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Inhibition of Carnitine Biosynthesis by Valproic Acid in Rats—The Biochemical Mechanism of Inhibition

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ABSTRACT. The anticonvulsive drug, valproic acid (VPA), inhibits the biosynthesis of carnitine, and may contribute in this way to carnitine deficiency associated with VPA therapy. The conversion of [^{3}H]-butyrobetaine into [^{3}H]-carnitine was determined 60 min following a single intraperitoneal (i.p.) dose of 1.2 mmol/kg VPA in rats. The fraction of radioactivity found in [^{3}H]-carnitine in the liver decreased from 63.2 ± 1.50% to 39.2 ± 1.11% (mean ± SEM). Total carnitine in the liver also decreased, whereas the precursor butyrobetaine increased from 5.01 ± 0.71 nmol/g to 8.22 ± 0.82 nmol/g (mean ± SEM). VPA also exhibited a dramatic effect on the conversion of an unlabeled loading amount of butyrobetaine. The increment in total carnitine caused by butyrobetaine in liver was reduced from 161 ± 15.4 nmol/g to 53.2 ± 5.11 nmol/g (mean ± SEM). These data prove that VPA reduces the flux through butyrobetaine hydroxylase (EC 1.14.11.1.). The drug *in vitro*, however, did not inhibit the enzyme directly. Searching for the mechanism of action, we found that VPA decreased the level of α-ketoglutarate (α-KG; a cofactor of butyrobetaine hydroxylase) from 73.5 ± 2.90 nmol/g to 52.9 ± 2.2 nmol/g (mean ± SEM) in the liver. The level of L-glutamate showed a rather dramatic decrease in the liver. Moreover, α-KG proved to have a protective role against VPA in the [^{3}H]-butyrobetaine conversion experiment. BIOCHEM PHARMACOL 52;9:1429–1433, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. carnitine biosynthesis; carnitine deficiency; butyrobetaine; valproic acid; epilepsy; L-glutamate; α-ketoglutarate

VPA# (dipropyl-acetate) has generally been used for treatment of epilepsy. In addition to its anticonvulsive properties, the drug has many side effects such as hepatotoxicity, hyperammonemia and carnitine deficiency (for reviews, see [1, 2]). Drug-induced carnitine deficiencies are reasonably assigned to the secondary carnitine deficiencies, i.e. secondary to metabolic disorders associated with organic acidurias [3–5]. The common mechanism for the development of carnitine deficiency is the esterification of a high fraction of carnitine with fatty acids or with some organic acids. These acylcarnitines are preferentially excreted by the kidney, leading to an extra loss of carnitine. The mechanism of the development of drug-induced carnitine deficiencies is similar to those in acidurias, e.g. in the case of pivampicillin therapy, when a huge amount of pivaloylcarnitine is excreted into the urine [6].

By contrast, the mechanism for carnitine deficiency due to VPA treatment seems to be different. Although serum concentration and urinary excretion of acylcarnitines increased upon VPA administration in human [7–9] and animal studies [10–12], the VPA–carnitine ester detected by gas chromatography–mass spectrometry (GC-MS) accounted for only 1% of the administered dose of VPA [13]. The GC-MS analysis in the urine of VPA-treated patients detected mostly octanoyl- and hexanoylcarnitine esters [7]. VPA and/or its metabolites apparently act by inhibiting some steps of fatty acid oxidation [1, 8], thereby causing accumulation of middle and shortchain carnitine esters.

Although this mechanism of action of VPA with respect to carnitine deficiency is surely operative, it alone does not seem to explain the development of carnitine deficiency. In organic acidurias, the urinary excretion of total carnitine increased several fold [4, 5]. In rats given VPA, it increased approximately twofold [10], whereas in VPA-treated patients it slightly increased [9]. In our human study, total carnitine in the urine even decreased [14] during VPA therapy. Moreover, an inhibition of carnitine uptake into fibroblasts on the effect of VPA was recently reported [15]. This action, if operative *in vivo*, would result in an increased serum level and increased urinary excretion, provided that the biosynthesis of carnitine at the same time remained unchanged. These considerations led us to the assumption that some other mechanism, namely an im-

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Abbreviations: α-KG, α-ketoglutarate; CoA, coenzyme A; VPA, valproic acid; Bu, butyrobetaine; SCh, short-chain acylcarnitine; LCh, long-chain acylcarnitine.

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paired carnitine biosynthesis, is involved in VPA-induced carnitine deficiency.

MATERIALS AND METHODS

Animals and Treatment

Male Wistar rats weighing 200–250 g were used. The experiments were performed between 9:00 a.m. and 12:00 p.m. The animals were given 1.2 mmol or 2.4 mmol per kg VPA i.p. in 1.0 mL 0.9% NaCl solution that, as a single dose, falls within the range of the doses used in experiments with rodents [11, 12] (200 or 400 mg/kg of sodium valproate). Controls were injected with saline. After 30 min, all animals were injected i.p. with approximately 15×10^6 cpm [^{3}H]-Bu. Sixty minutes following VPA or saline administration (30 min following isotope administration), the animals were decapitated. Blood was saved in heparinized tubes, and the livers were rapidly removed and frozen in liquid N_2 and stored until work up.

Analyses

Total carnitine was determined by a radioisotopic method [16] using small columns [17]. Total free- and acetyl-CoA [18], α-KG [19] and l-glutamate [20] were determined in freeze-clamped liver samples by standard enzymatic analyses. Distribution of radioactivity between carnitine and Bu was evaluated by reversed phase ion-pairing high performance liquid chromatography (HPLC) as previously described [21, 22]. Bu was determined as previously described [21] by using modified HPLC conditions [22].

Chemicals

Sodium salt of VPA was a gift from Gerot Pharmazeutika (Vienna, Austria). Acetyl-CoA was prepared from acetic anhydride and CoA as described elsewhere [23]. [^{3}H]-Bu with approximately 10^6 cpm/nmol specific activity was synthesized from 4-(Dimethylamino)butyric acid (Aldrich Chemical, Steinheim, Germany) and [^{3}H]$_3$CJ (Amersham, Little Chalfont, UK) as described elsewhere [24]. [^{14}C]-acetyl-CoA was obtained from Amersham. Aldrich Chemical supplied the (3-carboxypropyl)trimethyl-ammonium (γ-butyrobetaine) and l-heptanesulfonic acid. The source of other fine chemicals, α-KG, carnitine acetyltransferase, benzoic acid, trimethylacetate (pivalic acid) and coenzyme A was obtained from Sigma Chemical (St. Louis, MO, USA).

RESULTS AND DISCUSSION

Conversion of Bu to L-Carnitine

The stereospecific hydroxylation of γ-Bu to L-carnitine is catalyzed by the γ-Bu hydroxylase (EC 1.14.11.1.) located in the soluble fraction of the cytosol. Because γ-butyrobetaine hydroxylase occurs in rats exclusively in the liver, an evaluation of the flux through the enzyme in the liver gives a sure measurement of the carnitine supply for the organism.

By the 30th min following administration of [^{3}H]-Bu, 30–35% of the injected radioactivity ($4.5–5.0 \times 10^6$ cpm) was usually recovered in the whole liver; in control animals, 60–70% of this was found in carnitine in accordance with previous studies [22, 25]. This amount can reasonably be assumed as a tracer due to the high specific activity of the injected Bu. VPA (tested in two doses) markedly reduced the conversion of [^{3}H]-Bu (Fig. 1). The reduction in conversion by the 1.2 mmol/kg dose was from 63.2 ± 1.5% to 39.2 ± 1.11% (mean ± SEM, $P < 0.01$), whereas 2.4 mmol/kg VPA decreased conversion from 61.6 ± 5.2% to 34.8 ± 3.8% (mean ± SEM, $P < 0.01$). The 1.2 mmol/kg dose probably exerted the maximum inhibition. Therefore, the smaller 1.2 mmol/kg dose was used in the subsequent studies.

In addition to the tracer experiments (Fig. 1), the inhibition of the flux through Bu hydroxylase by VPA was confirmed by using a loading amount of unlabeled butyrobetaine. VPA exhibited a rather dramatic effect on the conversion of a loading amount (200 μmol/animal) of Bu (Fig. 2). The increment in total carnitine caused by Bu in liver of the Bu-only control group was 161 ± 15.2 nmol/g, which dropped to 53.2 ± 5.11 nmol/g in the Bu-plus-VPA group (mean ± SEM). The corresponding values in the

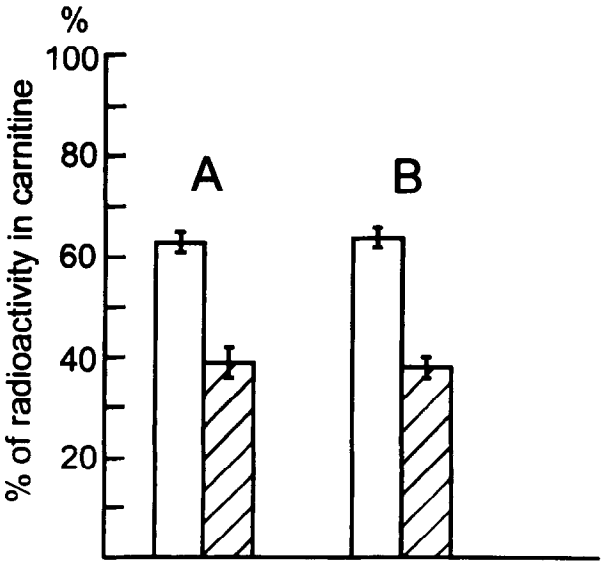

FIG. 1. [^{3}H]-Bu (containing 15×10^6 counts/min) was injected in 1.0 mL 0.9% NaCl i.p. 30 min following the injection 1.2 mmol (A) or 2.4 mmol (B) per kilogram of VPA. Controls were given saline instead of VPA. Thirty minutes after the administration of the isotope (60 min after administration of VPA), the animals were killed, and a 0.5-g portion of the liver was analyzed for the distribution of radioactivity between Bu and carnitine. Bars indicate percentage of radioactivity in carnitine (mean ± SEM) for five (A) or four (B) animals. White bar, control; hatched bar, VPA-treated animals.

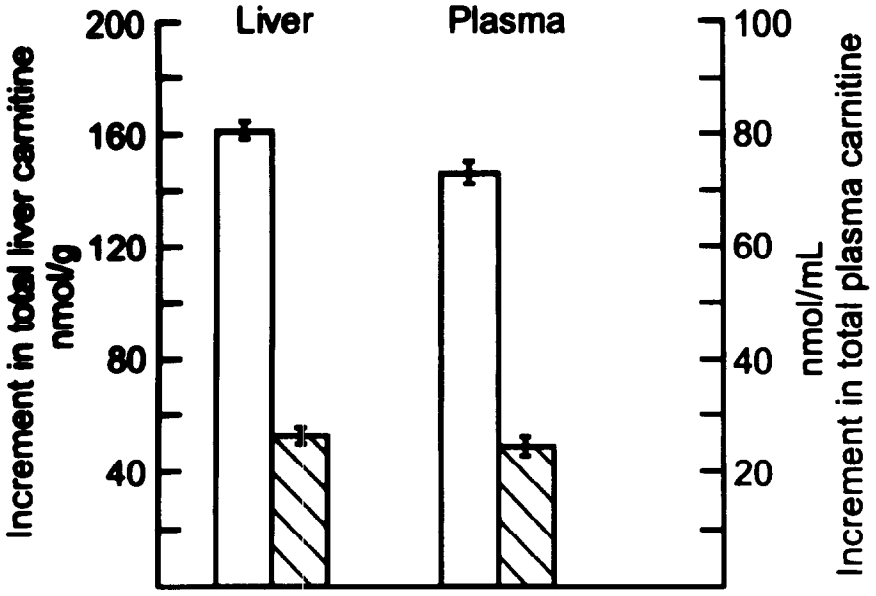

FIG. 2. Two hundred micromoles of Bu were injected into 1.0 mL 0.9% NaCl i.p. 30 min following the injection of 1.2 mmol/kg VPA or saline. Thirty minutes after the administration of the Bu (60 min after administration of VPA), the animals were killed, and total carnitine was measured in the liver and plasma by enzymatic analysis. The carnitine increments evoked by Bu were calculated as follows: in a control group (no treatment), the total carnitine in the liver and plasma was 181 nmol/g and 35.2 nmol/mL, respectively. These values were subtracted from the corresponding values of the butyrobetaine-treated group to obtain the increments. In the VPA-treated control group (white bar), these values in the liver and plasma were 152 nmol/g and 30.8 nmol/mL, respectively. These control values were subtracted from the corresponding values of the group given Bu plus VPA (hatched bar) to obtain the increments. Bars indicate nanomoles of carnitine in grams or milliliters (mean ± SEM) for five animals.

serum were 73.2 ± 4.50 nmol/mL vs. 24.4 ± 2.24 nmol/mL. In the calculation of the increment, the basal values of the corresponding control groups without Bu (with or without VPA) were subtracted (see caption to Fig. 2).

Analyses of Carnitine and Bu

The carnitine analyses in the serum and liver showed a decrease in total and free carnitine (Table 1) in accordance with other human and animal studies. Because the acid-soluble, short-chain carnitine esters increased (more in the liver and to a lesser degree in the plasma), the acyl:free-carnitine ratio must also have increased. A decrease in total liver carnitine was also found in another study in mice [11] but not in rats [10]. An important point demonstrated in Table 1 is that the Bu level showed a significant increase ($P < 0.01$) from 5.01 nmol/g to 8.12 nmol/g nmol in the VPA-treated animals. Because a higher fraction of radioactivity remained in the form of Bu in these animals (e.g. 60% instead of the 30% control value), the specific activity of the pool can be assumed to be unchanged. The accumulation of Bu coupled with the reduced amount of total carnitine also indicates a reduced flux through the Bu hydroxylase in agreement with Figs. 1 and 2.

Possible Mechanism of Action of VPA

After testing, VPA *in vitro* did not directly inhibit the Bu hydroxylase enzyme (data not shown). Therefore, VPA likely acts *in situ* in a rather complex way by affecting the cofactors of Bu hydroxylase, namely α-KG and/or ascorbic acid.

VPA decreases free CoA levels and VPA-CoA ester is formed [26, 27]. Speculatively, the CoA- and acetyl-CoA-depleting effect of VPA may play a role in the impairment of the flux through the Bu hydroxylase in a mediated way. To test this idea, we compared the effect of some agents on liver CoA levels and on Bu-to-carnitine conversion. VPA depleted the free CoA level and also impaired the conversion of Bu (Table 2). Pivalic acid, which severely depleted CoAs, did not inhibit the conversion of Bu. On the contrary, benzoic acid, which did not deplete CoA levels under this condition, inhibited Bu conversion. (In the metabolism of benzoic acid, CoA ester is formed in the first step

TABLE 1. Effect of valproate on the carnitine and Bu content of plasma and liver in rats

Animals (n)	Liver Bu (nmol/g)	Liver carnitine (nmol/g)				Plasma carnitine (nmol/mL)			
		Total	Free	SCh	LCh	Total	Free	SCh	LCh
Control (5)	5.01 ± 0.71	181.3 ± 11.8	109 ± 9.20	68.9 ± 7.02	3.26 ± 0.91	35.2 ± 1.24	23.1 ± 2.85	11.1 ± 2.20	1.00 ± 0.12
Treated (5)	8.22† ± 0.82	152.0* ± 8.11	65.9† ± 4.12	82.9 ± 7.11	3.30 ± 0.42	30.8 ± 3.11	15.8* ± 1.12	14.1 ± 2.65	0.89 ± 0.05

The animals were treated with 1.2 mmol/kg VPA. Numbers are mean ± SEM for number of animals shown in parentheses. Significantly different from controls: *$P < 0.02$, †$P < 0.01$.

TABLE 2. Effects of VPA and related compounds on CoA content and butyrobetaine-carnitine conversion in rat liver

Experiment	Acetyl-CoA (nmol/g)	Free CoA (nmol/g)	Distribution of radioactivity (% carnitine)
VPA			
Control (5)	41.1 ± 5.30	50.6 ± 5.41	68.7
Treated (4)	9.9 ± 1.11†	14.4 ± 0.81†	40.9
Benzoic acid*			
Control (4)	50.2 ± 6.42	46.5 ± 5.01	69.3
Treated (4)	48.2 ± 5.22	44.2 ± 6.32	49.3
Pivalic acid			
Control (5)	45.3 ± 3.22	12.8 ± 1.51	70.1
Treated (5)	34.2 ± 4.31	3.8 ± 0.81†	68.7

The agents were administered in 1.2-mmol/kg doses and 1 hr later the CoA content, acetyl-CoA content and the distribution of radioactivity were determined as indicated under Materials and Methods. Numbers are means ± SEM for number of animals shown in parentheses. Significantly different from controls: †$P < 0.01$. *The effect of benzoic acid on the CoA content was also tested 30 min following the application of the agent with the same negative result.

[27]; however, the steady-state level is so low that it does not deplete the tissue free CoA level.) Altogether the depleted free CoA and acetyl-CoA levels may not be related to the impairment of Bu conversion. A number of organic acids excreted in metabolic acidurias and associated with secondary carnitine deficiency (e.g. propionic acid, methylmalonic acid and glutaric acid) were tested for possible inhibitory effects on Bu–carnitine conversion with a negative result (data not shown).

In the subsequent experiments, we tested whether the levels of cofactors required for Bu hydroxylase were affected by VPA. We found a significant decrease in the α-KG level in the liver, whereas the ascorbic acid level remained unchanged (Table 3). Benzoic acid (inhibitory for Bu conversion) also decreased the α-KG level, whereas pivalic acid (not inhibitory) did not. These facts suggest that VPA affects Bu conversion via the α-KG level (Table 3). The effect of benzoic acid on carnitine biosynthesis is in accordance with a recent report showing that benzoic acid therapy caused carnitine deficiency in patients [28].

These data indicate that VPA impairs the conversion of Bu to carnitine, i.e. the flux through the Bu hydroxylase enzyme. As the mechanism of action, we propose that VPA lowers the level of α-KG, the coenzyme for Bu hydroxylase in the liver, in the only place where Bu-to-carnitine conversion occurs. The change, from 73.5 nmol/g control value to 52 nmol/g (from 98 μmol/L to 69 μmol/L), can be very effective in lowering the flux through the enzyme because this range falls far below the saturation level. In the case of rat liver Bu hydroxylase, a 0.5 mmol/L apparent K_m value was reported for α-KG [29]. These values were found in the whole cell. The changes in the cytosol, where Bu hydroxylase is located, may be rather marked.

TABLE 3. Effect of VPA and related compounds on the α-KG and L-glutamate level in rat liver

Experiment	Ascorbic acid (μg/g)	α-KG (nmol/g)	L-Glutamate (μmol/g)
Control (8)	172 ± 6.25	73.5 ± 2.93	2.73 ± 0.71
VPA			
30 min (8)	183 ± 17.2	52.4 ± 3.52*	1.14 ± 0.03†
60 min (8)	168 ± 25.0	52.9 ± 2.2*	1.24 ± 0.07†
Benzoic acid	171 ± 21.4	62.7 ± 1.10‡	2.19 ± 0.03*
Pivalic acid	165 ± 11.2	70.1 ± 5.11	2.51 ± 0.20

The rats were given 1.2 mmol/kg of each compound i.p. and killed 60 min later, unless otherwise stated. Numbers are mean ± SEM for number of animals shown in parentheses. Significantly different from controls: ‡$P < 0.05$, *$P < 0.02$, †$P < 0.01$.

Figure 3 shows that α-KG is protective against VPA in Bu conversion, thus supplying final evidence that a decreased α-KG level plays a role in this action of VPA (Fig. 3, VPA plus α-KG group). It is noteworthy that α-KG alone was able to facilitate Bu conversion (Fig. 3, α-KG group). In the pathway of carnitine biosynthesis, there is another hydroxylating step, catalyzed by the trimethyllysine hydroxylase enzyme, which works with the same coenzymes. The effect of VPA on the α-KG level probably means a severe arrest because the inhibition of trimethyllysine hydroxylation may also contribute to the impairment of carnitine biosynthesis *in vivo*.

Tracking down the mechanism, a reason for the lowered α-KG level may be the drop in the L-glutamate level (Table 3) because it is a partner substrate in the glutamate dehydrogenase reaction. The reason for the decreased liver L-glutamate level is still obscure, but an increased glutamate decarboxylase activity [30] may be responsible. The severe drop in the L-glutamate level also predicts a decrease in N-acetyl-glutamate. If so, this would explain hyperammonemia, the other side effect of VPA treatment because N-

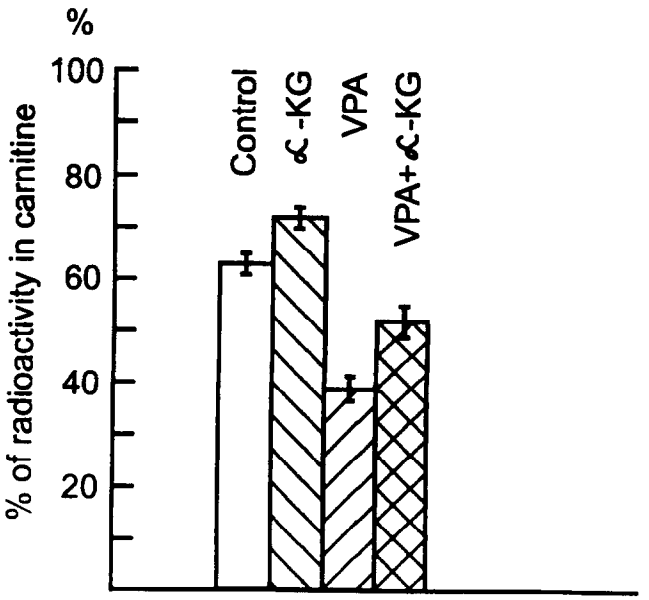

FIG. 3. Conditions were as those shown in Fig. 1A except that 0.3 mmol α-KG was given with the [^{3}H]-Bu where indicated. Bars indicate percentage of radioactivity in carnitine (mean ± SEM) for five animals.

acetyl-glutamate is an activator of the carbamyl phosphate synthase I.

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