (50 mg kg^{-1}) and γ-butyrobetaine (50 mg kg^{-1}) to wild-type and JVS mice. After administration of L-carnitine, the plasma concentrations in the two types of mice reached about 80 μg mL^{-1}, then decreased gradually to a plateau of about twice the respective control level after 1–2 h. After administration of γ-butyrobetaine, the plasma concentration of L-carnitine in JVS mice reached about 6 μg mL^{-1} at 15 min, then remained at about twice the JVS control level, whereas in wild-type mice the plasma concentration of L-carnitine was hardly changed.

Table 2 shows the concentrations of L-carnitine in the tissues of wild-type and JVS mice at 4 h after an intravenous administration of L-carnitine (50 mg kg^{-1}) or γ-butyrobetaine (50 mg kg^{-1}). In the saline group, the tissue concentrations of L-carnitine in JVS mice were much lower than those in wild-type mice. After admin-

Table 1 Plasma and liver concentrations of endogenous saturated and free fatty acids in wild-type and JVS mice

	Wild type mice		**JVS mice**	
	Plasma	Liver	Plasma	Liver
Free Fatty Acid	(μEq mL^{-1})	(μEq(g wet weight)$^{-1}$)	(μEq mL^{-1})	(μEq(g wet weight)$^{-1}$)
	0.85 ± 0.16	1.93 ± 0.38	0.95 ± 0.28	6.85 ± 1.42**
Saturated fatty acid	(μEq mL^{-1})	(μEq(g wet weight)$^{-1}$)	(μEq mL^{-1})	(μEq(g wet weight)$^{-1}$)
Myristic acid	4.08 ± 1.16	7.05 ± 4.8	4.49 ± 1.42	6.66 ± 3.07
Palmitic acid	38.8 ± 3.3	108.7 ± 20.1	53.2 ± 6.7*	261.9 ± 37.7**
Stearic acid	10.3 ± 4.2	40.7 ± 9.8	21.9 ± 4.8*	78.2 ± 17.6*
Arachidic acid	3.81 ± 0.73	0.66 ± 0.19	1.95 ± 0.74	0.65 ± 0.21

Each value represents the mean ± s.d. of three experiments.
*$P < 0.05$, **$P < 0.01$ significantly different from the wild-type mice.

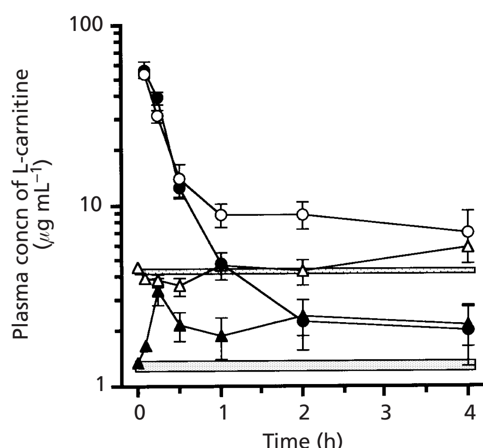

Figure 1 Time-course of plasma concentration of L-carnitine after intravenous administration of L-carnitine (50 mg kg^{-1}) (○) and γ-butyrobetaine (50 mg kg^{-1}) (△) to wild-type (open symbols) and JVS (closed symbols) mice. Each point with bar represents the mean ± s.d. of three experiments. ▭, control level of wild-type mice; ▭, control level of JVS mice.

istration of L-carnitine, the tissue concentrations of L-carnitine in both types of mice were only slightly changed and the tissue-to-plasma concentration ratio (Kp,app) was rather lower than that in the saline control. On the other hand, after administration of γ-butyrobetaine, the L-carnitine concentration in the liver of JVS mice was significantly increased, whereas it was hardly changed in wild-type mice. Similar increases in L-carnitine concentration after γ-butyrobetaine administration were also seen in the brain and kidney, which are organs synthesizing L-carnitine from γ-butyrobetaine (Vaz et al 1998).

Effect of γ-butyrobetaine on free fatty acid concentration in plasma and liver

Figure 2 shows the concentrations of FFA in plasma and liver at 4 h after intravenous administration of γ-butyrobetaine to wild-type and JVS mice. The plasma concentrations of FFA in both types of mice were not changed (Figure 2A). However, the concentration of FFA in the liver of JVS mice was significantly decreased to the wild-type control level by γ-butyrobetaine, whereas it was unchanged in wild-type mice (Figure 2B).

Effect of γ-butyrobetaine administration on saturated fatty acid concentration in plasma and liver

As shown in Figure 3, after intravenous administration of γ-butyrobetaine (50 mg kg^{-1}) the plasma concentrations of all saturated fatty acids were hardly changed in wild-type mice, but the plasma concentrations of palmitic acid and stearic acid in JVS mice were significantly decreased by about 65% at 4 h after administration ($P < 0.05$).

Table 3 shows the liver concentrations of saturated fatty acids at 4 h after intravenous administration of γ-butyrobetaine to wild-type and JVS mice. The concentrations of saturated fatty acids in the liver of wild-type mice were unchanged, whereas in JVS mice palmitic

Table 2 Plasma and tissue concentrations of L-carnitine at 4 h after i.v. administration of L-carnitine (50 mg kg^{-1}) and γ-butyrobetaine (50 mg kg^{-1}) to wild-type and JVS mice

Tissue	Wild-type mice			JVS mice		
	Saline	L-carnitine	γ-butyrobetaine	Saline	L-carnitine	γ-butyrobetaine
Plasma	4.53 ± 0.73	6.99 ± 1.91	5.78 ± 1.47	1.37 ± 0.95	2.02 ± 1.28	2.17 ± 0.74
Brain	40.1 ± 5.5	50.3 ± 9.8	45.8 ± 7.2	6.89 ± 3.50	6.39 ± 2.75	30.8 ± 10.2*
Liver	53.8 ± 7.9	58.5 ± 15.1	58.1 ± 16.1	5.61 ± 0.88	6.90 ± 2.70	14.9 ± 2.9*
Kidney	78.7 ± 18.8	91.1 ± 35.5	102.1 ± 18.0	8.57 ± 4.47	12.3 ± 3.4	18.8 ± 2.7*
Gut	6.64 ± 2.72	10.5 ± 6.9	11.5 ± 6.7	3.94 ± 2.62	7.84 ± 3.76	5.58 ± 6.64
Heart	71.5 ± 17.8	89.3 ± 7.6	111.3 ± 19.5	7.29 ± 4.23	9.20 ± 0.66	7.15 ± 0.81
Muscle	11.6 ± 2.5	12.8 ± 2.7	17.4 ± 6.7	3.51 ± 0.78	2.62 ± 1.66	2.90 ± 0.83
Lung	21.7 ± 4.8	30.8 ± 11.3	25.6 ± 7.6	4.01 ± 1.85	10.6 ± 6.4	7.90 ± 3.19
Spleen	5.44 ± 1.49	4.37 ± 1.84	4.54 ± 2.55	4.44 ± 3.03	3.50 ± 0.64	4.08 ± 2.36

Each value represents the mean ± s.d. (μg mL^{-1} or μg(g wet weight)$^{-1}$) of three experiments.
*$P < 0.05$ significantly different from the administration of saline.

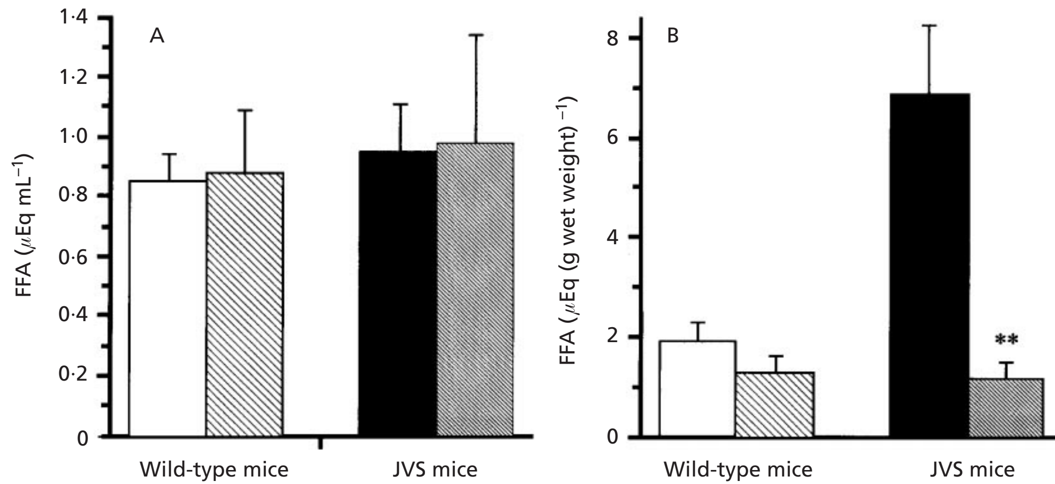

Figure 2 Concentrations of free fatty acids (FFA) in plasma (A) and liver (B) at 4 h after intravenous administration of saline (□, ■) and γ-butyrobetaine (50 mg kg^{-1}) (▧, ▧) to wild-type and JVS mice. Each column with bar represents the mean ± s.d. of three experiments.

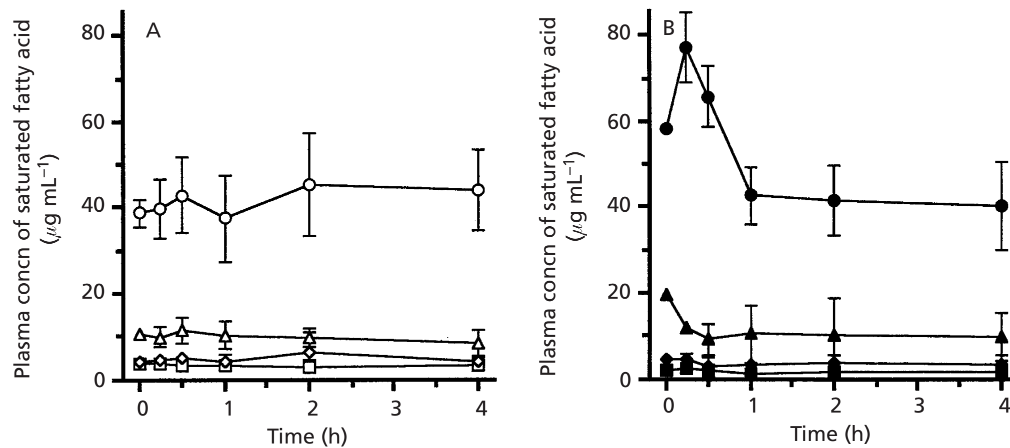

Figure 3 Time-course of plasma concentration of saturated fatty acids after intravenous administration of γ-butyrobetaine (50 mg kg^{-1}) to wild-type (A) and JVS (B) mice. Each point with bar represents the mean ± s.d. of three experiments. ◇, Myristic acid; ○, palmitic acid; △, stearic acid; □, arachidic acid.

Table 3 Liver concentrations of saturated fatty acids at 4 h after i.v. administration of γ-butyrobetaine (50 mg kg^{-1}) to wild-type and JVS mice

	Wild-type mice		**JVS mice**	
Saline	Saline	γ-butyrobetaine	Saline	γ-butyrobetaine
Myristic acid	7.05 ± 2.65	8.1 ± 2.1	6.66 ± 1.47	5.91 ± 1.13
Palmitic acid	109 ± 19	94.3 ± 12.9	262 ± 27	188 ± 34.6*
Stearic acid	40.8 ± 11.9	43.2 ± 17.6	78.2 ± 10.0	37.3 ± 6.4**
Arachidic acid	0.66 ± 0.23	0.55 ± 0.29	0.65 ± 0.21	0.44 ± 0.19

Each value represents the mean ± s.d. (μg mL^{-1} or μg(g wet weight)$^{-1}$) of three experiments.
*$P < 0.05$, **$P < 0.01$ significantly different from the administration of saline.

acid and stearic acid were decreased by 71 and 47%, respectively.

Discussion

It is important to clarify the effect of γ-butyrobetaine on changes in fatty acid metabolism to assess its possible usefulness for treating primary systemic carnitine deficiency diseases. Fat deposition in the liver of JVS mice was clearly observed, and among saturated fatty acids, the concentrations of stearic acid and palmitic acid in the plasma and liver of JVS mice were significantly higher than those in wild-type mice. These abnormalities of fatty metabolism in JVS mice could be quite selectively improved by a single intravenous administration of γ-butyrobetaine.

The plasma concentration of L-carnitine decreased more rapidly in JVS mice than in wild-type mice after L-carnitine administration. This result is consistent with previous reports that the renal reabsorption of L-carnitine is very low in JVS mice compared with that in wild-type mice (Horiuchi et al 1994; Yokogawa et al 1999a). JVS mice lack the function of octn2, a transporter of L-carnitine, due to a mutation in the gene (Nezu et al 1999). The L-carnitine concentration in the liver of wild-type and JVS mice was hardly changed after L-carnitine administration, whereas after administration of γ-butyrobetaine the L-carnitine concentrations were significantly increased in the brain, liver and kidney of JVS mice (Table 1). Moreover, fatty acid concentrations were significantly decreased by administration of γ-butyrobetaine in JVS mice, but not in wild-type mice (Figures 2 and 3, Table 2). These results suggest that tissues of wild-type mice do not need additional L-carnitine, whereas the liver of JVS mice can not take up exogenous L-carnitine due to the lack of L-carnitine transporter activity, but can take up γ-butyrobetaine, which is transformed to L-carnitine, leading to an improvement of fatty acid metabolism. Indeed, it has been reported that L-carnitine is biosynthesized from γ-butyrobetaine in the liver, brain and kidney (Vaz et al 1998). Horiuchi et al (1998) showed that the activity of γ-butyrobetaine hydroxylase in JVS mice is about twice that in wild-type mice.

In JVS mice, stearic acid and palmitic acid in plasma and liver were decreased after administration of γ-butyrobetaine (Figure 3 and Table 2). This indicates that the liver biosynthesized L-carnitine from γ-butyrobetaine and subsequently these long-chain fatty acids could be metabolized to short-chain fatty acids by β-oxidation. A question arises as to how the liver of JVS mice, lacking the L-carnitine transporter, can utilize γ-butyrobetaine. There are some reports that the L-carnitine transporter activity of the cells was effectively inhibited by γ-butyrobetaine (Stieger et al 1995; Tamai et al 1998; Yokogawa et al 1999b, c). However, Berardi et al (1998) reported that γ-butyrobetaine transport is significantly inhibited by propionylcarnitine, but not by L-carnitine or other acylcarnitines. The cellular uptake of γ-butyrobetaine may involve a carrier-mediated transport system different from the L-carnitine transporter, octn2. JVS mice, before weaning, must be given a subcutaneous injection of L-carnitine (50 mg kg^{-1}) daily for 1 month and thereafter every week. However, when JVS mice were similarly given γ-butyrobetaine instead of L-carnitine, they survived (data not shown). These results suggest that JVS mice can transport γ-butyrobetaine and can normally biosynthesize L-carnitine from γ-butyrobetaine.

Consequently, γ-butyrobetaine may be more useful than L-carnitine for the treatment of diseases due to primary carnitine deficiency.

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